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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

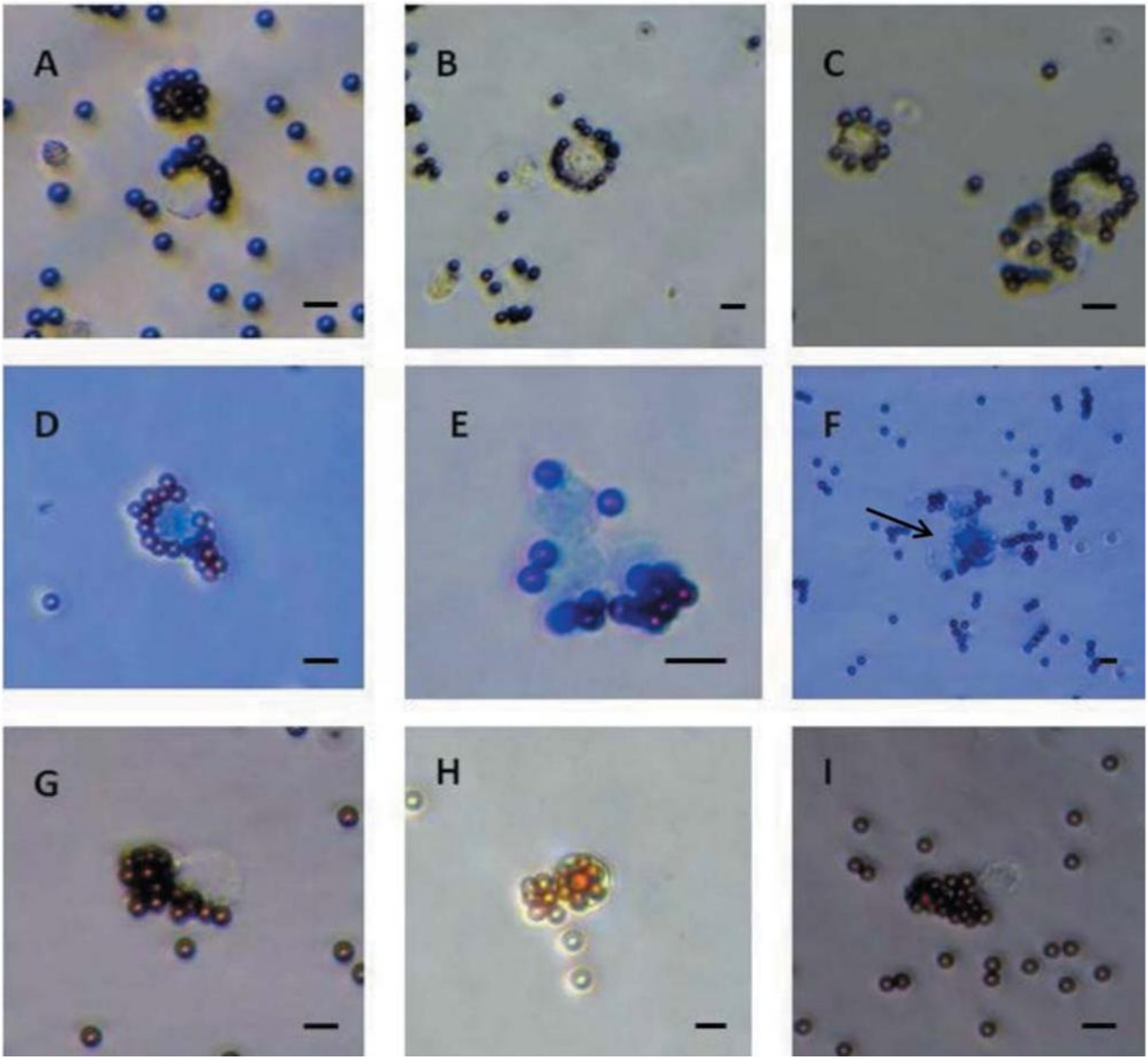

Figure 1. *Circulating tumor cells as observed under light microscopy after immunomagnetic separation from 1 ml of blood. We expect that the cancer cells are enveloped by immunomagnetic beads. The arrows are indicating beads. The cell viability was tested via Trypan blue exclusion method (D-E). In several 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.

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 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%)

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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References

. Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, Reuben JM, Doyle GV, Allard WJ, Terstappen LW and Hayes DF: Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med *351*: 781-791, 2004.

2 Cristofanilli M, Hayes DF, Budd GT, Ellis MJ, Stopeck A, Reuben JM, Doyle GV, Matera J, Allard WJ, Miller MC, Fritsche HA, Hortobagyi GN *et al*: Circulating tumor cells: a novel prognostic factor for newly diagnosed metastatic breast cancer. J Clin Oncol *23*: 1420-1430, 2005.

3 Fehm T, Hoffmann O, Aktas B, Becker S, Solomayer EF, Wallwiener D, Kimmig R and Kasimir-Bauer S: Detection and characterization of circulating tumor cells in blood ofprimary breast cancer patients by RT-PCR and comparison to status of bone marrow disseminated cells. Breast Cancer Res *11*: R59, 2009.

4 Giordano A1, Giuliano M, De Laurentiis M, Arpino G, Jackson S, Handy BC, Ueno NT, Andreopoulou E, Alvarez RH, Valero V, De Placido S, Hortobagyi GN, Reuben JM and Cristofanilli M: Circulating tumor cells in immunohistochemical subtypes of metastatic breast cancer: lack of prediction in HER2- positive disease treated with targeted therapy. Ann Oncol *23(5)*: 1144-1150, 2012.

5 Giuliano M1, Giordano A, Jackson S, Hess KR, De Giorgi U, Mego M, Handy BC, Ueno NT, Alvarez RH, De Laurentiis M, De Placido S, Valero V, Hortobagyi GN,Reuben JM and Cristofanilli M: Circulating tumor cells as prognostic and predictive markers in metastatic breast cancer patients receiving first-line systemic treatment. Breast Cancer Res *213*: R67, 2011.

6 Ignatiadis M, Georgoulias V and Mavroudis D: Circulating tumor cells in breast cancer. Curr Opin Obstet Gynecol *20*: 55-60, 2008.

7 Meng S1, Tripathy D, Frenkel EP, Shete S, Naftalis EZ, Huth JF, Beitsch PD, Leitch M, Hoover S, Euhus D, Haley B, Morrison L, Fleming TP, Herlyn D,Terstappen LW, Fehm T, Tucker TF, Lane N, Wang J and Uhr JW: irculating tumor cells in patients with breast cancer dormancy. Clin Cancer Res *10*: 8152-8162, 2004.

8 Criscitiello C, Sotiriou C and Ignatiadis M: Circulating tumor cells and emerging blood biomarkers in breast cancer. Curr Opin Oncol *22*: 552-558, 2010.

9 Hayes DF, Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Miller MC, Matera J, Allard WJ, Doyle GV and Terstappen LW: 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*: 4218-4224, 2006.

10 Allard WJ, Matera J, Miller MC, Repollet M, Connelly MC, Rao C, Tibbe AG, Uhr JW and Terstappen LW: Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. Clin Cancer Res *10*: 6897-6904, 2004.

11 Lankiewicz S, Rivero BG and Bocher O: Quantitative real-time RT-PCR of disseminated tumor cells in combination with immunomagnetic cell enrichment. Mol Biotechnol *34*: 15-27, 2006.

12 Hauch S, Zimmermann S, Lankiewicz S, Zieglschmid V, Bocher O and Albert WH: The clinical significance of circulating tumour cells in breast cancer and colorectal cancer patients. Anticancer Res *27*: 1337-1341, 2007.

13 Graves H and Czerniecki BJ: Circulating tumor cells in breast cancer patients: an evolving role in patient prognosis and disease progression. Patholog Res Int *2011*: 621090, 2011.

14 Ignatiadis M, Xenidis N, Perraki M, Apostolaki S, Politaki E, Kafousi M, Stathopoulos EN, Stathopoulou A, Lianidou E, Chlouverakis G, Sotiriou C, Georgoulias V and Mavroudis D: Different prognostic value of cytokeratin-19 mRNA positive circulating tumor cells according to estrogen receptor and HER2 status in early-stage breast cancer. J Clin Oncol *25*: 5194 -5202, 2007.

15 Zhao S, Liu Y, Zhang Q, Li H, Zhang M, Ma W, Zhao W, Wang J and Yang M: The prognostic role of circulating tumor cells (CTCs) detected by RT-PCR in breast cancer: a meta-analysis of published literature. Breast Cancer Res Treat *130*: 809-816, 2011.

16 Brandt B, Roetger A, Heidl S, Jackisch C, Lelle RJ, Assmann G and Zanker KS: Isolation of blood-borne epithelium-derived c-erbB-2 oncoprotein-positive clustered cells from the peripheral blood of breast cancer patients. Int J Cancer *76*: 824-828, 1998.

17 Wulfing P, Borchard J, Buerger H, Heidl S, Zanker KS, Kiesel L and Brandt B: HER2-positive circulating tumor cells indicate poor clinical outcome in stage I to III breast cancer patients.Clin Cancer Res *12*: 1715-1720, 2006.

18 Harris L, Fritsche H, Mennel R, Norton L, Ravdin P, Taube S, Somerfield MR, Hayes DF and Bast RC Jr.: American Society of Clinical Oncology 2007 update of recommendations for the use of tumormarkers in breast cancer. J Clin Oncol *25(33)*: 5287-5312, 2007.

19 Puglisi F, Fontanella C, Numico G, Sini V, Evangelista L, Monetti F, Gori S and Del Mastro L: Follow-up of patients with early breast cancer: Is it time to rewrite the story? Crit Rev Oncol Hematol Mar 22. pii: S1040-8428(14)00050-X. doi: 10.1016/j.critrevonc.2014.03.001, 2014.

20 Litière S, de Vries EG, Seymour L, Sargent D, Shankar L and Bogaerts J;The components of progression as explanatory variables for overall survival in the Response Evaluation Criteria in Solid Tumours 1.1 database.Eur J Cancer Apr 10. pii: S0959-8049(14)00269-X. doi: 10.1016/j.ejca.2014.03.014, 2014 .

21 Ignatiadis M, Georgoulias V and Mavroudis D: Micrometastatic disease in breast cancer: clinical implications. Eur J Cancer *44*: 2726-2736, 2008.

22 Mikulova V, Kolostova K and Zima T: Methods for detection of circulating tumour cells and their clinical value in cancer patients. Folia Biol (Praha) *57*: 151-61, 2011.

23 Kolostova K, Pinterova D, Hoffman RM and Bobek V.: Circulating human prostate cancer cells from an orthotopic mouse model rapidly captured by immunomagnetic beads and imaged by GFP expression. Anticancer Res *31*: 1535-1539, 2011.

24 Menen RS, Pinney E, Kolostova K, Bobek V, Suetsugu A, Zhang N, Bouvet M and Hoffman RM: A rapid imageable *in vivo* metastasis assay for circulating tumor cells. Anticancer Res *31*: 3125-3128, 2011

25 Xenidis N, Ignatiadis M, Apostolaki S, Perraki M, Kalbakis K, Agelaki S, Stathopoulos EN, Chlouverakis G, Lianidou E, Kakolyris S, Georgoulias V and Mavroudis D: Cytokeratin-19 mRNA-positive circulating tumor cells after adjuvant chemotherapy in patients with early breast cancer. J Clin Oncol *27*: 2177-2184, 2009.

26 Wiedswang G, Borgen E, Schirmer C, Karesen R, Kvalheim G, Nesland JM and Naume B: Comparison of the clinical significance of occult tumor cells in blood and bone marrow in breast cancer. Int J Cancer *118*: 2013-2019, 2006.

27 Stathopoulou A, Mavroudis D, Perraki M, Apostolaki S, Vlachonikolis I, Lianidou E and Georgoulias V: Molecular detection of cancer cells in the peripheral blood of patients with breast cancer: comparison of CK-19, CEA and maspin as detection markers. Anticancer Res *23*: 1883-1890, 2003.

28 Rack B, Andergassen U, Neugebauer J, Salmen J, Hepp P, Sommer H, Lichtenegger W, Friese K, Beckmann MW, Hauner D, Hauner H and Janni W: The German SUCCESS C Study -the first European lifestyle study on breast cancer. Breast Care *5*: 395- 400, 2010.

29 Pierga JY, Bonneton C, Magdelenat H, Vincent-Salomon A, Nos C, Pouillart P and Thiery JP: Clinical significance of proliferative potential of occult metastatic cells in bone marrow of patients with breast cancer. Br J Cancer *89*: 539-545, 2003.

30 Jotsuka T, Okumura Y, Nakano S, Nitta H, Sato T, Miyachi M, Suzumura K and Yamashita J: 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, 2004.

31 Giatromanolaki A, Koukourakis MI, Kakolyris S, Mavroudis D, Kouroussis C, Mavroudi C,Perraki M, Sivridis E and Georgoulias V: 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, 2004.

32 Gaforio JJ, Serrano MJ, Sanchez-Rovira P, Sirvent A, Delgado-Rodriguez M, Campos M, de la Torre N, Algarra I, Duenas R and Lozano A: 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, 2003.

33 Demel U, Tilz GP, Foeldes-Papp Z, Gutierrez B, Albert WH and Bocher O: 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, 2004.

34 Benoy IH, Elst H, Philips M, Wuyts H, van DP, Scharpe S, Van ME, Vermeulen PB and Dirix LY: 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, 2006.

35 Sandri MT, Zorzino L, Cassatella MC, Bassi F, Luini A, Casadio C, Botteri E, Rotmensz N, Adamoli L and Nole F: Changes in circulating tumor cell detection in patients with localized breast cancer before and after surgery. Ann Surg Oncol *17*: 1539-1545, 2010.

36 Bidard FC, Peeters DJ, Fehm T, Nolé F, Gisbert-Criado R, Mavroudis D, Grisanti S, Generali D, Garcia-Saenz JA, Stebbing J, Caldas C, Gazzaniga P, Manso L, Zamarchi R, de Lascoiti AF, De 37. Mattos-Arruda L, Ignatiadis M, Lebofsky R, van Laere SJ, Meier-Stiegen F, Sandri MT, Vidal-Martinez J, Politaki E, Consoli F, Bottini A, Diaz-Rubio E, Krell J, Dawson SJ, Raimondi C, Rutten A, Janni W, Munzone E, Carañana V, Agelaki S, Almici C, Dirix L, Solomayer EF, Zorzino L, Johannes H, Reis-Filho JS, Pantel K, Pierga JY and Michiels S: Clinical validity of circulating tumour cells in patients with metastatic breast cancer: a pooled analysis of individual patient data. Lancet Oncol *15(4)*: 406-414, 2014.

38 Giordano A and Cristofanilli M: CTCs in metastatic breast cancer.Recent Results Cancer Res *195*: 193-201, 2012.

39 Giuliano M1, Giordano A, Jackson S, Hess KR, De Giorgi U, Mego M, Handy BC, Ueno NT, Alvarez RH, De Laurentiis M, De Placido S, Valero V, Hortobagyi GN, Reuben JM and Cristofanilli M: Circulating tumor cells as prognostic and predictive markers in metastatic breast cancer patients receiving first-line systemic treatment. Breast Cancer Res *13(3)*: R67, 2011.

40 Tewes M, Aktas B, Welt A, Mueller S, Hauch S, Kimmig R and Kasimir-Bauer S: 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. Breast Cancer Res Treat *115*: 581-590, 2009.

41 Mayer JA, Pham T, Wong KL, Scoggin J, Sales EV, Clarin T, Pircher TJ, Mikolajczyk SD, Cotter PD and Bischoff FZ: FISH-based determination of HER2 status in circulating tumor cells isolated with the microfluidic CEE platform. Cancer Genet *204*: 589-595, 2011.

42 Pestrin M, Bessi S, Galardi F, Truglia M, Biggeri A, Biagioni C, Cappadona S, Biganzoli L,Giannini A andDi LA: Correlation of HER2 status between primary tumors and corresponding circulating tumor cells in advanced breast cancer patients. Breast Cancer Res Treat *118*: 523-530, 2009.

43 Tokudome N1, Ito Y, Takahashi S, Kobayashi K, Taira S, Tsutsumi C, Oto M, Oba M, Inoue K, Kuwayama A, Masumura K, Nakayama Y, Watanabe C and Hatake K: Detection of circulating tumor cells in peripheral blood of heavily treated metastatic breast cancer patients. Breast Cancer *18*: 195-202,2011.

44 Apostolaki S, Perraki M, Kallergi G, Kafousi M, Papadopoulos S, Kotsakis A, Pallis A, Xenidis N, Kalmanti L, Kalbakis K, Agelaki S, Kalykaki A, Stournaras C,Stathopoulos E, Georgoulias V and Mavroudis D: Detection of occult HER2 mRNA-positive tumor cells in the peripheral blood of patients with operable breast cancer: evaluation of their prognostic relevance. Breast Cancer Res Treat *117*: 525-534, 2009.

45 Bidard FC, Mathiot C, Delaloge S, Brain E, Giachetti S, de CP, Marty M and Pierga JY: Single circulating tumor cell detection and overall survival in nonmetastatic breast cancer. Ann Oncol *21*: 729-33, 2010.

46 Molloy TJ, Bosma AJ, Baumbusch LO, Synnestvedt M, Borgen E, Russnes HG, Schlichting E, van't Veer LJ and Naume B: The prognostic significance of tumour cell detection in the peripheral blood versus the bone marrow in 733 early-stage breast cancer patients. Breast Cancer Res *13*: R61, 2011.

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Characterization of circulating tumor cells in early breast cancer patients receiving neoadjuvant chemotherapy

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Running title: Character of CTCs in early breast cancer

Key words: CTCs, circulating tumor cells, breast cancer, cultivation, *in vitro*, gene expression, neodadjuvant treatment

Abstract

Introduction: Neoadjuvant chemotherapy (NACT) is indicated in locally advanced breast cancer (LABC) to reduce tumor burden prior to surgical intervention. Under chemotherapeutic pressures, minimal residual disease (MRD) in some patients can persist and give rise to clonally advanced and potentially resistant disease. Circulating tumor cells (CTCs) offer an attractive approach to not only screen for early evidence of MRD but also to assess chemosensitivity and thus response to treatment regimens.

Aim: The aim of this study was to evaluate a CTC navigated therapy model for tumor response during NACT with anthracyclines (AC) and/ or taxanes (TAX). Comparison between tumor volume measurement using ultrasound as compared to the presence and molecular characterization of CTCs over multiple time intervals corresponding with treatment was assessed.

Methods: Twenty patients with diagnosed breast cancer (BC) of different histotypes undergoing NACT were evaluated and monitored during a period of 6-24 months. Peripheral blood draws were collected for CTC evaluation at a minimum of 4 time points (before NACT start, after 2-3 chemotherapy cycles, before surgery, after surgery). CTCs were enriched using a size- based filtration method (MetaCell®) and evaluated in a two-step manner. First by cytomorphological evaluation of the captured viable cells, followed by qPCR analysis of the enriched cell population containing CTCs. A set of tumor-associated (TA) genes and genes associated with chemoresistance (CA) were analyzed.

Results: Overall, the presence and molecular characterization of CTCs outperformed the standard approach of measuring tumor volume by ultrasound in predicting overall response. CTCs were detected by cytomorphologic criteria in 17 of 20 (85 %) patients before NACT, in 15 of the 17 (88 %) patients during NACT, in 13 of 18 (72 %) patients before planned surgery, and in 13 of 14 (86 %) patients shortly after surgery. ll patients were re-evaluated after 24 months, including adjuvant therapy periods. The most significant tumor volume reduction during NACT was documented in triple negative BC (TNBC) patients, more significantly after AC than after TAX administration. We also observed a significant discordance of ER and HER2 status between primary disease and CTCs. In patients with ER+ primary tumor, up to 81-100 % of CTCs were ER-, and in HER2+ primary tumors, 10-40 % of CTCs were HER2- during NACT and 50-58 % after surgery. Chemoresistance among non-responders involved 2 or more genes while responders were observed with single gene chemoresistance.

Conclusion: The results of this study demonstrate feasibility in the use of a CTC navigated therapy model to better understand and characterize chemosensitivity from a liquid biopsy. Successful recovery of viable CTCs in early stage BC could enable detection of MRD as well as real–time monitoring of tumor-derived molecular characterization to guide therapy administration. Implementation of such models into the clinic could aid personalized treatment and permit access to a broader armamentarium of cytostatics for patients when indicated.

Introduction

Neoadjuvant chemotherapy (NACT) is indicated mainly in locally advanced breast cancer (LABC), which involves stage IIB (T2N1, T3N0) and stage III, including inflammatory breast cancer (BC). NACT is implemented to either reduce tumor volume in an effort to convert inoperable to operable tumors or to convert patients from requiring a mastectomy to breast-conserving surgery (BCS). NSABP B-18 and EORTC 1092 studies reported that application of NACT is connected mainly to down staging of the disease (1-3). However, subgroup analysis of both studies observed the trend towards better outcome in patients under 50 of age (1). The same benefit of adjuvant chemotherapy in young premenopausal women (4) and women under 50 years (5) was seen. Moreover, early indication of NACT and response to doxorubicin (A) and cyclophosphamide (C) regimen yielded better disease free survival (DFS) and overall survival (OS) in comparison to patients undergoing adjuvant docetaxel (D) treatment in the B27 study (6).

The current indication of NACT is based on tumor biology and is considered as a treatment option in BC patients with high tumor dissemination risk and worse prognosis (7). The most important finding among neoadjuvant studies is the association between pathologic complete response (pCR) observation and long-term clinical outcome. pCR mainly occurs in patients with aggressive BC subtypes (8); tumors exhibiting high proliferation (Luminal B, HER2+, TNBC) have higher rates of pCR compared to Luminal A (9). Post-treatment residual disease and Ki67 levels seem to also have prognostic significance (10-11); mainly patients with extensive residual disease and Ki67 > 35 % after NACT have significantly worse outcome. The need for new biomarkers reflects the shortcomings of existing therapy options. Although AC- and TAX- regimens in NACT are considered standard clinical practice, recurrence due to clonal expansion and/or resistance of residual tumor cells due to treatment selection pressures occur.

The metastatic potential of BC disease could be stratified not only by primary disease subtype, but also measured by CTC presence. Although their occurrence is very rare in blood, CTCs have significant prognostic power both for patients with primary (PBC) and metastatic breast cancer (MBC) (21-23). Yet, clinical utility based on the predictive power of CTC enumeration remains uncertain. The genomic characteristics of CTCs may indeed be more important for therapy recommendation as suggested by the SWOG S0500 trial (24) as well as enhance patient outcomes (25-26).

Many enrichment methods have been successfully implemented into laboratory practice to enrich, detect and isolate CTCs from a simple blood draw. The most commonly used isolation techniques are immunomagnetic or size- based (27-30). CTCs can be detected by immunofluorescent staining (31), laser scanning cytometry (32) or quantitative polymerase chain reaction (qPCR) (33). In the case of BC, several markers are known to have different expression ratios if comparing non-malignant normal cells to tumor cells, such as cytokeratin 19 (KRT19), human epidermal growth factor receptor 2 (HER2), epithelial cell adhesion molecule (EpCAM), mucin 1 (MUC1), epidermal growth factor receptor (EGFR), mammaglobin (MGB), and maspin. Individually, these markers could be used for CTC identification at the molecular level. However, even greater specificity can be achieved by using a multi-marker assay to profile each marker simultaneously (34-37). In previous studies, the size-based MetaCell filtration system has been shown to not only reliably recover viable CTCs from BC patients for cytomorphologic evaluation but also permit downstream CTC molecular characterization.

Despite clinical advances, MRD in some patients can persist and give rise to clonally advanced and resistant disease. Although we know that phenotypic differences between primary tumor and CTCs exist (38-42), patients are still treated according to primary tumor characteristics. Recent therapy indications do not reflect the dynamic changes that occur among tumor cells, which are subject of chemotherapy pressure. In this study, we compared tumor response during NACT with anthracyclines (AC) and/or taxanes (TAX) as measured by tumor volume to the presence and characterization of CTCs over multiple time intervals corresponding with standard treatment cycles. We report the use of real-time molecular characterization of chemoresistance and tumor related genes following size-based MetaCell enrichment of CTCs in predicting therapeutic decisions and overall survival.

Patients and methods

Study design:

In total, 20 BC patients undergoing NACT were included in the presented study. The analysis comprised of 116 testing blood samples obtained during regular medical examinations between years 2014-2016. CTC examination was executed before the NACT start, during the NACT and/or before surgery and after surgery. If NACT comprised sequential AC and TAX administration, the blood withdrawals were usually done before the first AC-cycle, before the first TAX-cycle and before the last TAX-cycle. CTC were enriched from peripheral blood (8 ml) by size- based filtration method (MetaCell®, Czech Republic).

Patient characteristics:

Patient characteristics can be found in **Table 1.** The median age of the group was 39 years. Only one patient in the study was postmenopausal (71 years of age at the time of diagnosis). Of the NACT indicated patients, 16 patients were treated because of LABC with lymph node involvement; in 4 out of 20 patients negative lymph nodes (N0) were reported.

Based on histological evaluation, BC was classified as invasive carcinoma of not specified type (NST) in 5 cases or invasive ductal carcinoma (IDC) in 14 cases and medullary carcinoma in 1 case. 16/20 carcinomas had poor degree of differentiation, i.e. grade 3 (G3), 3/20 carcinomas had middle grade (G2) and 1/20 carcinoma had low degree of differentiation (G1).

All patients had very aggressive disease according proliferation parameters (Ki67) of tumor cells. The value of Ki67 was in one case 20 %, all other primary tumors exhibited minimally 40 % of Ki67 expression. Altogether, 12/20 tumors were estrogen receptor positive (ER+) and/or progesterone receptor positive (PR+), 6/20 patients were human epidermal growth factor receptor type 2 positive (HER2+). 9/20 patients were classified as TN. Patient no 7 was also classified as TNBC given that postoperative ER and PR status was negative for both. According the subtype classification, 5 of 20 patients were HR+/HER2- (luminal B), 4 of 20 patients were HR+/HER2+ (luminal B, HER2+), 2 of 20 patients were HR-/HER2+ (HER2-

amplified/overexpressed), and 9 of 20 patients were TNBC (ER-, PR- and HER2-). Clinicopathologic features for every patient involved into the study are reported in details (N=20) **in Table 2.** For more individual risks of patients see notices in **Table 2.** NACT regimens and cytostatics dosing please see **Suppl. file 1** Methods.

Table 1: Clinicopathological features of patients in study (N=20)

Characteristics	**Number of patients**	**(%)**
Total	20	100
Tumor size		
T1	6	30
T2	14	70
Nodal status		
N0	4	20
N1	15	75
N2	0	0
N3	1	5
Grading		
G1	1	5
G2	3	15
G3	16	80
ER status		
Positive	11	55
Negative	9	45
PR status		
Positive	14	70
Negative	6	30
HER2 status		
Positive	6	30
Negative	14	70
Menopausal status		
Premenopausal	19	95
Postmenopausal	1	5

Table 2: Clinicopathological features of patients in study (N=20) reported individually

No	**Age**	**Stage before therapy**	**Histology Ki67 (%)**	**Receptors from biopsy (%)**	**Regimen of NACT**	**Notices**	**Stage post-surgery**	**Histology Ki67 (%) post-surgery**	**Receptors post-surgery (%)**
1	37	T2N1	IDC, G3, Ki 40	ER90, PR60, HER2-	4xAC-4xD	BRCA1 mutation	T2N2	IDC, G3, Ki 10	ER70, PR10, HER2-
2	43	T2N1	IDC, G1, Ki 50	ER100, PR50-60, HER2-	4xAC-4xD		T1aN0	IDC, Ki 30, G?	ER20, PR0, HER2-
3	71	T2N1	IDC, G3, Ki 40	ER100, PR60, HER2+	3xFEC-3xDH		T1cN0	IDC, G3, KI 3	ER100, PR100, HER2+
4	33	sin T1cN1 dx. T1aN0	IDC, G3, Ki 40	ER50, PR5, HER2+	4xAC-4xDH	CHEK2 mutation	sin. T1aN0 dx. pCR	IDC, G2, Ki 1	ER75, PR5, HER2-
5	39	T2N1	NST, G2, Ki 20	ER80, PR90, HER2+	4xAC-4xDH		T1cN1mi	NST, G2, Ki 10	ER70, PR30, HER2+
6	38	T2N0	IDC, G2, Ki 90	ER0, PR0, HER2-	3xFEC-3xD		pCR		

7	31	T2N1	IDC,G3,Ki 75	ER50,PR10, HER2-	4xAC, 1xD, 9xT	dg during lactation	pCR		
8	36	T1cN1	IDC,G3,Ki 55	ER0, PR5, HER2+	4xAC-4xDH		T1aN0	IDC,G3,Ki 5	ER0, PR0, HER2+
9	31	T2N1	NST,G3,Ki 70	ER50, PR70, HER2+	5xA	dg 18.w. of pregnancy 4x DH adjuvantly	T1cN0	NST,G1,**Ki 60**	ER0, PR0, HER2+
10	36	T2N0	NST,G3,Ki 75	ER0, PR2, HER2- classified as TNBC	3xFEC-3xD		T2N0	NST,G3, **Ki 95**	ER0, PR2, HER2- classified as TNBC
11	31	T2N1	IDC,G3,Ki 80	ER5, PR7, HER2- classified as TNBC	4xACdd-4xD		T1aN0	IDC,G3,Ki 20	ER0, PR0, HER2-
12	33	T2N1	NST, G3, Ki 50	ER0, PR10, HER2+	4xACdd-1xDH, 9xTH		T2N1a(1/14)	NST, G3, Ki 20	ER0, PR0, HER2+
13	41	BC: T2N1 Ovary IIIc	IDC,G3, Ki 100 Serous Ca,G3	ER0, PR0, HER2- ER75	2xFEC75, 5xCBDCA +paklitaxel	vs BRCA1+	T1cN0 OC: T3bN1	Metaplastic ca, G3, Ki50 OC: same	ER5, PR1, HER2- classified as TNBC
14	41	T1cN1	IDC, G3, Ki 50	ER80-90, PR20-25, HER2-	4xAC- 4xD		T2N3	IDC, G3, **Ki 50**	ER100, PR25, HER2-
15	39	T1mN1	NST, G2, Ki 30	ER90, PR5, HER2-	4xAC-1xD, 9xT	dg during lactation	T2N1c	NST, G3, Ki0	ER100, PR10, HER2-
16	44	T1cN0	IDC, G3, Ki40	ER0, PR0, HER2-	6xFEC		T1bN0	NST, G1, Ki5	ER0, PR0, HER2-
17	44	T2N1	Medullary, G3, Ki70	ER0, PR0, HER2-	4xAC-12xT		pCR		
18	52	T2N1	IDC, G3, Ki50	ER100, PR50-75, HER2-	4xAC-4xD		T2N1	NST, G2, Ki5	ER100, PR1, HER2-
19	40	T2mN3	IDC, G3, Ki 60	ER0, PR0, HER2-	1xACdd, 3xAC,12xT	BRCA1 mutation	Died before surgery		
20	40	T1cN0	IDC, G3, Ki 95	ER0, PR0, HER2-	3xFEC-9xT		pCR		

CTC examination:

CTCs were enriched from peripheral blood (EDTA/ 6-8 ml) by a size-based filtration method (MetaCell®, Czech Republic). Isolated enriched cells were incubated 3-5 days in vitro (37°C, 5% CO_2) and evaluated in a two -step manner.

First, cytomorphologic evaluation of the captured viable cells followed by qPCR analysis of RNA isolated from captured cytomorpholoic CTC–like cells for expression of TA- and CA-genes. For more detail, please see **Suppl. file 1 Methods**.

Patients' blood collections were classified as CTC positive by combined microscopic cytomorhologic evaluation and molecular analysis. Within the process of cytomorphological analysis, fluorescently stained viable cells were scored for various CTC criteria (nucleus size, nuclear membrane irregularity, prominent nucleoli, nucleoli count, cell size, presence of 2D and 3D cell sheets, etc.). Recovered size-enriched fractions of cells captured on the membrane were lysed by RLT+ β-mercaptoethanol buffer and stored at -20°C for subsequent RNA - analysis.

Differences between the whole blood leucocyte fraction enriched CTC fractions (with and without *in vitro* incubation) were detected by qPCR analysis of TA and/or CA genes. TaqMan™ Gene Expression Assays (Thermo Fisher Scientific, USA) were used for gene expression monitoring in all samples. ActB (control), CD24, CD44, CD45, CD68, KRT19, EpCAM, MUC1, MGB, HER2, ESR, PGR as TA genes and MRP1, MRP2, MRP4, MRP5, MRP7, MDR1, ERCC1 as CA genes were tested to determine expression levels each CTC-enriched sample (Table 3).

Samples with elevated expression levels in two or more TA-markers among the cultured CTC- fraction compared to their matched whole blood leucocyte fraction were characterized as CTC positive based on gene expression analysis.

Statistical analysis:
The qPCR data evaluation was based on standard ddCT method (43). qPCR results were analyzed by means of GenEx Professional software (MultiD) enabling multifactorial comparisons between the involved group. Relative RNA levels are displayed graphically in clusters. The differences between the tested samples were compared by Mean - Whitney testing (significance level $p < 0,05$ if not set automatically by GenEx).

Supplementary files METHODS

Therapy specification and side effects / file I:
All patients underwent NACT based on antracyclins (regimen AC, i.e. doxorubicin (A) 60mg/m2 and cyclophosphamid (C) 600mg/m2 (n=12), A alone in case of pregnant woman (n=1), FEC, i.e. flurouracil (F) 500mg/m2, epirubicin (E) 100mg/m2 if not stated otherwise, cyclophosphamid (C) 500mg/m2 (n=5), all in interval q3w) and/or taxanes (docetaxel (D) 100mg/m2 q3w or paklitaxel (T) 80mg/m2 weekly, in case of breast and ovarian cancer (n=1) T 175mg/m2 in combination with carboplatin (CBDCA) AUC5, both q3w). In 2 of 20 patients, AC dose dense regimen (ACdd) was used, which is based on standard doses of cytostatics in interval q2w and with support of growth factor (pegfilgrastim). Pegfilgrastim was also used with D 100mg/m2 (n=11).
In patient with HER2 positivity, taxanes in combination with trastuzumab were administered, i.e. Herceptin (H) 600 mg subcutaneously, cardiac functions were monitored within 3 months. Toleration of Herceptin was excellent without cardiac complications.

The toleration of therapy was good in general. We haven't noticed G3 or G4 side effects (neu < 1x 10^9/L, Hb < 50 g/L, ptc < 50 x 10^9/L, GIT toxicity with the need for hospitalization, prolonged infused reaction with the need for hospitalization, skin rash > 30 % of BSA, decrease in EF > 20 % or residual EF < 40 %, alteration of liver test with the level > 5-20x ULN). None of the patients had to discontinue the therapy, none of the NACT cycles was delayed. The worse treatment toleration we registered in patient no. 13 with ovarian cancer (OC) in parallel. As the OC was diagnosed later on, we started therapy of BC with FEC75 (flurouracil (F) 500mg/m2, epirubicin (E) 75mg/m2, cyclophosphamid (C) 500mg/m2) and then continued with carboplatin and paclitaxel with great response in both tumor localizations.

The most commonly observed side effects were nausea, fatigue and hot flushes. In patients with skin rash after D we switched D to T. Neuropathy wasn't frequent. The pregnancy of 1 patient went to term without complication. The child is healthy and doing well. Surgery for the primary tumor was performed after childbirth, lactation was stopped and the patient continued with adjuvant CT and targeted anti-HER2 therapy. The lactation of 2 other patients diagnosed after delivery was finished before the start of CT.

Whole peripheral blood processing – Leucocyte fraction preparation

200 µl of peripheral blood and 800 µl of Buffer EL (Qiagen, Germany) were mixed together and incubated for 10-15 min on ice. Suspension was centrifuged at 400 x g for 10 min at 4°C. Supernatant was completely removed and discarded. 400 µl of Buffer EL was added to the cell pellet and resuspended by pipetting. Suspension was centrifuged at 400 x g for 10 min at 4°C and supernatant was completely removed and discarded. 600 µl of Buffer RLT supplemented by β-mercaptoethanol (VWR, USA) was added to pellet; suspension was mixed by pipetting and stored at -20°C.

Enrichment of CTCs for RNA isolation

6 ml of peripheral blood were filtered through membrane (8 µm pores) of the Metacell® (Metacell s.r.o., Czech Republic) device. Separated fraction of cells captured on the membrane was disrupted by 600 µl of Buffer RLT+ β-mercaptoethanol and suspension was stored at -20°C. REFERENCES

Enrichment of CTCs for cultivation

6ml of peripheral blood were filtered through membrane (8 µm pores) of the Metacell® device. Membrane with captured cells was washed by RPMI media (SIGMA-ALDRICH, USA) and transferred into cultivation plate. 4 ml of RPMI media supplemented by fetal bovine serum (10%) (SIGMA-ALDRICH, USA), Amphotericin B (SIGMA-ALDRICH, USA) and Penicillin-streptomycin (SIGMA-ALDRICH, USA) antibiotics to avoid contamination were added on the membrane. Captured cells were cultured in vitro under standard conditions (37°C, 5% CO_2) for 3-5 days.

Cytomorphological analysis

After 3-5 days of cultivation, nucleus and cytoplasm of viable cells were stained by vital fluorescent dyes Nucblue® Live ReadyProbes® Reagent (Thermo Fisher Scientific, USA) and Celltracker™ Green CMFDA Dye (Thermo Fisher Scientific, USA), respectively. Stained cells were captured (magnification x40) C software, Olympus IX51 fluorescent microscope with built in camera, Olympus U-RFL-T power supply unit) and identified according to usual cytopathologic criteria for CTCs including nuclei larger than 10µm, proliferation, presence of tridimensional sheets, high nuclear/cytoplasmic ratio, prominent nucleoli, irregular nuclei, visible cytoplasm. Cultured cells growing on the membrane and under the membrane were then disrupted by 600 µl of Buffer RLT+ β-mercaptoethanol and stored at -20°C.

RNA isolation and reverse transcription

RNeasy Mini Kit (Qiagen, Germany) was used for RNA isolation in samples. Fractions of peripheral blood processing (1. whole peripheral blood, 2. enriched cells, 3. cultured cells on the membrane and 4. cultured cells under the membrane) mixed with Buffer RLT supplemented by β-mercaptoethanol were thawed. 600 µl of 70% ethanol (Fagron a.s., The Czech Republic) were added and mixed by pipetting. Whole volume including any precipitate was transferred into QIAamp spin column in 2 ml collection tube and centrifuged for 15 s at 8000 x g. Liquid waste under the column was removed and discarded. 700 µl of Buffer RW1 were added into QIAamp spin column and centrifuged for 15 s at 8000 x g. Liquid waste under the column was removed and discarded. 500 µl of Buffer RPE supplemented by ethanol were added into QIAamp spin column and centrifuged for 15 s at 8000 x g. Liquid waste under the column was removed and discarded. 500 µl of Buffer RPE supplemented by ethanol were added into QIAamp spin column and centrifuged for 2 min at full speed. Liquid waste under the column and collection tube were removed and discarded. QIAamp spin column was placed into a new 2 ml collection tube and centrifuged at full speed for 1 min. QIAamp spin column was transferred into a new 1.5 ml microcentrifuge tube and 30 µl of RNase-free water was pipetted directly onto the QIAamp membrane. After 2 min of incubation, QIAamp spin column was centrifuged for 1 min at 8000 x g. 30 µl of RNase-free water was pipetted onto the QIAamp membrane and after 2 min of incubation, QIAamp spin column was centrifuged for 1 min at 8000 x g. Concentration of RNA was measured by nanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, USA).

High-Capacity RNA-to-cDNA™ Kit (Thermo Fisher Scientific, USA) was used for cDNA synthesis. Reaction components were added according to manufacturing instructions ***(Table XX).***

Table XX: Instructions for preparing reverse transcription reaction mix (per 20 µl reaction)

Component	**Volume/Reaction (µl)**
2X RT Buffer	10
20X RT Enzyme Mix	1
Sample	Up to 9 µl
Nuclease-free H_2O	To 20 µl
Total per Reaction	20

Tubes with reaction mix were centrifuged and placed to the thermal cycler (ELISABETH PHARMACON, spol. s r.o., The Czech Republic). Conditions in thermal cycler were set according to manufacturing instructions (**Table XX1).**

Table XX1: Instructions of optimized conditions for High-Capacity RNA-to-cDNA™ Kit

Step	**Temperature (°C)**	**Time (min)**
Step 1	37	60
Step 2	95	5
Step 3	4	∞

Gene expression analysis

Differences between whole blood, isolated and cultivated CTCs fraction were detected by qPCR analysis of tumor- and/or resistance-associated genes. TaqMan™ Gene Expression Assays (Thermo Fisher Scientific, USA) were used for gene expression monitoring in the samples. ActB (control), CD24, CD44, CD45, CD68, KRT19, EpCAM, MUC1, MGB, HER2, ESR, PGR as tumor-associated and MRP1, MRP2, MRP4, MRP5, MRP7, MDR1, ERCC1 as chemoresistance-associated genes were tested to find out their expression level in CTCs (Table XX2).

Table XX2: List and Hs codes of monitored genes.

Name	Assay	Hs number
MDR1	ABCB1	Hs00184500_m1
MRP1	ABCC1	Hs01561502_m1
MRP2	ABCC2	Hs00166123_m1
MRP4	ABCC4	Hs00988717_m1
MRP5	ABCC5	Hs00981087_m1
MRP7	ABCC10	Hs00375701_m1
ActB	ACTB	Hs01060665_g1
CD24	CD24	Hs02379687_s1
CD44	CD44	Hs01075861_m1
CD68	CD68	Hs02836816_g1
EpCAM	EPCAM	Hs00158980_m1
Her2	ERBB2	Hs01001580_m1
ERCC1	ERCC1	Hs01012158_m1
ESR	ESR1	Hs00174860_m1
KRT18	KRT18	Hs01920599_gH
KRT19	KRT19	Hs01051611_gH
MUC1	MUC1	Hs00159357_m1
PGR	PGR	Hs01556702_m1
CD45	PTPRC	Hs04189704_m1
MGB	SCGB2A2	Hs00935948_m1

Gene expression analysis was measured by COBAS ® 480 (Roche s.r.o., Czech Republic). Temperature conditions in were set according to manufacturing instructions of TaqMan® Fast Advanced Master Mix (Thermo Fisher Scientific, USA) (Table XX3).

Table XX3: Thermal-cycling conditions for qPCR analysis.

Step	Cycles	Temperature (°C)	Time (min:s)
Step 1	1	50	02:00
Step 2	1	95	00:20
Step 3	45	95	00:03
Step 4	45	60	00:30

Results

CTC and regression of tumor volume during NACT:

The effects of chemotherapy (CT) were observed clinically by bimanual palpation of breast and by ultrasound examination roughly at the same time of blood draw. Regression of tumor mass was assessed as very significant (response rate 3) if regression was reported as more than 50 %, as moderate (response rate 2) if the degree of regression was 50 %, and as minimal (response rate 1) if the degree of regression was less then 50 %. For no observed tumor regression or even progression a number 0 is used.

CTC positivity was verified following a two-step protocol; All CTC-positive cytomorphologic features that were observed among captured malignant cells had to be confirmed by expression of relevant TA -genes. Finding of minimally 1 CTC was reported as positive. Report on CTC positivity and tumor volume reduction during NACT is shown in **table 4.**

Good treatment response appeared to be linked to the effect of AC more often then to TAX (40 % of patients were responding significantly - response rate 3). Effect of TAX was evaluated as significant in 4 of 17 (23 %) patients. CTC positivity was detected in 87.5 % of AC-responders and 75 % of TAX-responders. Among non- responders (response rate 0 - 1), 85 - 100 % CTC positivity was observed. We did not observe a correlation between tumor volume reduction and CTC positivity rate. Overall, NACT did not appear to significantly effect CTC positivity (72 % of CTC+ patients before operation vs 85 % positivity at start of NACT).

Tumor volume and CTC changes before, during and after NACT:
Complex information on the NACT effects mirrored in captured CTC is shown in detail in **Table 5.**

Chemoresistance to AC, TAX, 5-fluorouracil, cisplatin, carboplatin and other cytostatics was tested in CTCs (**Table 3**). Results suggest that expression of CA-genes may reflect the effects of NACT on treatment response. Our patients were treated only by AC and/ or TAX- based therapy and observed elevation in gene expression of CA- genes MRP 1 (association with chemoresistance to AC), MRP 2 and/or MRP 7 (associated with chemoresistance to TAX) which could be the basis for cross-resistance causing zero effect of later administered cytostatics.

Table 3: Resistatnce to cytostatics according to MRP genes expression

Resistance to:	**Genes associated with chemoresistance:**						
anthracyclines	MRP1	MRP2					
taxanes		MRP2			MRP7		
irinotekan / topotekan	MRP1	MRP2	MRP4				
alkylating agents	MRP1	MRP2					
5-fluorouracil				MRP5			
platinum derivates		MRP2		MRP5		ERRC1	
metothrexat		MRP2	MRP4	MRP5			
vinca-alkaloids	MRP1				MRP7		
multi-drug resistance as defined by MDR1 (P-glykoprotein)							MDR1
gemcitabine							RRM1 / RRM2

Table 4: CTC positivity rate and regression of tumor volume during NACT

	CTC	**Regression of tumor volume (Response rate- RR)**							
	Positivity (%)	**RR = 0 (%)**	**CTC in group RR =0 (%)**	**RR = 1 (%)**	**CTC in group RR =1 (%)**	**RR = 2 (%)**	**CTC in group RR =2 (%)**	**RR =3 (%)**	**CTC in group RR =3 (%)**
Before AC	17/20 (85)								
After AC	16/18 (88)	1/20 (5)	1/1 (100)	7/20 (35)	6/7 (85)	4/20 (20)	3/4 (75)	8/20 (40)	7/8 (87.5)
After TAX (before surgery)	13/18 (72)	3/17 (18)	3/3 (100)	9/17 (53)	7/8 (87.5)	1/17 (6)	1/1 (100)	4/17 (23)	3/4 (75)

Table 5: Effect of NACT in comparison to CA- genes expressions

Patient	CTC before NACT			Clinical effect of AC	CTC after AC			Clinical effect of TAX	CTC before operation (after TAX)			Effect of completed NACT
	YES (1)/ NO (0)	Genes with elevated relative RNA levels			YES (1)/ NO (0)	Genes with elevated relative RNA levels		YES (1)/ NO (0)	Genes with elevated relative RNA levels			
		TA-genes	CA-genes			TA-genes	CA-genes		TA- genes		CA - genes	
1	1	EPCAM, KRT19, MUC1	MRP1, MRP4, MRP5, MRP7	1	1	EPCAM, KRT19, MUC1, HER2, CD24		0	**not evaluated**	-	-	1
2	0	**CTC negative**	**-**	**3**	**1**	EPCAM, KRT29, MUC1, HER2	-	1	**0**	**CTC negative**	**-**	**3**
3	1	EPCAM, KRT19	MRP7	2	1	KRT19, HER2	MRP1, MRP7, ERCC1	1	1	EPCA, HER2, KRT19	MRP2, MRP4	2
4	1	HER2, KRT19	-	**3**	**1**	HER2, KRT19, EPCA,MUC1, CD24,CD44	MRP1, MRP5, MRP7, ERCC1	2	1	KRT19, CD24, CD44,	-	**3**
5	1	HER2, KRT19	-	**3**	**1**	KRT19, HER2, CD24	-	0	**0**	**CTC negative**	**-**	2
6	1	EPCAM, KRT19, HER2	MRP5	**3**	**0**	**CTC negative**	**-**	1	1	HER2, MUC1, KRT19 ,MGB, CD24, CD44,	MRP5, MRP7, ERCC1	**3**

7	1	EPCAM, KRT19	-	2	1	HER2, ESR1, KRT19, MUC1, CD24,CD44,	MRP1	**3**	**0**	**CTC negative**	**-**	**3**
8	0	**CTC negative**	-	2	1	HER2, KRT19, MUC1, CD24, ESR1	-	1	1	KRT19, MGB, CD24, HER2	MRP1, MRP5, MRP7	**3**
9	1	KRT19, CD24, HER2	-	**3**	**not evaluated**	-	-	no taxanes	1	EPCAM, HER2, KRT19, CD24	MRP1, MRP7	2
10	1	KRT19, CD24, HER2	MRP1, MRP4, MRP5, MRP7, ERCC1	1	1	HER2, KRT19, MGB, MUC1, CD24, CD44,	MRP1, MRP5, MRP7,	0	1	CD24, CD44, KRT19	MRP1, MRP4, MRP5, ERCC1	1
11	1	KRT19, CD24, HER2	MRP1, MRP5, MRP7	2	**0**	**CTC negative**	**-**	**3**	**1**	KRT18, KRT19, CD24	MRP1 MRP7	**3**
12	1	KRT19, MGB, ESR1, CD24, CD44,	MRP1, MRP4	1	1	KRT19, HER2, CD24, ESR1,	MRP1	1	1	CD24, CD44, KRT18, KRT19	MRP1, MDR1	1
13	1	KRT19, MGB, CD24, CD44	MRP1, MRP5, MRP7	1	**not evaluated**	-	-	**1**	**0**	**CTC negative**	**-**	**3**
14	**0**	**CTC negative**	**-**	1	1	KRT18, KRT19, CD24, CD44,	ERCC1, MRP1	1	**not evaluated**	-	-	0
15	1	KRT18, KRT19, CD24	MRP1, MRP4	0	1	KRT18, KRT19, CD24	MRP1, MRP7	1	1	KRT18, KRT19, CD24	-	0
16	1	KRT18, KRT19, MUC1, HER, CD24, CD44,	ERCC1, MDR1, MRP1, MRP5, MRP7	*3*	1	CD24, CD44, KRT18, KRT19, MUC1	ERCC1, MRP1, MRP2, MRP5	no taxanes	**not evaluated**	-	-	*3*
17	1	KRT18, KRT19, CD24, CD44,	MRP1, MRP4, MRP7	*3*	1	KRT18, KRT19, CD24	-	3	**0**	**CTC negative**	**-**	*3*
18	1	EpCAM, KRT18, KRT19, CD24	MRP1	1	1	KRT18, KRT19, CD24	MRP4	1	1	KRT18, KRT19, MUC1, CD44	MRP1, MRP2, MRP7	3

19	1	KRT18, KRT19, CD24, CD44,	ERCC 1, MRP1	*3*	1	KRT18, KRT19, CD24, CD44,	MRP1 , MRP7	3	1	KRT18 , KRT19	MRP1 , MRP2, MRP7	Died
20	1	KRT18, KRT19, CD24	ERCC 1, MDR1, MRP1, MRP4	1	1	KRT18, KRT19, MUC, CD24, CD44,	MRP1	**3**	**1**	KRT18 , KRT19 , CD24, CD44	MRP4	**3**

In table 5, significant response to therapy appears in bold italics. Epithelial signs of CTC (TA- gene expression) and minimal CA- genes expression during NACT appear to correlate with positive clinical effect of AC or TAX. Interestingly, CTC negativity during NACT was seen only in patients with relevant response rate (no 2 or 3) and clinical effect of the therapy.

In responders (response rate 2 - 3), a unique effect of AC (patients 2 - 9, 16, 17 and 19) is documented. Table 2 also reports on the histopathologic subtypes of these patients (2x ER+, 5x HER2+, 4x TNBC). The best overall response (response rate 2 - 3) to AC and/ or TAX was more frequently demonstrated in TNBC (7x) and HER2+ (5x) patients, less frequently in ER+ (3x) patients.

On the other hand, among non-responders (response rate 0 - 1) two or more CA- genes were frequently expressed. As the characteristics of CTC dynamically changed during the NACT, in some cases we observed expression of only one CA-gene before cytostatic therapy and two or more during or after that.

Developing chemoresistance is documented for TAX in patients no 1, 3, 6, 8, 10, 12, 14, 15 and 18. Expression of CA- genes MRP2, MRP7 or MDR1 was detected before or during the TAX therapy. Please see more in detail in Suppl. (CTC presence and character during disease monitoring)

We may conclude that as the proportion or volume of resistant CTC present elevated in the captured CTC-fraction, a worse therapy outcome was observed clinically. Resistant patients have elevated mainly MRP1 during AC therapy and MRP1 and MRP7 genes during TAX therapy**.**

Some more complicated cases with unanticipated therapy effect not correlating with CTC characteristics are also described **in table 5** (italic). For example, in patients no 2 and no 5, presence of CTC during TAX therapy was not detected, yet the response to therapy was small among them. In patients no 16, 17 and 19 we detected a multi-resistant gene profile in CTCs before the AC therapy, yet these patients responded to the treatment very well.

Changes of tumor histological characteristics during and after NACT:

After completing NACT and planned surgery, definitive histology was compared with the pre-treatment biopsy (see table 2). In most cases, the effect of NACT was linked to significant decrease of Ki 67. Only in 2 cases the decline of Ki 67 was very small or none (patients no 9 and 14). BC phenotype changed significantly in one patient (no 9; pregnant during NACT). We hypothesize the loss of ER and PR expression could be caused by the termination of pregnancy. Insufficient length of NACT and AC monotherapy could be the cause of a small decrease in Ki67.

HER2+ CTCs in a patient with HER2- primary tumor (no 14) and the absence of anti-HER2 therapy could be another reason for MRD persistence. In patient no 14, the up- staging of tumor disease was observed. Massive lymphatic node involvement and CTC presence with stem cell characteristics (CD44/CD24) could be the cause of the high Ki67 value.

In another case (no 10) the increase of Ki 67 from 75 to 95% was documented. This patient had chemoresistant tumor (response 0) with characteristics of TNBC. However, we confirmed elevated HER2 – expression positivity repeatedly in the CTC fraction (please see Suppl. File 2 CTC presence and character during disease monitoring). Expression of many CA- genes including MDR1 was probably the reason of treatment failure.

Discordancy of ER and HER2 status among primary tumor and CTC:

The discordancy in ER and HER2 expression in primary tumors and CTCs can be seen **in table 6.** The distribution of ER+, HER2+ and TN BC was as follows: 25 % ER+, 30 % HER2+ and 45 % TNBC.

The distribution of CTCs during NACT was discordant in patients with ER positive primary tumors (only 8 % CTC were ER+). In cases of TNBC and HER2+ primary tumors the concordance was even higher: during NACT only 67 % of TNBC patients had triple negative CTCs and 80 % of HER2+ patients had HER2+ CTC. Interestingly, phenotypic evaluation of CTCs at any time throughout observation, concordance is seen only for TNBC (68 % of TNBC patients had ER- and HER2- CTCs). For more detailed stratification see **table 7.**

Table 6: Concordancy of primary tumors and CTCs in status of ER and HER2.

	Primary tumor (%)	**CTCs+ samples at all (%)**	**CTCs+ samples during NACT (%)**	**CTCs+ samples at any time (%)**
ER+	5/20 (25)	27/30 (90)	1/13 (8)	1/27 (4)
HER2+	6/20 (30)	38/42 (91)	12/15 (80)	18/38 (47)
TNBC	9/20 (45)	28/37 (76)	12/18 (67)	19/28 (68)

Table 7: Concordance and discordancy in ER and HER2 status of CTC in comparison to primary tumor subtypes

Primary tumor	CTCs			
	ER+	ER-	HER2+	HER2-
	during NACT any time	during NACT any time	during NACT any time	during NACT any time
ER+ HER2-	**1/13 (8 %)** **1/27 (4 %)**	12/13 (92 %) 26/27 (96 %)	4/13 (31 %) 7/27 (26 %)	**9/13 (69 %)** **20/27 (74 %)**
ER- HER2-	0/18 0/28	**18/18 (100 %)** **28/28 (100 %)**	6/18 (33 %) 9/28 (32 %)	**12/18 (67 %)** **19/28 (68 %)**
ER+ HER2+	**0/10** **5/26 (19 %)**	10/10 (100 %) 21/26 (81 %)	**9/10 (90 %)** **13/26 (50 %)**	1/10 (10 %) 13/26 (50 %)
ER- HER2+	3/5 (60 %) 3/12 (25 %)	**2/5 (40 %)** **9/12 (75 %)**	**3/5 (60 %) 5/12** **(42%)**	2/5 (40 %) 7/12 (58 %)

In luminal B tumors, concordance rate with ER positivity was only 4 - 8 % and in HER2 negativity it was 69 - 74 %. In ER+ HER2+ patients, only 0 - 19 % of samples were ER+ with concordance in HER2+ during NACT at 90 %. Interestingly we have observed interchanged (? Not sure what this means) HER2+ in 50 % of samples monitored during patients observation.

We can conclude, that during NACT we monitored CTC with a quite high rate of concordance in TNBC and HER2+ BC patients. **The lowest concordance was seen with ER+ status independent of HER2 status.** The monitoring of CTC status before and after surgery suggests that the majority of CTCs are ER- and HER2- in HER2- primary BC. However, in HER2+ primary BC, CTC discordance of HER2 status is observed in nearly 50% cases. Thus, CTCs predominantly retain more aggressive properties.

CTC after NACT and surgery:
CTCs were observed in 100 % of patients after surgery at different time points. In comparison to CTC positivity before operation (after NACT), detection of MRD increased from72 % to 100 %. The presence of CTCs after tumor resection or after completion of adjuvant therapy (AT) could lead to disease relapse given their presence long time after tumor resection. In addition, CA-positive expression of the MRP1 gene was observed after AC- based therapy. In contrast, the expression of MRP7 (TAX- associated resistance) was not so frequent. Moreover, multi-resistant CTC (with expression of MDR1 or two or more MRP genes including ERCC1) detection supports a possible scenario of stem cells-like CTC selection after cytostatic therapy.

Although we didn't assess stem cell markers beyond CD24/ CD44 in cases of ER+ disease, we detected CTCs with epithelial characteristics more often before/during NACT as compared to CTC with stem cell CD24/CD44 properties after operation. Yet, aggressive CTC (DEFINE AGGRESSIVE) subtypes are more frequently CD24/CD44 positive from the beginning of the disease.

During the follow-up (1 - 2.5 years) we observed relapse in four patients, three of whom died. In patient no 1 (HER2-, BRCA+) liver metastases developed 1.5 year after diagnosis; in patient no 5 and 12 (HER2+) as well as no 19 (TNBC), CNS metastases were diagnosed between 1.5 and 2 years after diagnosis. Patient no 12 is still undergoing therapy, currently showing signs of remission. Patients no 1 and 12 were classified as non-responders during NACT (response rate 1), and patient no 5 as moderate responder (response rate to AC 3 but no response to TAX therapy). Patient no 19 responded very well (response rate 3) despite the unfavorable primary tumor staging (stage III).

In patient no 1, CTCs with either epithelial keratin expression or stem cell properties were both positive for MRP1 expression. Patient no 5 was treated with Tamoxifen (TMX) and Herceptin (H) adjuvantly; during this treatment period, CTCs were ESR and HER2+. During the TMX monotherapy and in time of generalisation CTCs were not sensitive to hormonal therapy (ESR-), we also detected expression of CD24/CD44 and of multidrug-resistance genes (MRP1, MRP5, ERCC1- MRP7- MRP1, MRP2 and MDR1 expression in different samples).

Patient no 12 underwent H monotherapy adjuvantly but CTCs were not HER2+ during this treatment period. HER2+ CTCs appeared again after the therapy of CNS metastases (not seen in suppl data), moreover we also detected CTC with expression of ESR1 and clusters of CTC. Although primary disease was ER- (only 10 % of cells were PR+), dynamic CTC changes were documented with characteristics likely reflecting the therapy they were treated with.

The 1y-OS of our group of patients was 95 % (1/20 died), 2y- OS is 78 % (3/14 died). Median OS (mOS) was 23.5 months.

Monitoring of the BC treatment during and after surgery:
We have described the comprehensive disease course of all the patients included into the study (**see Suppl. File 2 CTC presence and characterization during disease monitoring**). Among all cases, those who achieved pCR after NACT appeared to be most interesting.

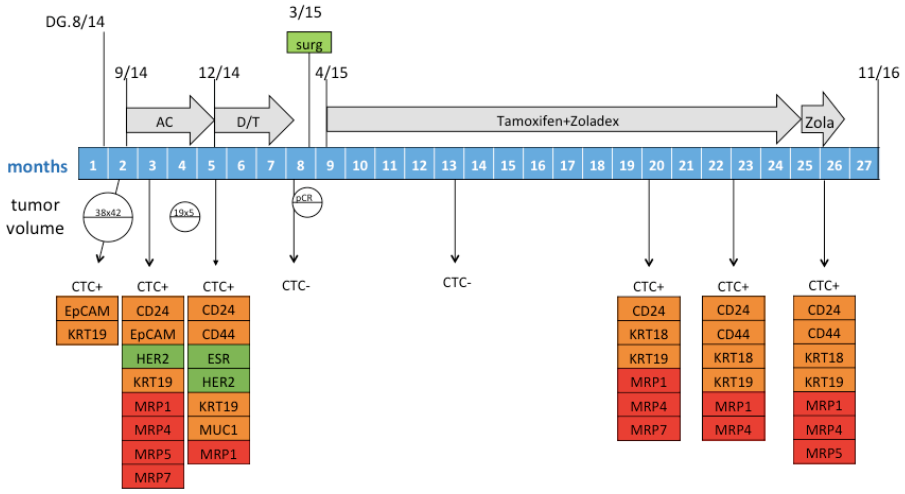
In picture 1 **(Figure 1)** the case of a young patient with LABC (medullary character) is described (patient no 7). She underwent standard AC- TAX therapy with a very good clinical effect (response rate 3), which was seen mainly after TAX therapy. Although CTCs before therapy displayed no markers of chemoresistance, expression during and after AC, one with multi-resistant and second with only AC-resistant CTC phenotype, were present. Interestingly, HER2+ and ESR1 expressing CTCs were present in either both or one of the later blood draws, respectively.

Effect of TAX was affirmed both at the level of pathologic examination and MRD with results of pCR and CTC negativity. As the primary tumor was ER and PR positive, patient continued and to date remains on adjuvant hormonal therapy. We observed that the first follow-up blood analysis (at about 5 months after surgery) was CTC negative, but the second at one year after surgery and a subsequent test at 15 months after surgery, detected MRD based on CTC presence. Our data suggest that CTCs were not sensitive to hormonal therapy given their positive expression of chemoresistance markers.

Although the patient remains in clinical remission, the persistence of chemoresistance gene expression in CTCs warrants close monitoring.

Figure 1: CTC- monitoring during and after NACT in BC patient achieving pCR.

Patient no. 7	Clinicopathological features	
Age: 31	Pre-surgery	Post-surgery
TNM	T2N1M0	pCR
Histology	ESR50, PGR10, HER2-, G3, Ki75	pCR



AC – doxorubicin+cyclophosphamid; D/T – docetaxel/paklitaxel; surg – surgery; Zola - Zoladex

Discussion

NACT is a standard approach in therapy of LABC and CTCs are believed to be associated with tumor aggressivness. Studies have reported CTC frequency in PBC to be lower in comparison to MBC, with positivity rates shown to be in the range of 22-23 % before and 10-17 % after neoadjuvant therapy (44-46) and 19-43 % in adjuvant setting (47-50). Lavrov et al. detected CTC in 38 % of patients with triple negative early disease and 42 % of triple negative locally advanced disease (51). By using multi-cytokeratin-specific antibodies to detect CTCs, Serrano et al. observed CTCs in 70 % of patients before and 54 % after NACT (52). Camara et al. observed an even higher frequency of 83 % among patients before NACT (53).

Similarly, in our study, CTCs were detected in 85 % of patients before and 72 % after NACT. The relatively high detection rate of CTC involvement among patients with advanced disease can be stratified by clinically: 95 % young premenopausal woman, 75 % HER2+ and 45% TNBC, 100 % of tumors with high Ki67 and 80 % with G3, and 75 % of patients having LABC with lymphatic node involvement.

Another possible reason for high CTC detection rates may be due to the method used to detect CTCs. Our two-step detection protocol combining size- based filtration with both cytomorphological and molecular characterization may identify more CTCs that go beyond the limited epithelial definition based only on EpCAM and Keratin expression (e.g. out of the 116 samples EpCAM elevated expression has been conformed in only 16 cases, expression of KRT 18/19 in 90 samples, HER2 in 34, MUC1 in 31 and MMG in 12 samples).

The presence of CTC before or after chemotherapy was associated with worse outcome in comparison to patients who were persistently CTC-negative in the SUCCES trial (54). The prognostic impact of CTC positivity before therapy and CTC- negativity after the therapy has not been fully evaluated yet for BC but in the work of Lorente, et al. (55) patients having a decline in CTC count reported after the therapy, have better survival outcomes in mestatic castration resistant prostate cancer.

In our study, most patients remained CTC positive during the NACT (88 % of CTC+ patient during NACT, 72% after NACT). It is expected that by NACT tumor cell mobilization occurs and the number of proliferating CTC released is decreases. On the other hand, CTC release from the primary tumor induced by NACT can lead to increasing number of CTC or their fragments detected in circulation. Additionally, it was shown that systemic response to treatment is independent from local response (54). The only significant correlation was found between the number of CTC and AC. Patients with a minimal reduction in CTC count during the AC regimen had a significantly higher probability to develop early distant metastases in comparison to the patients with good response (more than 10-fold reduction of CTC count). Sequential therapy with TAX was connected to tumor cell dissemination, although a reduction in primary tumor size was observed by ultrasound measurement.

The presence of CTCs was correlated with AC and TAX response, but as the method we use for CTC detection does not rely on CTC- counting we can't confirm correlation of tumor shrinkage with CTC number decrease. TNBC seemed to be more sensitive to NACT and the effect of AC was more significant than the effect of TAX if tumor volume reduction was compared.

Responders were found mainly in the group of patients with CTC expressing epithelial markers and CTC with a minimal CA- genes expression. CTC negativity during NACT was seen only in patients with relevant clinical effect (tumor volume reduction measured by ultrasound). In non- responders, two or more CA- genes were usually frequently overexpressed in CTC fraction. We observed high CTC detection rate during the whole therapy course, and the expression of CA- gene MRP1 correlated quite well with the effect of AC.

To answer the question of how long NACT should be administered in the case of persistent CTC presence, we would need to know if the presence of CTC has more prognostic or predictive value. pCR is usually defined as the absence of both invasive and non-invasive carcinoma in breast tissue and is most often seen in HER2+ or TNBC disease. If we assume aggressive tumor cells exist in these subtypes, we could hypothesize that pCR may be explained by the eradication of, sometimes-significant amounts, of highly proliferating tumor cells. In both aspects, the number and characteristics of tumor cells could be equally important. We assume that CTC characterization is more powerful then only the number of CTCs. If so, the high incidence of CTC in blood could be the result of the release of cells from the primary tumor but their presence would not indicate worsen patient outcome.

Several neoadjuvant clinical trials have evaluated the benefit of combining additional chemotherapy (e.g. capecitabine) or targeted therapy (e.g. bevacizumab) with a standard AC or TAX-based chemotherapy regimen. Although outcomes of combination regimens in HER2 negative tumors are unconvincing (12-14), targeted anti-HER2 therapy added to standard NACT improved significantly the outcome for HER2 positive patients (15-17). In these studies, higher incidence of pCR in ER-/HER2+ (HER2 enriched) subtype in comparison to ER+/HER2+ BC was also shown. For patients with TNBC participating in the CALGB and GeparSixto trials, significant improvements in pCR rates in the breast and axilla were demonstrated when carboplatin was added to more complex neoadjuvant AC- and TAX- based regimens (18-19). In the GeparTrio trial (20), the benefit of switching NACT in non-responding patients was evaluated. Patients lacking clinical response to neoadjuvant TAC (docetaxel, doxorubicin, cyclophosphamide) were randomized to four additional cycles of the same regimen or four cycles of vinorelbine and capecitabine. Although a benefit for ER+ patients was outlined (DFS, OS), future studies will be needed to specify treatment according the molecular profile of the disease. Thus, the need for new biomarkers reflects the shortcomings of existing therapy options. Although AC- and TAX- regimens in NACT are considered standard clinical practice, recurrence due to clonal expansion and/or resistance of residual tumor cells due to treatment selection pressures occur.

We also know from clinical trials that NACT has the same benefit for the prognosis of patients as adjuvant therapy. So the higher volume of MRD in patients treated with NACT should have no effect on the risk of disease relapse. On the other hand, the prognostic power of CTCs is based on their rate in blood. However, to find the connection between primary disease and CTC frequency is not easy. pCR was correlated with better outcome in some studies (56) but not in others (57-58). The use of pCR as a surrogate end point for survival remains unclear. In TNBC, association between genomic signature, pCR, and survival after chemotherapy was found only in patients with basal-like subtype (59). Another biomarker with the same importance for luminal tumors doesn't exist.

In our study, pCR was achieved in 4/9 TNBC; one could predict uniform biological subtypes of these 4 tumors. Despite pCR in case of these TNBC patients, all of their blood samples were evaluated as CTC positive. The validation of prognostic significance of pCR is needed before we can say that the eradication of CTCs could be the new goal of treatment instead of pCR.

CTC can survive as non-proliferating, dormant“cells, and associated with higher resistance to chemotherapy (60). After the completion of NACT, we observed few cases with persistent multi-resistant CTC refractory to therapy. Taking into account the higher significance of CTC characteristics, the question of how to treat resistant disease arises. One option is CTC navigate therapy, while another other option could be removal of the primary tumor rather than gambling with systemic treatment.

Negative results of SWOG 0500 study could be based on the lack of CTC characterization. We noticed a significant discordance of ER and HER2 status in comparison to primary disease and MRD. CTC were more frequently ER- in HER2 negative primary BC. HER2+ primary tumors had HER2+ CTC mainly during NACT, after which they lost sensitivity to antiHER2 therapy, very often during adjuvant trastuzumab therapy. HER2 status changed very dynamically and CTCs retained aggressive properties.

Treatment for 3 of 20 patients was generalized during adjuvant therapy. Adjuvant treatment strategy determination is the most difficult, and thus we treat patients blindly. We assume that targeting therapy based on CTC characterization could better the prognosis of patients.

The comparison of responders and non-responders (patients developing disease relapse during adjuvant therapy) at the molecular level could complete our knowledge of CTC significance in post-operative observation of patients. Prolongation of adjuvant hormonal therapy was shown to have clinical significance, confirming that the persistency of MRD long after the operation exist and require longer treatment.

We observed some patients for more than two years; dynamic changes of ER and HER2 status were described above. We assume that CTC monitoring in time could predict the risk of disease relapse earlier and more accurately than standard tumor markers and imaging tests. Both the number and CTC characteristics can thus play a critical role.

CTC guided therapy is not clinically indicated today and only treatment response monitoring via CTCs is possible. Promising designs of new studies focused on the predictive power of CTCs and study of large numbers of patients are a main prerequisite for defining the predictive value of CTCs (61-66). That CTCs could be used as a tissue source for testing sensitivity to different drugs in future, is highly attractive (67).

Conclusion

The connection between CTCs and of tumor histopathology with therapy sensitivity and/or pCR was discussed. We believe that monitoring of CTCs by molecular characterization, including TA- genes and CA- genes, could predict therapy outcome. We observed the connection of non-responding tumors with the presence of two or more genes associated with chemoresistance.

Ultrasound monitoring alone is perhaps insufficient where the combination of ultrasound observation and CTC monitoring could prove to be beneficial and enable earlier detection of disease relapse. Thus, in cases of non-responding tumors based volume and the presence of chemoresistant CTCc, it may be more effective to reconsider the length of NACT and possibly to switch from AC to TAX or to stop NACT and proceed to surgery. We don't have data on prolongation of NACT beyond the standard duration. Results from adjuvant trials with metronomic dosing of chemotherapy or adjuvant systemic treatment over the NACT may clarify the connection between persistent MRD and patients outcome. We believe that CTC-navigated therapies may improve patient outcome and until the end of prospective trials targeting predictive power of CTCs, CTC utility and information is limited to ranges of known (palliative) indications or as a support of adjuvant therapy choice (i.e. indication of adjuvant chemotherapy in TNBC to treat residual disease after NACT).

References

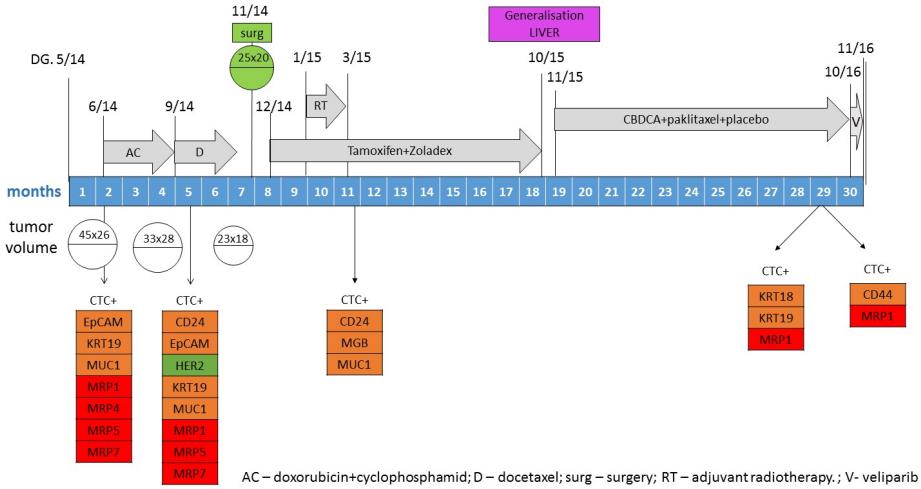
1.Rastogi P, Anderson SJ, Bear HD, et al. Preoperative chemotherapy: updates of National Surgical Adjuvant Breast and Bowel Project Protocols B-18 and B-27. J Clin Oncol. 2008;26:778–785
2.Van der Hage JA, van de Velde CJ, Julien JP, Tubiana-Hulin M, Vandervelden C, Duchateau L. Preoperative chemotherapy in primary operable breast cancer: results from the European 3. Organization for Research and Treatment of Cancer trial 10902. J Clin Oncol. 2001;19:4224–4237.
Mauri D, Pavlidis N, Ioannidis JP. Neoadjuvant versus adjuvant systemic treatment in breast cancer: a meta-analysis. J Natl Cancer Inst. 2005;97:188–194
4. Colleoni M, Bonetti M, Coates AS, et al. Early start of adjuvant chemotherapy may improve treatment outcome for premenopausal breast cancer patients with tumors not expressing estrogen receptors: the International Breast Cancer Study Group. J Clin Oncol. 2000;18:584–590.
5. Early Breast Cancer Trialists' Collaborative Group (EBCTCG) Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet. 2005;365:1687–1717
6. Bear HD, Anderson S, Smith RE, et al. Sequential preoperative or postoperative docetaxel added to preoperative doxorubicin plus cyclophosphamide for operable breast cancer: National Surgical Adjuvant Breast and Bowel Project Protocol B-27. J CIin Oncol. 2006;24:2019–2027
7. Sorlie T, Tibshirani R, Parker J, et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. PNAS 2003; 100(14):8418-8423
8. Cortazar P, Zhang L, Untch M, et al. Pathological complete response and long-term clinical benefit in

breast cancer: the CTNeoBC pooled analysis. Lancet. 2014;384:164–172
9. Von Minckwitz G, Untch M, Blohmer JU, et al. Definition and impact of pathologic complete response on prognosis after neoadjuvant chemotherapy in various intrinsic breast cancer subtypes. J Clin Oncol. 2012;30:1796–1804.
10. Symmans WF, Peintinger F, Hatzis C, et al. Measurement of residual breast cancer burden to predict survival after neoadjuvant chemotherapy. J Clin Oncol. 2007;25:4414–4422.
11. Von Minckwitz G, Schmitt WD, Loibl S, et al. Ki67 measured after neoadjuvant chemotherapy for primary breast cancer. Clin Cancer Res. 2013;19:4521–4531
12. Von Minckwitz G, Rezai M, Loibl S, et al. Capecitabine in addition to anthracycline- and taxane-based neoadjuvant treatment in patients with primary breast cancer: phase III 12. GeparQuattro study. J Clin Oncol. 2010;28:2015–2023.
13. Von Minckwitz G, Eidtmann H, Rezai M, et al. Neoadjuvant chemotherapy and bevacizumab for HER2-negative breast cancer. N Engl J Med. 2012;366:299–309
14. Miller K, O'Neill AM, Dang CT, et al. Bevacizumab (BV) in the adjuvant treatment of HER2-negative breast cancer: final results from Eastern Cooperative Oncology Group E5103. J Clin Oncol. 2014;32(Suppl) abstract 500.
15. Baselga J, Bradbury I, Eidtmann H, et al. Lapatinib with trastuzumab for HER2-positive early breast cancer (NeoALTTO): a randomised, open-label, multicentre, phase 3 trial. Lancet. 2012;379:633–640
16. Gianni L, Pienkowski T, Im YH, et al. Efficacy and safety of neoadjuvant pertuzumab and trastuzumab in women with locally advanced, inflammatory, or early HER2-positive breast cancer (NeoSphere): a randomised multicentre, open-label, phase 2 trial. Lancet Oncol. 2012;13:25–32
17. Schneeweiss A, Chia S, Hickish T, et al. Pertuzumab plus trastuzumab in combination with standard neoadjuvant anthracycline-containing and anthracycline-free chemotherapy regimens in patients with HER2-positive early breast cancer: a randomized phase II cardiac safety study (TRYPHAENA) Ann OncoI. 2013;24:2278–2284.
18. Sikov WM, Berry DA, Perou CM, et al. Impact of the addition of carboplatin and/or bevacizumab to neoadjuvant once-per-week paclitaxel followed by dose-dense doxorubicin and cyclophosphamide on pathologic complete response rates in stage II to III triple-negative breast cancer: CALGB 40603 (Alliance) J Clin Oncol. 2015;33(1):13–21
19. Von Minckwitz G, Schneeweiss A, Loibl S, et al. Neoadjuvant carboplatin in patients with triple-negative and HER2-positive early breast cancer (GeparSixto; GBG 66): a randomised phase 2 trial. Lancet Oncol. 2014;15:747–756
20. Von Minckwitz G, Kummel S, Vogel P, et al. Intensified neoadjuvant chemotherapy in early-responding breast cancer: phase III randomized GeparTrio study. J Natl Cancer Inst. 2008;100:552–562.
21. Cristofanilli M, Budd G, Ellis M, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. The New Engl. J of Med. 2004; 351(8): 781-791
22. Zhang L, Riethdorf S, Wu G, et al. Meta-analysis of the prognostic value of circulating tumor cells in breast cancer. Clin. Cancer Res. 2012; 18:5701–5710
23. Bidard FC, Peeters D, Fehm T, et al. Clinical validity of circulating tumour cells in patients with metastatic breast cancer: a pooled analysis of European studies. Lancet Oncol. 2014; 15(4):406-14
24. Smerage JB, Barlow WE, Hortobagyi GN, et al. Circulating Tumor Cells and Response to Chemotherapy in Metastatic Breast Cancer: SWOG S0500. JCO 2014
25. Liu Y, Liu Q, Wang T, et al. Circulating tumor cells in HER2-positive metastatic breast cancer patients: a valuable prognostic and predictive biomarker. BMC Cancer. 2013; 13: 202.
26. Bidard FC, Fehm T, Ignatiadis M, et al. Clinical application of circulating tumor cells in breast cancer: overview of the current interventional trials. Cancer Metastasis Rev. 2013; 32: 179-188.
27. Wu S, Liu S, Liu Z. et al. Classification of Circulating Tumor Cells by Epithelial-Mesenchymal Transition Markers. PLoS One 2015; 10(4); e0123976.
28. Wallwiener M, Hartkopf AD, Riethdorf S,et al. The impact of HER2 phenotype of circulating tumor cells in metastatic breast cancer: a retrospective study in 107 patients. BMC Cancer 2015; 15:403.

29. Mego M, Giordano A, De Giorgi U, et al. Circulating tumor cells in newly diagnosed inflammatory breast cancer. Breast Cancer Res. 2015;17(1):2.
30. Yusa A, Toneri M, Masuda T, et al. Development of a New Rapid Isolation Device for Circulating Tumor Cells (CTCs) Using Palladium Filter and Its Application for Genetic Analysis. PLoS One 2014; 9(2): e88821.
31. Peeters DJ, van Dam PJ, Van den Eynden GG, et al. Detection and prognostic significance of circulating tumour cells in patients with metastatic breast cancer according to immunohistochemical subtypes. Br J cancer 2014; 110(2): 375-83
32. Lee SK, Kim GS, Wu Y, et al. Nanowire Substrate-based Laser Scanning Cytometry for Quantification of Circulating Tumor Cells. Nano Lett. 20112; 12(6):2697–2704
33. Zhang JL, Yao Q, Chen Y Wang JH, et al. Effects of Herceptin on circulating tumor cells in HER2 positive early breast cancer. Genet. Mol. Res. 2015;14(1):2099-103
34. Bredemeier M, Edimiris P, Tewes M, et al. Establishment of a multimarker qPCR panel for the molecular characterization of circulating tumor cells in blood samples of metastatic breast cancer patients during the course of palliative treatment. Oncotarget 2016; 7(27):41677-41690
35. Lasa A, Garcia A, Alonso C, et al. Molecular detection of peripheral blood breast cancer mRNA transcripts as a surrogate biomarker for circulating tumor cells. PLoS One 2013; 8(9): e74079
36. Molloy TJ, Devriese LA, Helgason HH, et al. A multimarker QPCR-based platform for the detection of circulating tumour cells in patients with early-stage breast cancer. Br J Cancer. 2011 Jun 7;104(12):1913-9
37. Bitisik O, Saip P, Saglam S, et al. Mammaglobin and maspin transcripts in blood may reflect disease progression and the effect of therapy in breast cancer. Genet Mol Res 2009; 9(1): 97-106
38. Fehm T, Hoffmann O, Aktas B, et al. Detection and characterization of circulating tumor cells in blood of primary breast cancer patients by RT-PCR and comparison to status of bone marrow disseminated cells. Breast Cancer Res. 2009;11(4):R59
39. Banys M, Krawczyk N, Becker S, et al The influence of removal of primary tumor on incidence and phenotype of circulating tumor cells in primary breast cancer. Breast Cancer Res Treat 2012;132(1): 121–9
40. Fehm T, Krawczyk N, Solomayer EF, et al. ERalpha-status of disseminated tumour cells in bone marrow of primary breast cancer patients. Breast Cancer Res 2008; 10(5): R76
41. Aktas B, Müller V, Tewes M, et al. Comparison of estrogen and progesterone receptor status of circulating tumor cells and the primary tumor in metastatic breast cancer patients. Gynecol Oncol. 2011;122(2):356-60
42. Krawczyk N, Banys M, Neubauer H, et al. HER2 status on persistent disseminated tumor cells after adjuvant therapy may differ from initial HER2 status on primary tumor. Anticancer Res 2009;29(10): 4019-24
43. Livak KJ et Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 200;25(4):402-8
44. Pierga JY, Bidard FC, Mathiot C, et al. Circulating tumor cell detection predicts early metastatic relapse after neoadjuvant chemotherapy in large operable and locally advanced breast cancer in a phase II randomized trial. Clin Cancer Res. 2008;14:7004–7010
45. Bidard FC, Delaloge S, Giacchetti S, et al. Long term independent prognostic impact of circulating tumor cells detected before neoadjuvant chemotherapy in non-metastatic breast cancer: 70 months analysis of the REMAGUS02 study
46. Riethdorf S, Müller V, Zhang L, et al. Detection and HER2 expression of circulating tumor cells: prospective monitoring in breast cancer patients treated in the neoadjuvant GeparQuattro trial. Clin Cancer Res 2010;16:2634-45.
47. Xenidis N, Perraki M, Apostolaki S, et al. Differential effect of adjuvant taxane-based and taxane-free chemotherapy regimens on the CK-19 mRNA-positive circulating tumour cells in patients with early breast cancer. Br J Cancer. 2013;108:549–556
48. Franken B, de Groot MR, Mastboom WJ, et al. Circulating tumor cells, disease recurrence and survival in newly diagnosed breast cancer. Breast Cancer Res. 2012;14:R133
49. Pachmann K, Camara O, Kavallaris A, et al. Monitoring the response of circulating epithelial tumor

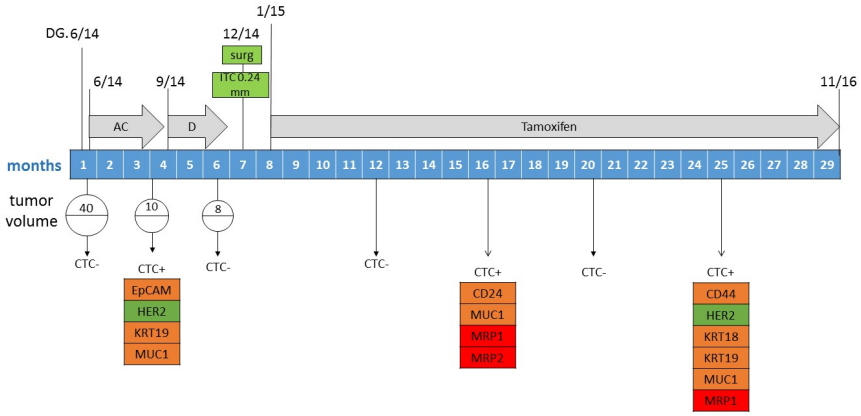
cells to adjuvant chemotherapy in breast cancer allows detection of patients at risk of early relapse. J Clin Oncol. 2008;26:1208–1215.
50. Xenidis N, Perraki M, Kafousi M, et al. Predictive and prognostic value of peripheral blood cytokeratin 19 mRNA positive cells detected by real time polymerase chain reaction in node negative breast cancer patients. J Clin Oncol. 2006;24:3756–3762
51. Lavrov AV1, Zubtsova ZhI, Zubtsov DA, et al.: Analysis of Circulating Tumor Cells in Patients with Triple Negative Breast Cancer during Preoperative Chemotherapy. Bull Exp Biol Med. 2014 May;157(1):159-61)
52. Serrano MJ, Rovira PS, Martínez-Zubiaurre I, et al. Dynamics of circulating tumor cells in early breast cancer under neoadjuvant therapy. Exp Ther Med 2012;4:43-8
53. Camara O, Rengsberger M, Egbe A, et al.The relevance of circulating epithelial tumor cells (CETC) for therapy monitoring during neoadjuvant (primary systemic) chemotherapy in breast cancer. Ann Oncol (2007) 18 (9): 1484-1492
54. Rack BK, Schindlbeck C, Andergassen U, et al. Use of CTCs in peripheral blood of breast cancer patients before and after adjuvant chemotherapy to predict risk of relapse: the SUCCESS trial. J Clin Oncol 2010; 28: Abs. 1003
55. Lorente D, Olmos D, Mateo J, et al. Decline in Circulating Tumor Cell Count and Treatment Outcome in Advanced Prostate Cancer. Eur Urol. 2016; 70(6):985-992
56. Bear HD, Anderson S, Smith RE,et al. Sequential preoperative or postoperative docetaxel added to preoperative doxorubicin plus cyclophosphamide for operable breast cancer: National Surgical Adjuvant Breast and Bowel Project protocol B-27. J Clin Oncol 2006;24:2019-2027
57. Evans TR, Yellowlees A, Forster E, et al. Phase III randomized trial of doxorubicin and docetaxel versus doxorubicin and cyclophosphamide as primary medical therapy in women with breast cancer: an Anglo-Celtic Cooperative Oncology Group study. J Clin Oncol 2005*;23:*2988-2995
58. Von Minckwitz G, Raab G, Caputo A, et al.. Doxorubicin with cyclophosphamide followed by docetaxel every 21 days compared with doxorubicin and docetaxel every 14 days as preoperative treatment in operable breast cancer: The GEPARDUO study of the German Breast Group. J Clin Oncol 2005*;23:*2676-2685
59. Prat A, Lluch A, Albanell J, et al. Predicting response and survival in chemotherapy-treated triple-negative breast cancer. British Journal of Cancer. 2014;111(8):1532–1541
60. Müller V, Stahmann N, Riethdorf S, et al. Circulating tumor cells in breast cancer: correlation to bone marrow micrometastases, heterogeneous response to systemic therapy and low proliferative activity. Clin Cancer Res. 2005 May 15;11(10):3678-85
61. Bidard FC, Fehm T, Ignatiadis M, et al. Clinical application of circulating tumor cells in breast cancer: overview of the current interventional trials. Cancer Metastasis Rev. 2013; 32: 179-188
62. Bidard FC, de Rycke Y, Asselain B, et al. CirCe T-DM1 phase II trial: Assessing the relevance of HER2-amplified circulating tumor cells as a tool to select HER2-negative metastatic breast cancer for treatment with T-DM1 Cancer Res 2013; 73(24 Suppl): Abs OT1-1-10. NCT01349842
63. Fehm T, Müller V, Aktas B, et al. HER2 status of circulating tumor cells in patients with metastatic breast cancer: a prospective, multicenter trial. Breast Cancer Res Treat. 2010 Nov;124(2):403-12
64. SABCS.org [homepage on the Internet] The San Antonio Breast Cancer Symposium. Dec, 2013. [Accessed February 21, 2014]. Available from: http://www.sabcs.org.
65. Bidard F, Baffert S, Hajage D, et al. Circulating tumor cells to guide the choice between chemotherapy and hormone therapy as first line treatment for hormone receptors positive metastatic breast cancer patients: the STIC CTC METABREAST trial. Cancer Research 2012; 72(24 Suppl): Abs OT3-4-06. NCT01710605
66. Characterization of Circulating Tumor Cells (CTC) From Patients With Metastatic Breast Cancer Using the CTC-Endocrine Therapy Index (COMETI P2). ClinicalTrials.gov Identifier:
NCT01701050
67. Yu M, Bardia A, Aceto N, et al.Cancer therapy. Ex vivo culture of circulating breast tumor cells for individualized testing of drug susceptibility. Science 2014; 11;345(6193):216-20

Patient no. 1	Clinicopathological features	
Age: 37	Pre-surgery	Post-surgery
TNM	T2N1M0	T2N2
Histology	ESR90, PGR60, HER2-, G3, Ki40	ESR70, PGR10, HER2-, G3, Ki10



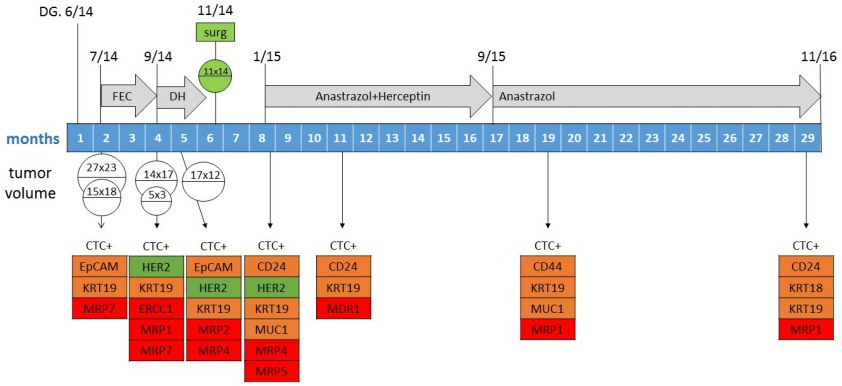
AC – doxorubicin+cyclophosphamid; D – docetaxel; surg – surgery; RT – adjuvant radiotherapy. ; V- veliparib

Patient no. 2	Clinicopathological features	
Age: 43	Pre-surgery	Post-surgery
TNM	T2N1M0	T1aN0
Histology	ESR100, PGR50-60, HER2-, G1, Ki50	ESR20, PGR0, HER2-, G?, Ki30



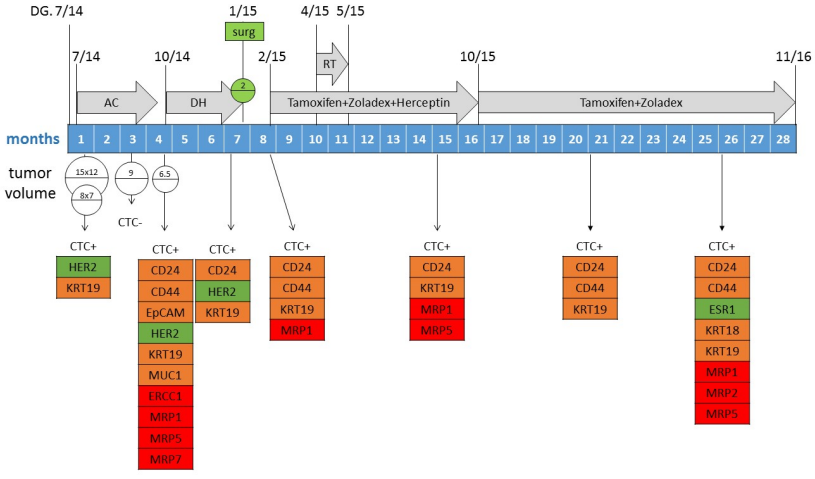
AC – doxorubicin+cyclophosphamid; D – docetaxel; surg – surgery; ITC – isolated tumor cells

Patient no. 3	Clinicopathological features	
Age: 71	Pre-surgery	Post-surgery
TNM	T2N1M0	T1cN0
Histology	ESR100, PGR60, HER2+, G3, Ki40	ESR100, PGR100, HER2+, G3, Ki3



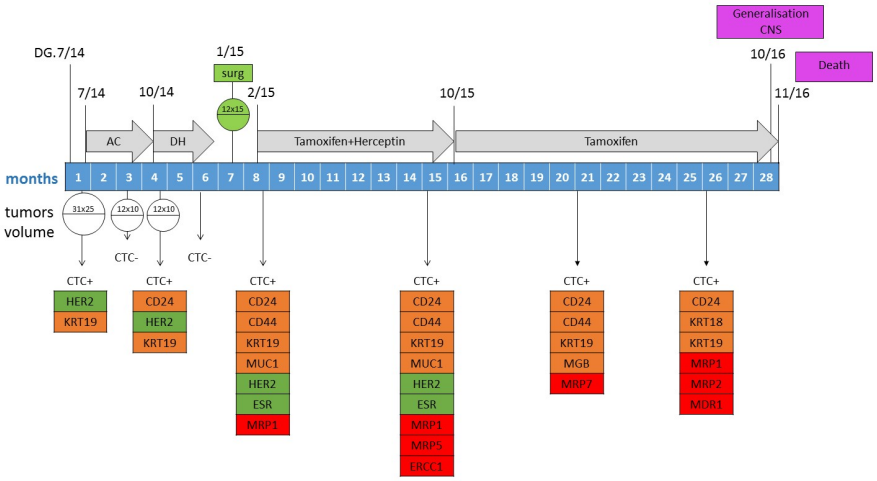
FEC –fluorouracil+epirubicin+cyclophosphamid; DH – docetaxel+herceptin; surg – surgery

Patient no. 4	Clinicopathological features	
Age: 33	Pre-surgery	Post-surgery
TNM	sin. T1N1, dx. T1N0	sin. T1bN0, dx. pCR
Histology	ESR50, PGR5, HER2+, G3, Ki40	ESR75, PGR5, HER2-, G2, Ki1



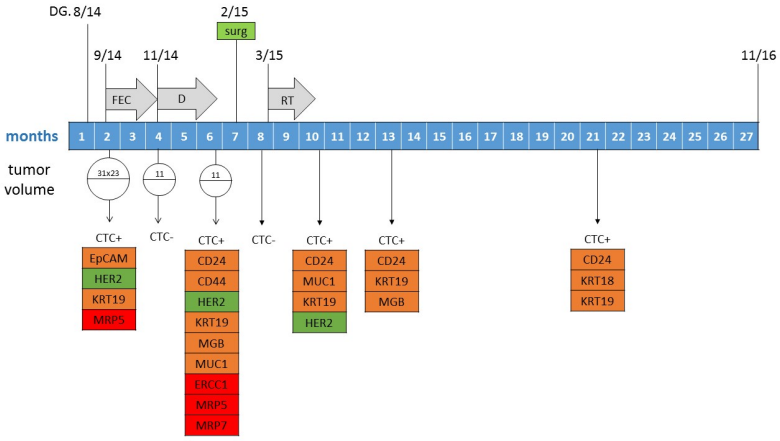
AC – doxorubicin+cyclophosphamid; DH – docetaxel+herceptin; surg – surgery; RT – radiotherapy (sin.)

Patient no. 5	Clinicopathological features	
Age: 39	Pre-surgery	Post-surgery
TNM	T2N1M0	T1cN1mi
Histology	ESR80, PGR90, HER2+, G2, Ki20	ESR70, PGR30, HER2+, G2, Ki10



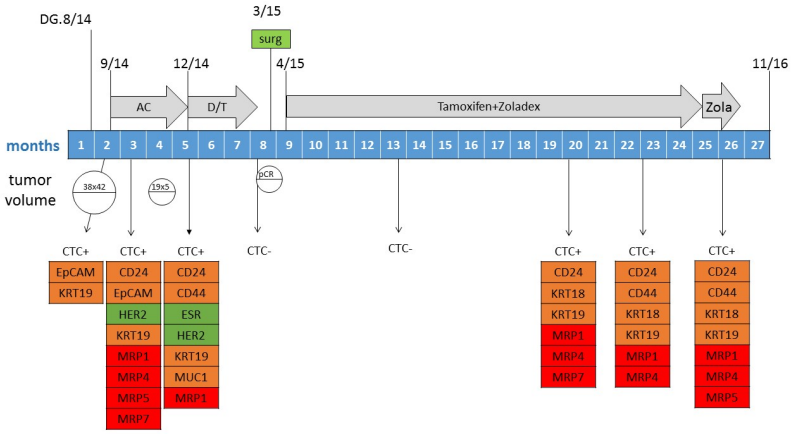
AC – doxorubicin+cyclophosphamid; DH – docetaxel+herceptin; surg – surgery

Patient no. 6	Clinicopathological features	
Age: 38	Pre-surgery	Post-surgery
TNM	T2N0M0	pCR
Histology	TNBC, G2, Ki90	pCR



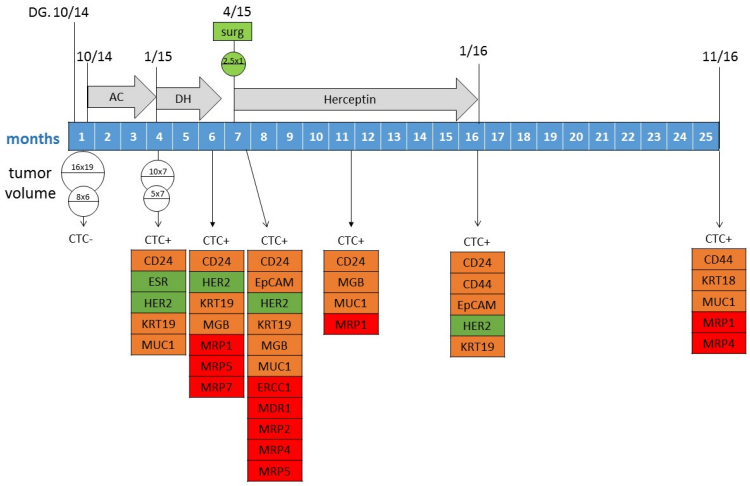
FEC – fluorouracil+epirubicin+cyclophosphamid; D – docetaxel; surg – surgery; RT – radiotherapy

Patient no. 7	Clinicopathological features	
Age: 31	Pre-surgery	Post-surgery
TNM	T2N1M0	pCR
Histology	ESR50, PGR10, HER2-, G3, Ki75	pCR



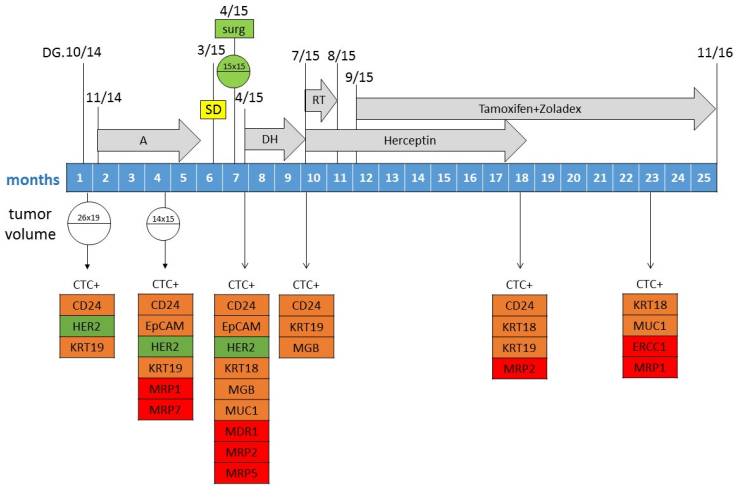
AC – doxorubicin+cyclophosphamid; D/T – docetaxel/paklitaxel; surg – surgery; Zola - Zoladex

Patient no. 8	Clinicopathological features	
Age: 36	Pre-surgery	Post-surgery
TNM	T1cN1M0	T1aN0
Histology	ESR0, PGR5, HER2+, G3, Ki55	ESR0, PGR0, HER2+, G3, Ki5



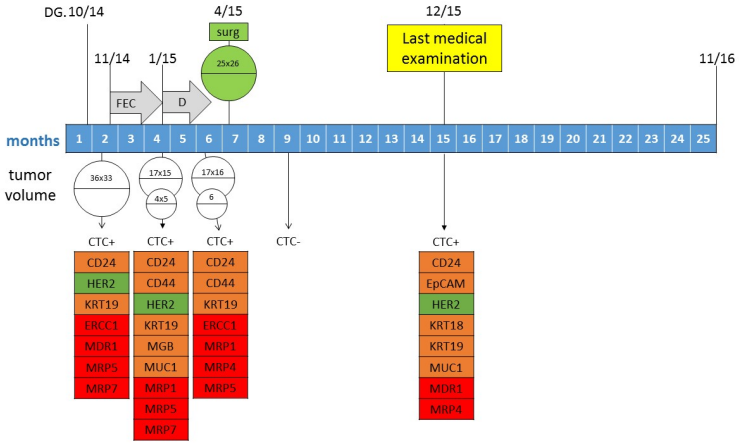
AC – doxorubicin+cyclophosphamid; DH – docetaxel+herceptin; surg – surgery

Patient no. 9	Clinicopathological features	
Age: 31	Pre-surgery	Post-surgery
TNM	T2N1M0	T1cN0
Histology	ESR50, PGR70, HER2+, G3, Ki70	ESR0, PGR0, HER2+, G1, Ki60



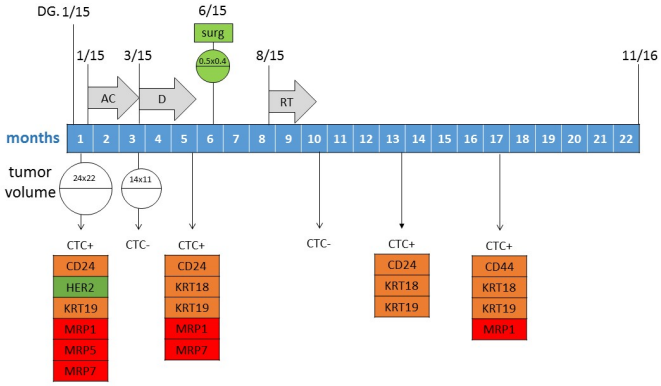
A – doxorubicin; SD – spontaneous delivery; DH – docetaxel+herceptin; surg – surgery; RT – radiotherapy

Patient no. 10	Clinicopathological features	
Age: 36	Pre-surgery	Post-surgery
TNM	T2N0M0	T2N0
Histology	ESR0, PGR2, HER2- (TNBC), G3, Ki75	ESR0, PGR2, HER2- (TNBC), G3, Ki95



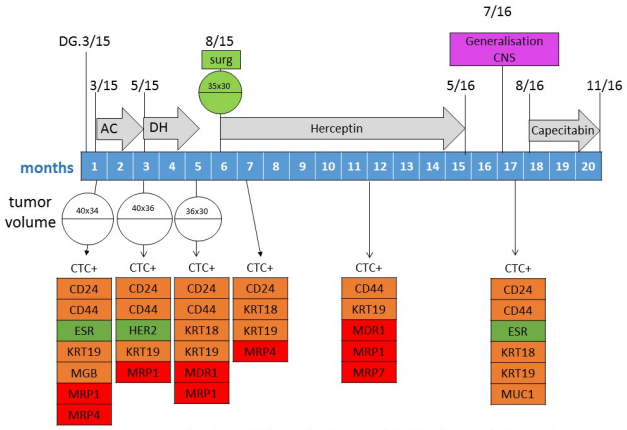
FEC – fluorouracil+epirubicin+cyclophosphamid; D – docetaxel; surg – surgery

Patient no. 11	Clinicopathological features	
Age: 31	Pre-surgery	Post-surgery
TNM	T2N1M0	T1aN0M0
Histology	ESR5, PGR7, HER2- (TNBC), G3, Ki80	TNBC, G3, Ki20



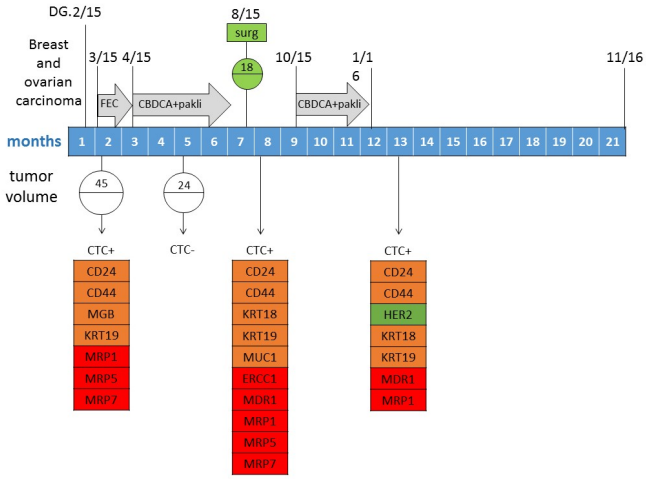
AC – doxorubicin+cyclophosphamid; D – docetaxel; surg – surgery; RT- radiotherapy

Patient no. 12	Clinicopathological features	
Age: 33	Pre-surgery	Post-surgery
TNM	T2N1	T2N1a
Histology	ESR0, PGR10, HER2+, G3, Ki50	ESR0, PGR0, HER2+, G3, Ki20



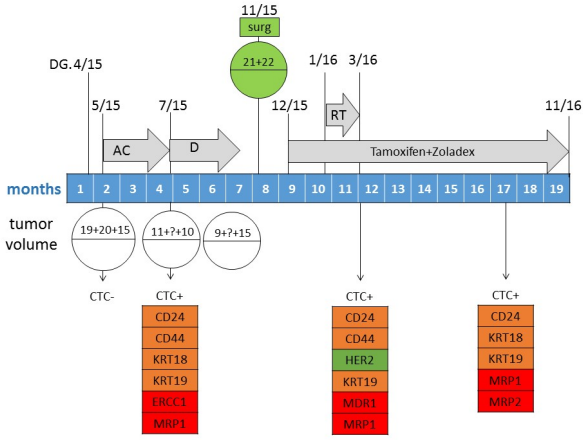
AC – doxorubicin+cyclophosphamid; DH – docetaxel+herceptin; surg – surgery

Patient no. 13	Clinicopathological features	
Age: 41	Pre-surgery	Post-surgery
TNM	Breast T2N1M0, ovary IIIC	Breast T1cN0, Ovary T3bN1
Histology	TNBC, G3, Ki100	ESR5, PGR1, HER2- (TNBC), G3, Ki50



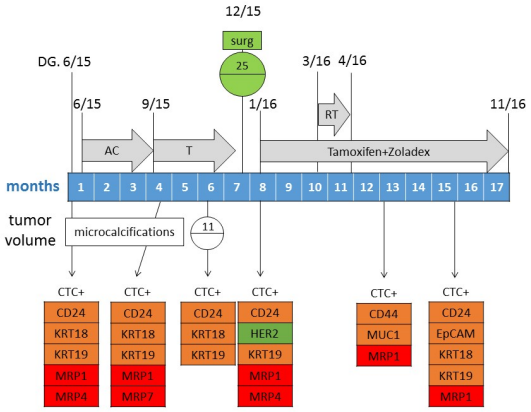
FEC – fluorouracil+epirubicin+cyclophosphamid ; CBDCA - carboplatin; pakli – paklitaxel; surg – surgery

Patient no. 14	Clinicopathological features	
Age: 41	Pre-surgery	Post-surgery
TNM	T1cN1	T2N3
Histology	ESR80-90, PGR20-25, HER2-, G3, Ki50	ESR100,PGR25, HER2-, G3, Ki50



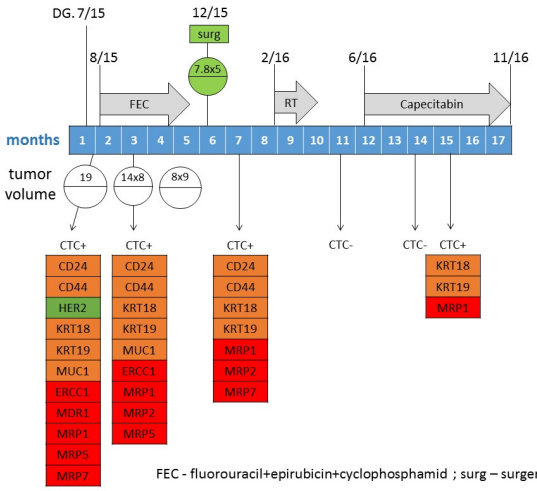
AC – doxorubicin+cyclophosphamid; D – docetaxel; surg – surgery; RT – radiotherapy

Patient no. 15	Clinicopathological features	
Age: 39	Pre-surgery	Post-surgery
TNM	T1N1	T2N1c
Histology	ESR90, PGR5, AR30, HER2-, G2, Ki30	ESR100, PGR10, HER2-, G3, Ki0



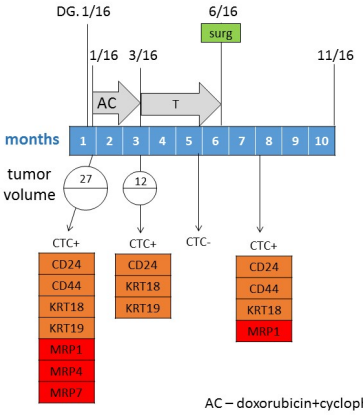
AC – doxorubicin+cyclophosphamid; T- paklitaxel; surg – surgery; RT – radiotherapy

Patient no. 16	Clinicopathological features	
Age: 44	Pre-surgery	Post-surgery
TNM	T1cN0	T1bN0
Histology	TNBC, G3, Ki40	TNBC (ER10), G1, Ki5



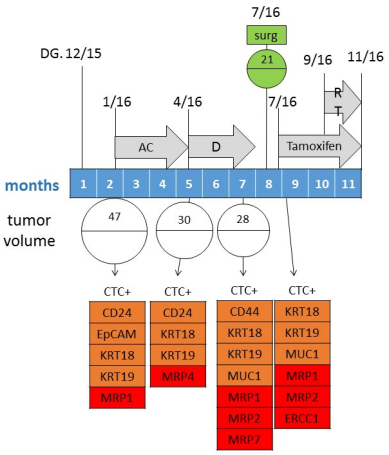
FEC - fluorouracil+epirubicin+cyclophosphamid ; surg – surgery; RT – radiotherapy

Patient no. 17	Clinicopathological features	
Age: 44	Pre-surgery	Post-surgery
TNM	T2N1	pCR
Histology	TNBC, G3, Ki70	pCR



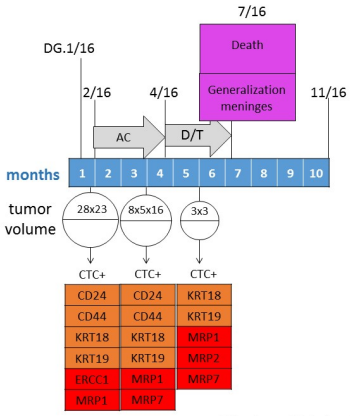
AC – doxorubicin+cyclophosphamid; T- paklitaxel; surg – surgery

Patient no. 18	Clinicopathological features	
Age: 52	Pre-surgery	Post-surgery
TNM	T2N1	T2N1
Histology	ESR100, PGR50-75, HER2-, G3, Ki50	ESR100, PGR1, HER2-, G2, Ki5



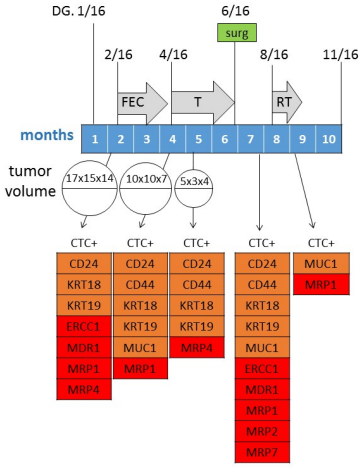
AC – doxorubicin+cyclophosphamid; D - docetaxel; surg – surgery, RT – radiotherapy

Patient no. 19	Clinicopathological features	
Age: 40	Pre-surgery	Post-surgery
TNM	T2N3M0	-
Histology	TNBC, G3, Ki60	-



AC – doxorubicin+cyclophosphamid; D/T – docetaxel/paklitaxel;

Patient no. 20	Clinicopathological features	
Age: 40	Pre-surgery	Post-surgery
TNM	T1cN0M0	pCR
Histology	TNBC, G3, Ki95	pCR



FEC – fluorouracil+epirubicin+cyclophosphamid; T –paklitaxel; surg – surgery; RT – radiotherapy

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Original Article

Circulating tumor cells: what we know, what do we want to know about them and are they ready to be used in clinics?

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Abstract: Circulating tumor cells (CTC) present in peripheral blood are assigned precursors of advanced tumor disease. Simplicity of blood withdrawal procedure adds practicallyan unlimited possibility of the CTC-monitoring and the advantages of the repeated biopsies over time. CTC got prognostic, predictive and diagnostic status with the technologic advance. Although the clinical utility of CTC has reached the high evidence, the significance of CTC testing was presented in the treatment strategy mostly with palliative intention. We report on the experienceswith the CTC-testing in the CLIA -like laboratory working with the size-based CTCseparation and *in vitro* culture. The data is presented in the form of case reports in patients with breast (BC), colorectal (CRC), prostate (PC) and lung cancer (NSCLC) to support the clinical utility of CTC during the neoadjuvant, adjuvant and palliative treatment. The pre-sented findings supportthe evidence for liquid biopsy clinical implementation and enhance the ability of malignant disease monitoring and the treatment efficacy prediction.

Keywords: Circulating tumor cells, breast cancer, colorectal cancer, non-small-cell lung cancer, prostate cancer, chemoresistance

Introduction

The count of CTC in blood of oncological patients is very low [1]. Detection of CTC is also limited by their heterogeneity. Finally, the capacity of various malignancies to release CTC into the peripheral circulation is different depending on the stage of the disease and also on the type of malignancy [2-12]. The chance of CTC positivity isin general notably higher in metastatic than in the primary disease, e.g. 2-55% in primary BC vs. 40-80% in metastatic BC [13].

Technologies try to overcome the rare occurrence of CTC by using the enrichment step to separate CTC from blood cells. The negative selection is based on the elimination of leukocyte fraction (e.g. by using anti-CD45 antibodies) from blood. The positive selection utilizes surface features of CTCor their physical properties such as cells size or density. The most commonly used methods are based on the immunomagnetic selection of CTC with epithelial fea-tures. But these methods are impoverished to detect CTC with the lack of epithelial character-istics, e.g. cancer cells whose phenotype has been altered by the process of epithelial -mes-enchymal transition (EMT) [14] or which have the character of stem cells [15]. Combinations of different methods and new approaches [16], in particular microfluidic systems, focus on increasing the sensitivity and specificity of CTC selection and detection.

The standardization of technologies at all levels of CTC identification and results interpretation based on different approaches is still a prob-

Table 1. Clinical indications to CTC examination

Prediction of disease response to neoadjuvant chemotherapy Indication of "additional" adjuvant therapy in residual disease Observation after adjuvant therapy
CTC monitoring after adjuvant therapy and during metastatic disease CTC-testing after resection of metastases and early prediction of disease relapse Assessment of KRAS mutation status from CTC
Strategy of using CTC for the palliative treatment guidance Typing of tumors with unknown primary site or duplicate tumors

lem. The rare occurrence of CTC in non-metastatic disease is the reason why the threshold for the CTC positivity is different in the primary disease in comparison to the metastatic malignancy. Therefore the prognostic significance of CTC demonstrated by the use of various approaches has not the same weight.

The only FDA (U.S. Food and Drug Administration) approved the methodfor the detection of CTC (Cellsearch®) is based on the eparation of EpCAM (epithelial cell adhesion molecule) posi-tive cells. Published data declare the prognos-tic significance of CTC detected by Cell search in metastatic BC [17], metastatic CRC and PC [2, 3].

The size-based enrichment protocol of CTC reported in our study enables capturing and *in vitro* cultivation of viable CTC (MetaCell®). CTC can be further analysed by the downstream molecular analysis (e.g. gene expression testing by qPCR). Previous studies indicateda fast and simple enrichment in various cancer types [4, 18-20].

In general, the prognostic significance of CTC is supported by several studies on the level of primary disease: inprimary BC pre- and post-operatively [21, 22], in early CRC preoperatively [23], from the postoperative lavage of the peri-toneal cavity [24] and after the adjuvant che-motherapy [25], or in early NSCLC [26].

The clinical utility of CTC is still the subject of clinical studies [27-30]. Well known SWOG s0500 trialdid not support the assumption of the clinical benefit of early chemotherapy change in patients with the metastatic BC and persistent CTC after the first cycle of therapy [31]. The characterisation of CTC was not per-formed in this study so the "real" predictive sig-nificance of CTC was not considered. Conve-rsely, the use of anti-HER2 antibody therapy in patients with metastatic BC and HER2-positive (HER2+) CTC resulted in the prolongation of the time to progression co-mpared to the patients who were not treated with the targeted therapy [32]. CTC detection and characterisation in patients with metastatic castrate- resistant PC (CRPC) can select a group of patients who will most probably not benefit from the hormonal therapy [33]. Splice variant 7 of the androgen receptor (AR-V7) detected in CTC predicts tumor response to the hormonal therapy and taxanes. The patients with AR-V7 positive CTC treated with taxanes survived longer than those treated with the hormonal therapy [34].

The assumption is that the characteristics of CTC are more important than the total CTC number. Below presented individual case re-ports describeour experience with CTC exami-nation in patients with BC, CRC, PC and NSCLC and the potential for their use in the clinical practice. Every case report is documented in the appropriate figure. The scheme of the dis-ease course, patients' characteristics and re-sults of CTC-testing are shown in pictures sche-matically. Questions asked by clinicians that lead to the indication of CTC-testing, arein rela-tion to several therapy relevantpoints (**Table 1**). CTC examination was indicated only as an addi-tional test to a standard diagnostics.

Material and methods

Before the blood collection, patients were informed about the purpose and nature of the examination and their agreement with testing was reaffirmed by the signing of a consent. Minimum of 7.5 ml peripheral blood was obtained (1.6 mg EDTA/1 ml of blood as anticoagulant) for the CTC examination once or repeatedly during 6-24 months.

Enrichment, cultivation and detection of CTC

Blood was subjected to the three -step analysis, consisting of the size- based capturing of cells (MetaCell®), the evaluation of cytomorphological parameters of captured and culturedviable cells by the fluorescent microscopy and by the gene-expression analysis (molecular detection).

Table 2. Genes associated with chemoresistance

Resistance to:	Genes associated with chemoresistance:							
Anthracyclines	MRP1	MRP2						
Taxanes		MRP2			MRP7			
Irinotekan/topotekan	MRP1	MRP2	MRP4					
Alkylating agents	MRP1	MRP2						
5-fluorouracil				MRP5				
Platinum derivates		MRP2		MRP5		ERRC1		
Metothrexat		MRP2	MRP4	MRP5				
Vinca-alkaloids	MRP1				MRP7			
Multi-drug resistance as defined by MDR1 (P-glykoprotein)								MDR1
Gemcitabine								RRM1/RRMM2

CTC were enriched by the filtration of the peripheral blood using Metacell® separation tool (MetaCell s.r.o., Czech Republic). Captured cells were cultured *in vitro* under standard condi-tions (37°C, 5% CO_2) for 3-5 days. Subsequent cytomorphological analysis is based on the characteristics of captured and culturedcells stained by vital fluorescent dyes (Nucblue® Live Ready Probes® Reagent and Cell tracker™ Green CMFDA, Thermo Fisher Scientific, USA). The cells were evaluated by standard cytopa-thologic criteria under the fluorescent micro-scope (e.g.cells size >15 μm, nucleus size >10 μm, irregular nuclear contour, high nuclear/ cytoplasmic ratio, prominent and/or irregular nucleoli, cell proliferation presence, tri-dimensional cell sheets growth). The digital documentation of the captured cells is available for each patient. If the cells with CTC-character were detected, further molecular analysis was provided. CTC were lysed and stored in RLT- buffer with β-mercaptoethanol solution (-20°C).

RNA isolation and cDNA preparation

RNeasy Mini Kit (Qiagen, Germany) was used for RNA isolation from the frozen cell lysates (white blood cells (WBC) and CTC) stored in RLT-buffer. RNA quantity and quality has been checked by NanoDrop (Thermo Fisher, U.S.). High-Capacity RNA-to-cDNA™ Kit (Thermo Fisher Scientific, USA) for cDNA synthesis using minimum of 100 ng RNA load for single cDNA reaction.

qPCR analysis

The gene expressionof tumor-associated (TA) genes (disease specific), stem cell markers and control genes (ACTB) was evaluated. Additionally, the markers of white blood cells (CD45, CD68) were included. Subsequently, expres-sion of genes associated with chemoresistance (CA-genes) was tested. The predictive associa-tions of tested CA-genes MRP1, MRP2, MRP4, MRP5, MRP7, MDR1, ERCC1, RRM1, and RRM2 with the tumor chemoresistance are listed in **Table 2**. TaqMan™ Gene Expression Assays (Thermo Fisher Scientific, USA) were used for qPCR analysis in the samples analysed by PCR technology system Cobas® 480 (Roche s.r.o., Czech Republic).

The gene expression in particular cell-fractions (WBC, CTC) was evaluated forevery patient individually. The gene expression in WBC fraction, CTC-enriched fraction and CTC- enriched and cultured fraction were compared to confirmthe cancer cell presence. The qPCR data evalua-tion was based on $2^{-\Delta\Delta CT}$ methods used to calcu-late relative changes in the gene expression analysis [35]. Samples with relatively elevated expression of TA-markers (2 and more) in cul-tured CTC-fraction compared to WBC fraction were evaluated as CTC positive based on gene expression analysis. qPCR results were anal-ysed by the means of GenEx Professional soft-ware (MultiD, Sweden) enabling multifactorial comparison (e.g WBC vs. CTC) applying Mann-Whitney test ($P<0.002$) inparticular patients.

Clinical interpretation

The CTC-test result formsreport on information about the presence/absence of CTC including the following statements: CTC presence was confirmed by the cytomorphological evaluation (YES/NO), CTC presence was confirmed by the elevated gene expression of the following genes: e.g. KRT18, KRT19, MUC1, CD24, HER2,

Age in time of dg	Staging before operation	Histology from biopsy	Staging after operation	Postoperative histology
34	T2N1M0	IDC, G3, TNBC, Ki67: 70%	T2N1aM0	Metaplastic carcinoma, G3, TNBC, Ki67: 70%

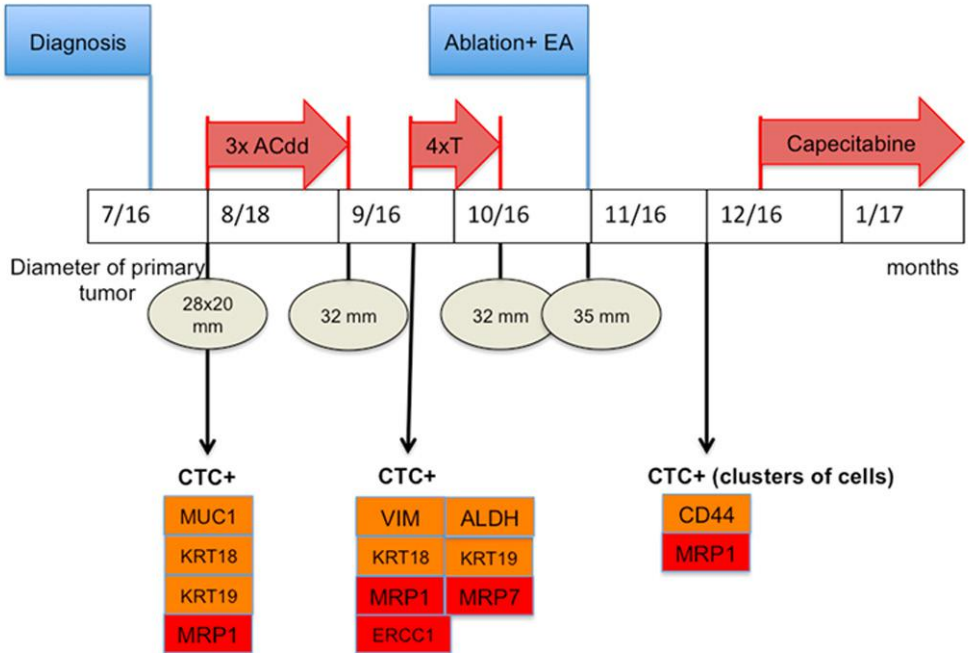


Figure 1. CTC monitoring during neoadjuvant chemotherapy in a patient with breast cancer. AC: doxorubicin + cyclophosphamide, dd: dose dense, T: paclitaxel, CTC+: CTC positivity, EA: axilla exenteration, dg: diagnosis, G: grade, TNBC: triple negative breast cancer, IDC: invasive ductal carcinoma, markers of stem cells: CD44/CD24, VIM (vi-mentin), ALDH (aldehyddehrogenase), markers of epithelial cells: KRT18/19 (keratins), MUC1 (mucin), markers of chemoresistance: see **Table 2**.

ESR. The chemoresistance of CTC may be predicted by the elevated gene expression of the following genes: e.g. MRP2, MRP7. The combination of MRP2 and MRP7 may indicate a resistance to taxanes. MRP2 itself indicates a resistance to platinum-derivatives. MRP7 could be involved into a resistance against vinca-alkaloids too. Repeated measurements enable monitoring of dynamic changeson CTC in time.

Results

Clinical implementation of CTC-examination: Prediction of disease response to neoadjuvant chemotherapy (NACT) in a patient with BC (Case report 1)

Hypothesis: CTC monitoring during NACT may help to predict the early failure of the cancer therapy.

State of the art: To reveal non-responders based on clinicopathological parameters are not entirely possible. Conversely, CTC proper-ties and, in particular the sensitivityto various cytostatics could be a predictor of the treat-ment response. The early shift of the chemo-therapy regimen based on the evolving chemo-resistance could boost the treatment efficacy. The characteristics of the primary disease usu-ally do not correlate with the presence of CTC [36]. The detection of CTC provides additive prognostic and predictive information. The dis-ease progression and the presence of CTC with the mesenchymal characteristics [37] could be the reason for the premature termination of NACT and the indication of a surgery. Whether the presence of CTC with mesenchymal fea-tures (stem cells like) can negatively influence the prognosis of the patients is not clear.

Predictive effect of pathologic complete remis-sion is not exclusive, too [38].

Patient report (1): the response to NACT in TNBC patient (34 years old, stage II) has been

Age in time of dg	Staging before operation	Histology from biopsy	Staging after operation	Postoperative histology
53	T1cN1M0	IDC, G3, ER0, PR0,HER2 neg., Ki67: 40%	T1bN0(0/5)M0	NST, G1, ER10, PR0, HER2 neg., Ki67: 5%

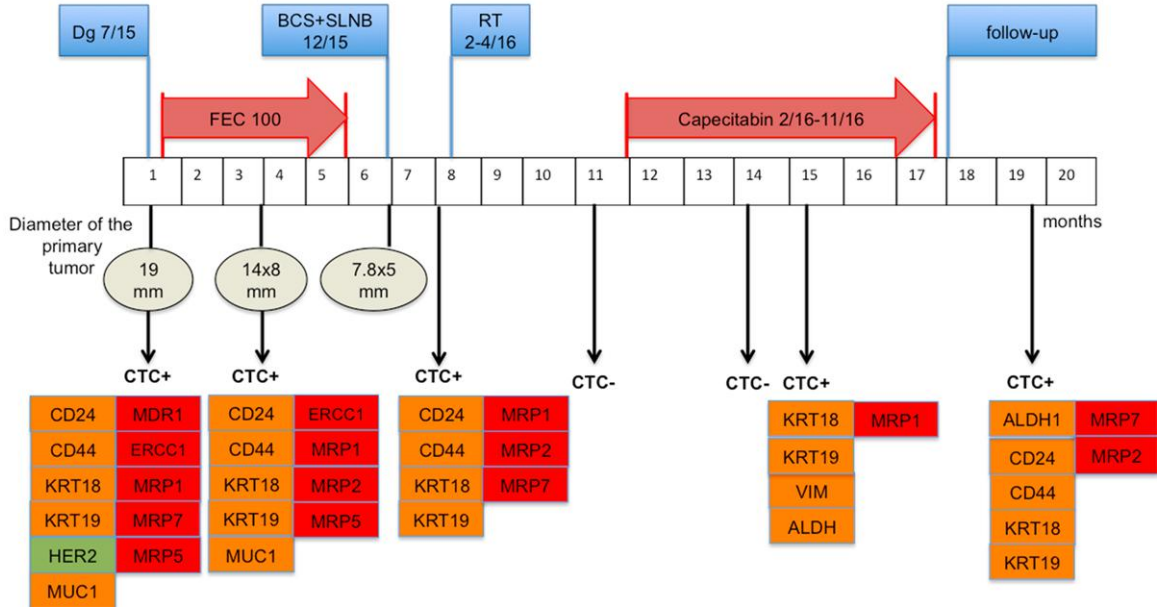


Figure 2. Indication of "additional" adjuvant therapy in a breast cancer patient with persisting CTC. CTC positivity: CTC+, CTC negativity: CTC-, FEC: flurouracil, epirubicin, cyclophosphamid, RT: radiotherapy, BCS: breast conserving surgery, SLNB: sentinel lymphatic node biopsy, dg: diagnosis, IDC: invasive ductal carcinoma, G: grade, markers of stem cells: CD44/CD24, ALDH (aldehyddehrogenase), VIM (vimentin), markers of epithelial cells: KRT18/19 (kera-tins), HER2: human epidermal growth factor receptor, MUC1 (mucin), markers of chemoresistance: see **Table 2**.

monitored. Tumor size was 28 mm at the beginning of the NACT; ultrasound examination described several pathological lymph nodes. CTC were present before NACT had started (**Figure 1**).

CTC displayed the expression of these TA-genes: MUC1, KRT18, KRT19 and CA-gene MRP1. After the 3rd therapy cycle with anthracycline (AC regimen), no therapeutic effect was obse-rved by the ultrasound examination. CTC test was positive again and the level of tumor cells resistance spread (expression of MRP1, MRP7 and ERCC1 was elevated). Expression of MRP7 is associated with the prediction of taxane chemoresistance. Nevertheless, the patient received 4 cycles of paclitaxelin a weekly mode. According to the ultrasound imaging, the tumor size remained at 32 mm.

Subsequently, NACT was terminated and the patient was indicated for a surgery. The final histology described a metaplastic carcinoma (35 mm in diameter). The postoperative blood test detected clusters of CTC. The elevated expressions of keratins were no longer demonstrated but CD44 positive cells were present.

To be discussed: Due to the existing anthracyclines resistance (MRP1), the age of patient and the adverse outcome of NACT, the patient continues with the adjuvant capecitabinetherapy (therapy choice is discussed below in the next case report).

Clinical implementation of CTC-examination: indication of "additional" adjuvant therapy (AT) in residual disease in a patient with BC (Case report 2)

Hypothesis: CTC molecular analysis during AT may help to predict the therapy efficiency and failure.

State of the art: AT administered after the removal of the primary tumor is one of the most difficult treatment strategies. The indication for the adjuvant chemotherapy (ACT) lacks person-

Age in time of dg	Staging of the disease	Histology
29	pT1c multi(3)pNO(0/2) M0	TNBC, G3, Ki67: 60%

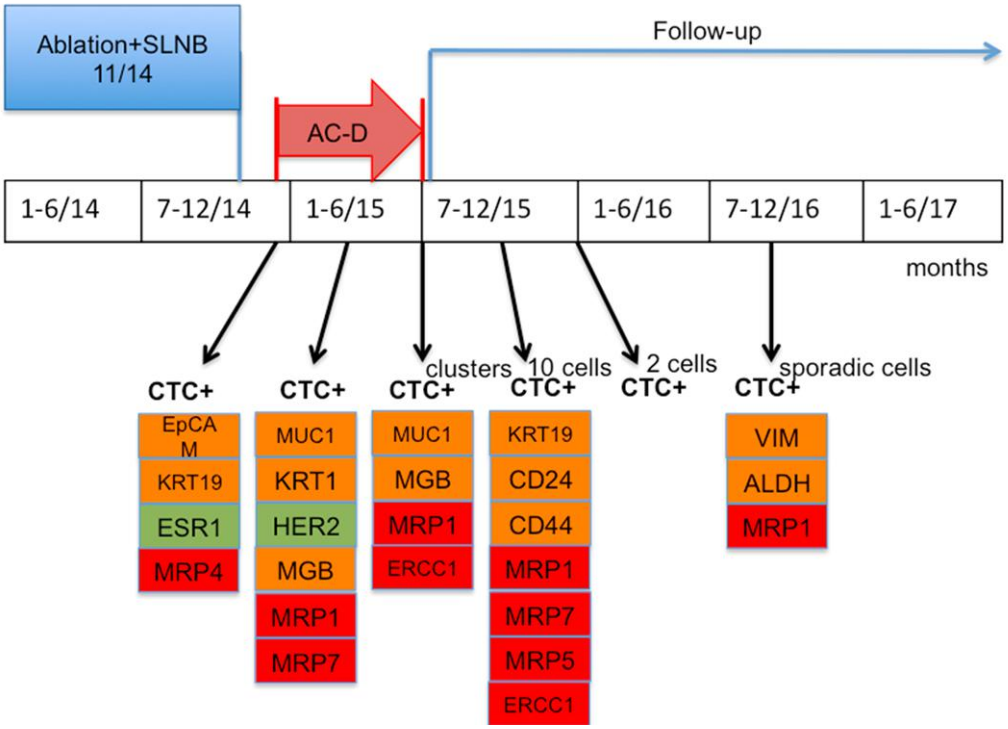


Figure 3. CTC monitoring during adjuvant therapy and in follow up period in a breast cancer patient. CTC positivity: CTC+, AC: doxorubicin + cyclophosphamide, D: docetaxel, SLNB: sentinel lymphatic node biopsy, dg: diagnosis, TNBC: triple negative breast cancer, G: grade, markers of stem cells: CD44/CD24, ALDH (aldehyddehrogenase), VIM (vimentin), markers of epithelial cells: KRT18/19 (keratins), HER2: human epidermal growth factor receptor, ESR1: oestrogen receptor gene, MUC1 (mucin), MGB: mammaglobin B, markers of chemoresistance: see **Table 2**.

alization and endangers the patient with an unnecessary treatment and a possible ineffective therapy. The correct indication of the correct therapy requires further identification of the residual disease.

There is a lack of data for ACT indication beyond the standard length of the therapy. Create-X study [39] is the only study addressing this question, however, with many questions regarding not only the primacy of data [40, 41]. Nevertheless, an "additional" treatment strat-egy is requested in the clinical practice.

Molecular typing of triple negative breast can-cer (TNBC) divided this diagnosis into several subtypes with a different prognosis [42]. Treatment guidelines of TNBC havenot accepted this fact yet, although the chemotherapy response differs in individual subtypes of TNBC [43]. The need for predictive markers in TNBC is therefore more than obvious and possible use of CTC is definite.

Patient's report (2): A case report of TNBC patient (44 years old, stage I) undergoing the additional ACT after the completion of NACT is reported (**Figure 2**). The ACT indication was based on the CTC persistence and primary disease residuum.

The first blood sample was tested before the start of NACT. Keratins (KRT18, KRT19), mucin (MUC1), human epidermal growth factor receptor (HER2) and MRP1 genes were overex-pressed in the CTC-enriched fraction (for more details about CTC during NACT see **Figure 2**).

CTC persisted postoperatively, as well as their chemoresistant character. Although no CTC were present after RT, additional ACT with capecitabine was started. The presence of CTC

after the 4th capecitabine cycle was not confirmed. After the 6th capecitabine cycle CTC were detected again, furthermore the cells exhibited clustering and overexpressed mark-ers associated with the mesenchymal charac-ter: vimentin (VIM) and aldehydehydrogenase (ALDH1). We assume that the super-selection of the aggressive clone arose during the course of the capecitabine therapy. The expression of HER2 was seen only at the beginning of NACT. Because of the persistent sensitivity to the current treatment we continued up to 8 cycles of the capecitabine therapy. The patient is cur-rently being monitored without any therapy and without any disease relapse.

To be discussed: In the presented case report we can demonstrate the aggressiveness of the tumor defined by persistent CTC long after the completion of the primary therapy and the possible therapeutic strategy of "watchful waiting" with the administration of systemic therapy apart from the completion of primary treat-ment. The indication of capecitabine according to the Create-X study is not entirely definite, as well as its inclusion into the AT treatment scheme in the period after the RT and in small T1b tumors. On the other hand, we know that the release of substantial quantities of CTC occurs early in tumors under 3 mm in the diam-eter [44]. Based on the observations in mice, the clusters of CTC have under observations in mice, 23- 50 × higher metastatic potential, their presence thus predicts the ability of cells to establish secondary lesions [45].

Clinical implementation of CTC-examination: observation after AT ina patient with TNBC (Case report 3)

Hypothesis: CTC molecular analysis after AT may help to predict disease relapse.

CTC presence after the tumor resection and/or after the completion of AT predicts higher risk of the disease relapse. Since none of the CTC-predictive use has been reliably demonstrated yet, there is the question of how to deal with the prognostic information offered by a regular CTC examination [46].

Patient's case (3): We enclose results of the postoperative CTC monitoring of a patient with TNBC (29 years old, stage I). CTC tests were provided during AT and subsequently in a follow-up period (**Figure 3**).

As shown in the figure, the presence of CTC with epithelial origin was detected during the AT course. Although the primary tumor was TNBC, CTC overexpressed oestrogen receptor (ESR) and HER2. Before the last docetaxel cycle (07/2015) during AT, we observed clustering of CTC and ER/HER2 lost. In the samples taken in 09/2015, 12/2015 and 09/2016 the number of CTC decreased and the characters of the cells changed from epithelial to mesenchymal (increased expression of VIM and ALDH). After the therapy completion, CTC remained resis-tant to antracyclines (epression of MRP1) for the rest of the time. We also registered elevat-ed ERCC1 expression, which seems to be con-nected with stem cells like phenotype of CTC quite often as published in 2016 by Kasimir-Bauer et al. [47].

To be discussed: The persistency of the low amount of CTC with the signs of the stem cells and MRP1 resistant behaviour during the follow-up period is reported, but the patient is still in remission clinically.

Clinical implementation of CTC-examination: CTC monitoring after AT and during metastatic disease in a patient with HER2+ BC (Case report 4)

Hypothesis: CTC molecular characterisation during the metastatic disease follow up period may help to predict the therapy indication.

State of the art: CTC-positivity and HER2+ are both negative prognostic markers in BC. Together with HER2 discordance between the prima-ry tumor and CTC (in studies 15%-35%), the disease becomes more aggressive and worse from the prognostic point of view [29, 48, 49]. The change of CTC phenotype is spontaneous [50] and behaviour of HER2+ CTC (proliferative potential) is different from those of HER2 negative (resistance to targeted therapy).

We observed the presence of CNS metastases in a patient (39 years old, stage II) with HER2+ locally advanced BC. CNS metastases were detected 11 months after the completion of trastuzumab therapy (**Figure 4**).

The patient started the NACT in 2014, the tumor responded to anthracyclines based ther-apy well but the effect of taxanes and trastu-zumab was quite poor. CTC were expressed HER2+ at the beginning of the disease therapy, but not later during the taxane-based therapy.

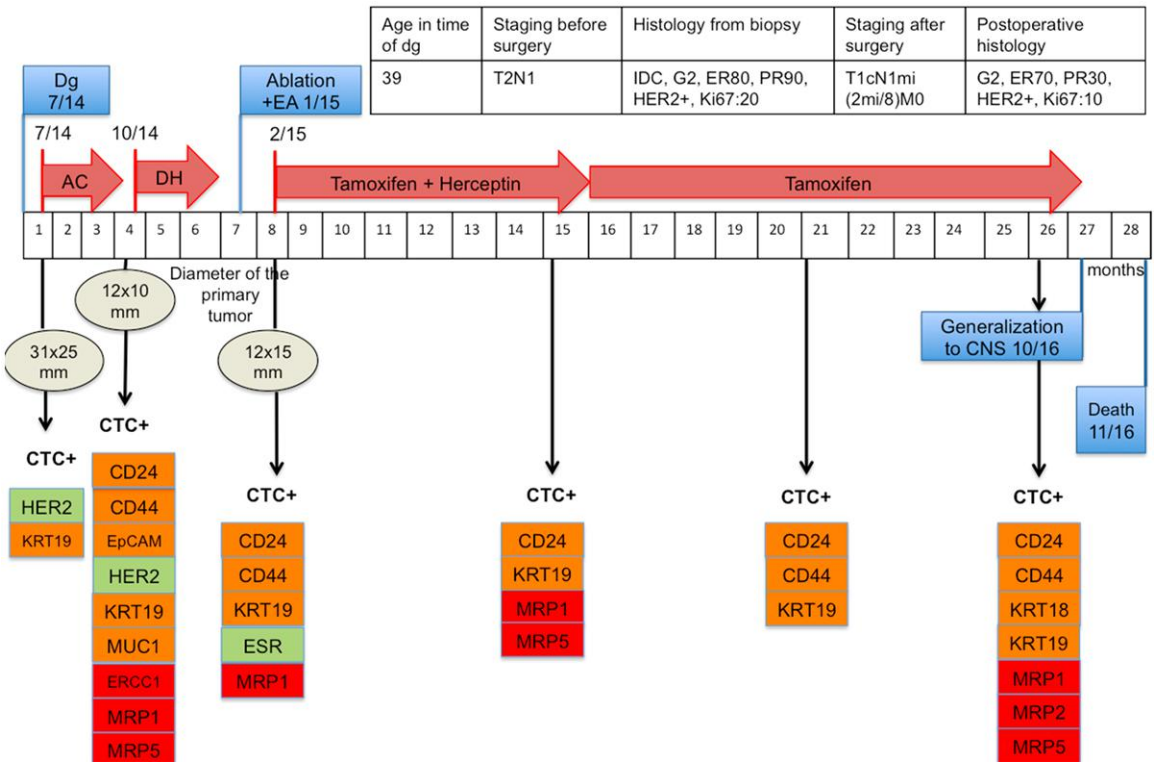


Figure 4. CTC monitoring in a patient with HER2- positive breast cancer. CTC positivity: CTC+, AC: doxorubicin + cyclophosphamide, DH: docetaxel + herceptin, EA: axilla exenteration, dg: diagnosis, ER: oestrogen receptor, PR: progesteron receptor, HER2: human epidermal growth factor receptor, G: grade, CNS: central nervous system, mark-ers of stem cells: CD44/CD24, markers of epithelial cells: KRT18/19 (keratins), EpCAM (Epithelial Cell Adhesion Molecule), MUC1 (mucin), markers of chemoresistance: see **Table 2**.

During the AT (tamoxifen+ herceptin) CTC positivity was confirmed regularly. HER2+ CTC were found during AT with trastuzumab. Expression of ESR was detected in only two of CTC postoperative samples (02/2015 and 04/2015).

The elevation of CTC count and chemoresistance had been documented again before the disease progression and brain metastasis were detected. The expression of KRT18 and CD44 was elevated. Shortly after the trastuzumab therapy completion, CTC expressing HER2 were not present anymore. The patient's death oc-curred very quickly after the diagnosis of brain metastases.

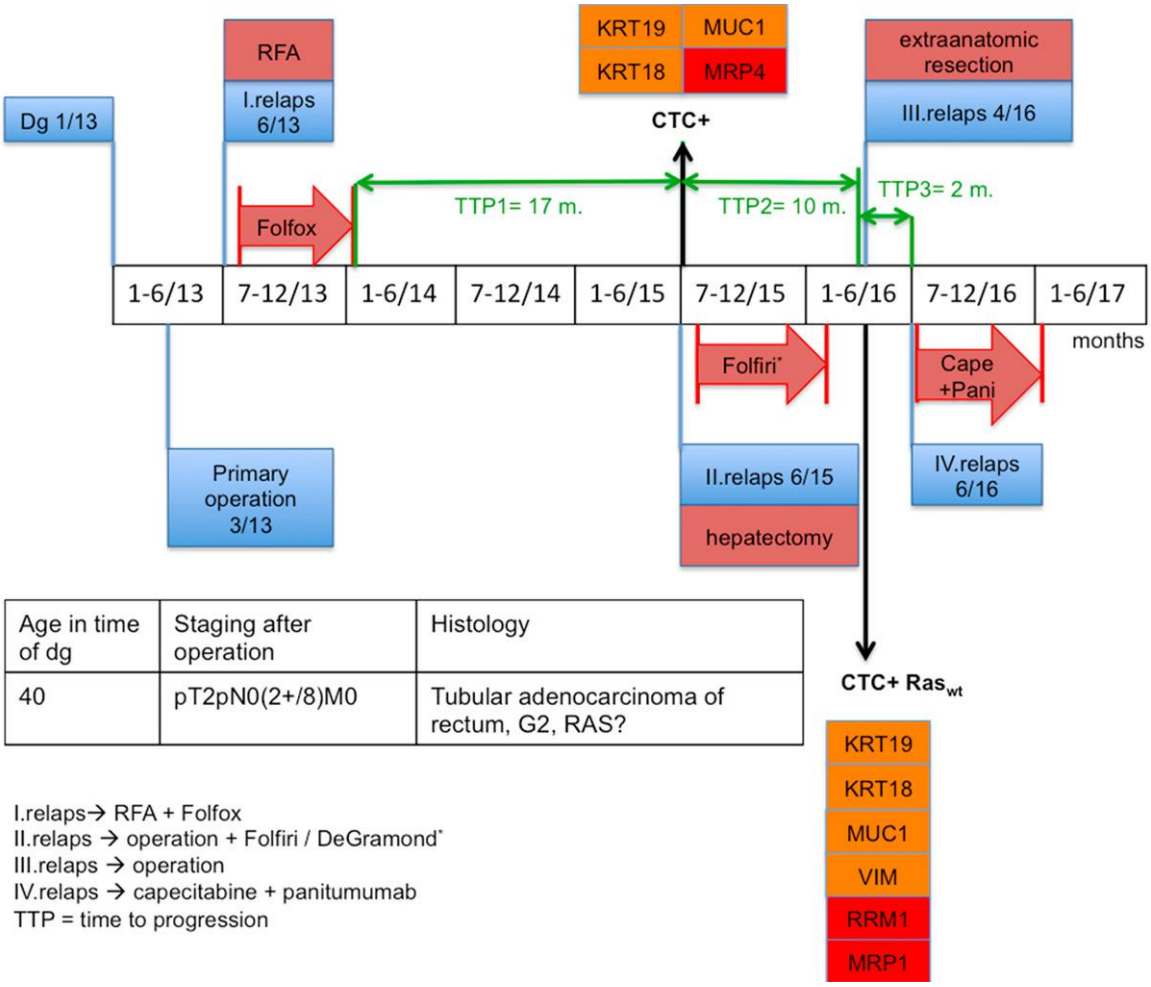
To be discussed: One could discuss the possibility of the re-administration of anti- HER2 therapy in the case of HER2+ CTC at the time of the brain metastases development. The effect of tamoxifen treatment could be redundant also as CTC did not express ESR. Such decisions do not reflect the existing recommendations and could be only used in clinical trials.

Clinical implementation of CTC-examination: CTC-testing after resection of metastases and early prediction of disease relapse in a patient with metastatic CRC

Hypothesis: CTC-examination including the che-moresistance profile analysis could help in the therapy indication in the metastatic disease course.

In patients with CRC and isolated metastatic liver disease, the presence of CTC has already been examined in several studies. The observa-tional period included the time before and/or after the resection or radiofrequency ablation (RFA) [51-53].

According to the type of the detection method, CTC were found in 10 -30% of patients before operation, 29-50% of patients during the sur-gery and in 5-28% of patients after the surgery. The presence of CTC in the time during or after the surgery had a prognostic significance.



Age in time of dg	Staging after operation	Histology
40	pT2pN0(2+/8)M0	Tubular adenocarcinoma of rectum, G2, RAS?

Figure 5. CTC-testing after resection of metastases in a patient with metastatic colorectal cancer. CTC positivity: CTC+, dg: diagnosis, G: grade, RAS: group of oncogenes, wt: wild type, RFA: radiofrequency ablation, m: months, cape: capecitabine, pani: panitumumab, FOLFOX/FOLFIRI: chemotherapy regimens (see main text), KRT18/19 (ker-atins), MUC1 (mucin), VIM (vimentin), genes of chemoresistance: see **Table 2**.

The monitoring of CTC in real-time and the observation of their dynamic behaviour would help to detectearly the disease relapse. Properties of CTC could also help to predict an individual risk of disease relapse [54] and to choose the therapy after the resection of liver metastases. The benefit of the targeted therapy in the adjuvant indication has not been dem-onstrated in CRC yet [55-58]. The presence of CTC with RAS wild type (RAS_{wt}) properties could change this situation by positive selection of CRC patients.

Patient's case (5): The patient (35 years of age) with CRC was undergoing a surgery because of adenocarcinoma of rectum. The postoperative staging was T2N0(0/10)M0, the status of RAS could not be examined because of heavy DNA fragmentation. Short time after the surgery, liver metastasis developed in the left liver lobe. The tumor was cured by RFA and ACT (FOLFOX regimen) (**Figure 5**).

18 months later the second liver relapse appeared. Liver metastasis was resected again. The patient was secured with a systemic thera-py (FOLFIRI) and blood collection for CTC examination was indicated. The results were showed CTC presence by molecular analysis, higher expression of keratins and MUC1 was confirmed, no mesenchymal markers were detected. A relatively small number (units of cells) of CTC could be the reason for quite a long time to the next progression (TTP2).

The third liver relapse developed after 10 months in 04/2016 and CTC test was positive again. Not only liver metastases but also CTC

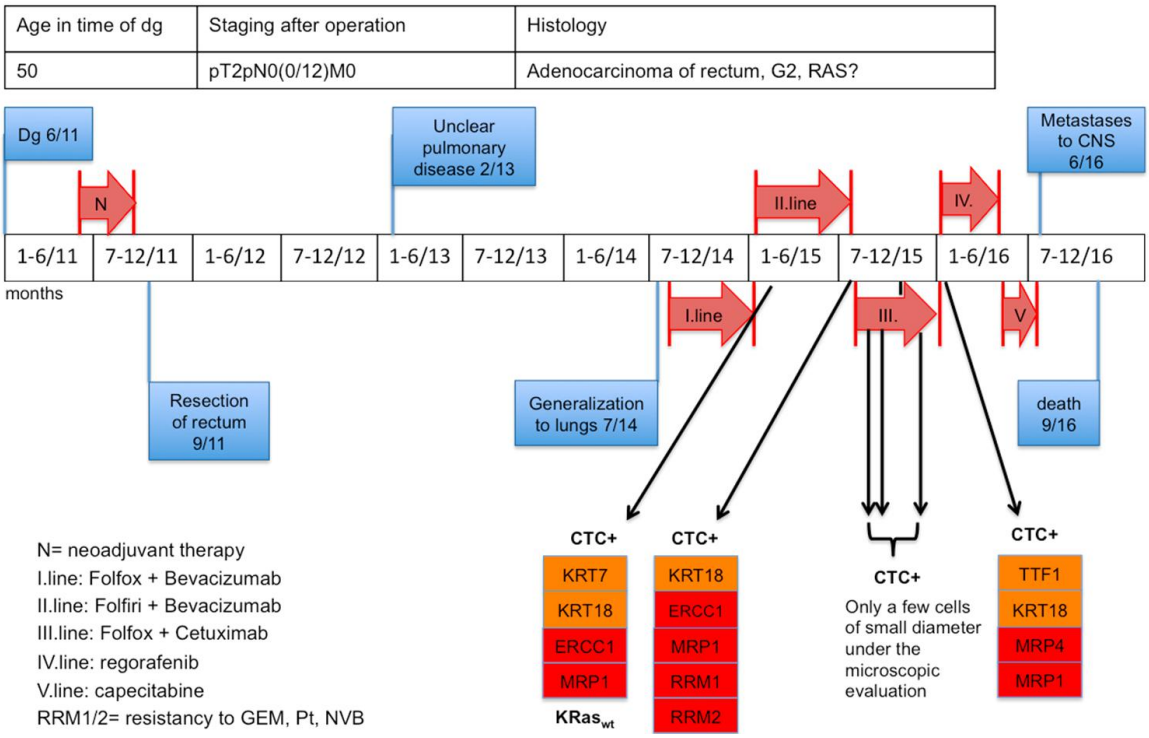


Figure 6. Assessment of KRAS mutation status from CTC in a patient with metastatic colorectal cancer. CTC positiv-ity: CTC+, dg: diagnosis, G: grade, RAS: group of oncogenes, wt: wilde type, CNS: central nervous system, FOLFOX/ FOLFIRI: chemotherapy regimens (see main text), KRT18/19 (keratins), TTF1 (Thyroid transcription factor 1), genes of chemoresistance: see **Table 2**.

were tested for the presence of RAS mutations with a negative result (RAS_{wt} was confirmed). The expression of VIM, clustering of CTC and a high number of CTC were signs for the high dis-ease relapse risk.

The 4th relapse developed in 2 months (TTP3). Because the patient refused any additional chemotherapy, an attempt was made by anoth-er liver resection, but with a short effect only. This patient was treated with anti-EGFR mono-clonal antibody and capecitabine from 07/2016 to 02/2017.

To be discussed: The disease volume and KRAS status could be controlled by monitoring of CTC after the surgical removal of metastases.

Clinical implementation of CTC-examination: assessment of KRAS mutation status fromCTC in patient with metastatic rectal adenocarci-noma

Hypothesis: CTC could present a relevant real-time information source displaying mutational status for genes relevant in the therapy indica-tion process.

State of the art: As we know from the clinical trials, the wild form of KRAS oncogene ($KRAS_{wt}$) is associated with the sensitivity to anti-EGFR therapy, especially in the tumors of the left colon. According to some published studies, the discordance in the state of KRAS in com-parison with the primary tumor and metasta-ses is relatively small [59-61]. Likewise, rela-tively good correspondence in the state of KRAS between the primary tumor and periph-eral blood is the reason for the effect of anti-EGFR therapy. Regardless the fact whether the result of KRAS is based on analysis of the pri-mary tumor or CTC [62, 63].

The problem may occur in advanced lines of treatment because of previous therapy, which can cause super-selection of aggressive tumor clones. Discrepancies at various disease levels (primary tumor, metastasis, blood) could be striking [64]. Another possible benefit of RAS oncogenes or other genes determination from CTC is in cases, in which we lost the option to analyse RAS directly from the primary or sec-ondary tumor.

Patient case (6): A case of a patient (50 years old, stage III) with rectal adenocarcinoma with unknown status of KRAS gene, because of low amount of primary tumor material, is presented (**Figure 6**).

The bulk in the left lung was discovered one year after the NACT therapy (02/2013). The patient has been under observation only because of bulklow diameter and the absence of other signs of an active disease. In 07/2014 lung metastases were confirmed by PET/CT and CTC were detected in the blood. The patient was treated with FOLFOX and bevacizumab in the 1st line and FOLFIRI and bevacizumab in the 2nd line but with only 6 and 3-month lasting effect. CTC examined after FOLFOX were resis-tant to oxaliplatin.

Before the initiation of the 3rd line of the therapy a biopsy from a newly discovered tumor mass in the liver was executed, but tumor cells were not aspirated. The analysis of KRAS was provided, based on CTC-material with the result of $KRAS_{wt}$. Nevertheless, combined FOLFOX and cetuximab therapy failed again. Only rela-tively small cells with several cancerousmor-phologic features were detected in the blood after the therapy completion. CTC expression profile was not done because of small amount of RNA.

We explain the therapeutic failure of the anti-EGFR therapy by tumor heterogeneity and by the administration of two previous therapy lines, which might cause the selection of chemoresistant cells subset (MRP1 and MRP4 expression).

The disease progressed macroscopically and new lesions in bones were discovered in 11/2015. We treated the patient with regorafenib and capecitabin in the next two lines but without any significant effect. The patient died in 9/2016 because of new CNS lesions. CNS metastases are not typical among CRC patients, and their presence explains the aggressiveness of the disease.

To be discussed: To influence the prognosis of the patient at the stage of generalization, the early treatment initiation is critical, but the verification of pulmonary focus (07/2014) could not be done, unfortunately. The liquid biopsy in such a case could replace screening, focused on the disease relapse verification. The molecular analysis of CTC including KRAS status analysis should be more perspective at the beginning of the disease. The effectiveness of anti-EGFR therapy was certainly affected by the previous treatment and by the chemoresis-tance of the disease, which was documented by examination of CTC. Increased EGFR expres-sion could also be the cause of non-effect of regorafenib as a possible escape mechanism of the tumor cells in CRC patient [65]. The data correlating the status of RAS mutations are usually obtained from patients receiving the first line therapy [66, 67], however it was shown that the monitoring of CTC could be relevant in the advanced lines of therapy, too [68].

Clinical implementation of CTC-examination: strategy of using CTC for the palliative treatment guidancein a patient with NSCLC

Hypothesis: CTC-examinations could be used for EGFR mutation detection during the therapy course in a patient with NSCLC.

State of the art: The dynamic changes of CTC could reflect the prognosis of patients with NSCLC [69]. The chemotherapy efficiency decreases with the sequential selection of the chemoresistant tumor clones and its success can be a guarantee only by using drugs with the new mechanism of the effect, which could tar-get on the slowly dividing cells and/or restoring the sensitivity of the tumor cells to cytostatics. The examination of CTC chemoresistance is one way of how to better choose the potentially effective cytostatic in palliative care. The muta-tional analysis of CTC may offer new infor-mation on EGFR-mutational status, identifying T790M mutation associated with anti-EGFR treatment resistance.

Patient's case (7): The case of 47-year old patient with stage IV NSCLC treated with combined carboplatin and pemetrexed therapy in the 1st line is presented. Her disease had the character of adenocarcinoma without muta-tions in genes EGFR, KRAS, NRAS, BRAF and ALK fusion was also not found in the primary tumor. The therapy was conducted from 01 to 05/2016. The examination in 02/2016 showed the presence of CTC with the expression of TA-associated genes EpCAM, MUC1, KRT18 and KRT19. CTC showed resistance to platinum (ERCC1) and cross-resistance to several other antineoplastic agents (MRP1).

CT (computer tomography) scan from 05/2016 showed a mild non-effect of the therapy. The control blood test was carried out at the same time, the characterization of CTC changed part-ly, and the expression of VIM was newly veri-fied. The disease developed more multi-resis-tant cells (expression of MRP1, MRP2, MRP4, MRP7, and ERCC1). Based on this result and based on the preserved sensitivity of the dis-ease on derivatives of 5 -fluorouracil (MRP5 expression has not been proven) and gem-citabine (expression level of RRM1 or RRM2 was not elevated), we indicated the treatment with gemcitabine and capecitabine in the 2nd line.

CT scan from 09/2016 showed a slight pro-gression of a one pulmonary node but also the regression of tumors in other locations. An unresponsive focus was subsequently irradiat-ed and after the completion of RT (09-10/2016) we continued with palliative treatment in the mentioned scheme till 12/2016. The control CT scan unfortunately revealed further bilateral progression of lung focuses. Despite of this result we declare the effect of the second-line treatment lasting for 6 months as successful.

To be discussed: CTC-assisted therapy supple-mented by chemoresistance testing may con-tribute to a better therapeutic effect.

Clinical implementation of CTC-examination: typing of tumors with unknown primary site (C80) or duplicate tumors

Hypothesis: CTC-examination could be used for diagnostics of tumors with unknown origin or for the differential diagnosis in patients with duplicate tumors.

State of the art: CTC could be beneficial in tumors of the unknown primary site (diagnosed as C80). The inter-individual heterogeneity or the tumor dedifferentiation delimitate success-ful typing of known origin tumors as well as of C80 [70]. Detailed analysis of DNA allows to find deviations and mutations of genes involved to the pathogenesis of C80 [71], which could be the aims for the targeted therapy [72]. CTC rep-resent a possible extension of the knowledge obtained from the tissue biopsies [73].

Patient's case (8): A 57-years old patient with duplicate CRC and PC, CTC examination was indicated to obtain prognostic information and to identify the type of CTC. CRC was resected; the post-operative stage was pT3pN0(14)M0, microsatellite stable (MSS). The PC stage T3b-N0-1, GS 3+4 was planned to examine by usingcholine- PET/CT. We discussed the need of AT in CRC. We considered both cancers as potentially aggressive; CRC because of their biological behaviour, PC because of the extent of the disease.

Detected CTC overexpressed following TA-genes: KRT18, KRT19, VIM, ALDH, VEGF, AMACR. The subset of genes confirmed pres-ence of the cells with epithelial origin (keratins), but the elevation of stem cell markers (VIM, ALDH1) was also demonstrated. Additionally VEGF expression supporting the tumorangio-genesis was elevated. The cells were exhibiting morphological features of the cells found in the patients with CRC, but the elevated expression of the AMACR gene couldbe ascribed to the cells of prostate origin. We concluded that probably the both cell types from both tumor types were present inthe patient's blood. The genes associated with the chemoresistance to anthracyclines (MRP1) and platinum (ERCC1) were detected.

We also indicated Oncotype DX Colon Cancer (Genomic Health, USA) examination with the result of the middle to high risk of the disease relapse according to the molecular print of the primary colon cancer (score of recurrence 39).

As RT of PC was planned, we recommended capecitabine as adjuvant monotherapy for CRC and dipherelin as the primary neoadjuvant treatment for prostate cancer.

To be discussed: The CTC examination helped us to distinguish the risk of relapse in two dif-ferent malignant diseases. Stage II CRC does-not always require ACT. The liquid biopsy could predict the need of the post-operative therapy in such cases. In comparison to molecular assays targeting the primary disease, the liquid biopsy offers a real-time monitoring of the CTC volume in time.

Discussion

The clinical evidence for the predictive value of CTC is still limited. Our two-step detection pro-tocol combining a size-based filtration with the both cytomorphological and molecular charac-teristics of CTC may identify CTC in patient sam-

ples, where they cannot be detected by other methods (e.g. EpCAM-based separations). In the reported CTC positive samples (24/34 i.e. 70.6%) EpCAM expression has been confirmed in 2samples (8%) only, expression of KRT 18/19 in 23 samples (95.8%) and MUC1 in 7 samples (29.2%). The changes in the number of CTC in responders compared to non-respond-ers suggest that CTC properties are different in the patients with the same disease undergoing the same treatment.

The highest possibility for the CTC clinical implementation is in the palliative indication. The CTC-navigated therapy based on the detec-tion of certain types of mutations in NSCLC patients is already part of a clinical care. We expect a similar use of CTC-testing in other diagnosis (e.g. the determination of RAS status in CRC or ARV7 in CRPC patients) and in the cases of the primary disease, where the tu-mor tissue is not approachable for a biopsy verification.

Considering the chances for CTC implementa-tion in AT, monitoring of MRD in the patients in remission after the completion of the primary treatment is the only way how to actively inter-vene to the course of this period. It is evident that a disease relapse may occur even years after the primary diagnosis. The persistence of CTC in the blood of the patients in remission after primary treatment significantly increases the risk of a relapse. Long observational peri-ods increase the already limited budgets for the cancer treatment. The liquid biopsy could be a promising inexpensive screening method (in the context of a comprehensive pharmaco-economic assessment) [74].

To sum up, in all the presented case reports it has been shown that the aggressiveness of the disease may be defined by the persistent CTC long after the completion of the primary therapy (e.g. NACT, AT). Subsequently, possible therapeutic strategy of “watchful waiting” com-bined with the administration of systemic ther-apy apart from the completion of the primary treatment could be of a help.

Considering the results of the CTC -chemore-sistance test (e.g. resistance to the anthracy-clines defined by MRP1), the patients could overcome the “watchful waiting periods” with a support of an “additional” AT, e.g. by cape-citabine administration after NACT in BC.

Similarly, if low amount of CTC with signs of the stem cells in the followup period is reported, the metronomic strategy of therapy could be considered.

Another situation has been described when HER2+ CTC were detected in the patients after the terminated anti HER2-therapy. One could discuss the possibility of a re-administration of the anti-HER2 therapy, especially at the time of the brain metastases development.

Our case reports show, monitoring of CTC, not only in BC but also in CRC and NSCLC, could control the disease volume after the surgical removal of metastases. In the last two men-tioned cases, CTC could be used for molecular subtyping of the tumor, which is in CRC and NSCLC a necessary condition which allow the anti-EGFR treatment indication.

In all of the cases the CTC-assisted therapy, supplemented by the chemoresistance testing may contribute to a positive therapeutic effect. In comparison to the molecular assays target-ing the primary disease, the liquid biopsy offers real-time monitoring of CTC volume in time.

Using CTC in the context of the disease diag-nostic in tumors of unknown primary site or in the patients with a duplicate tumor could accel-erate the therapeutic management of the can-cer patients in general.

Based on the data presented, we assume, that the liquid biopsy could significantly improvethe ability to monitor the malignant disease, to pre-dict the treatment efficacy and to provide us an additional base for the complementary use of CTC in concordance with conventional histology in the future.

Disclosure of conflict of interest

None.

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References

[1] Yu M, Stott S, Toner M, Maheswaran S, Haber DA. Circulating tumor cells: approaches to iso-lation and characterisation. J Cell Biol 2011; 192: 373-382.

[2] Huang X, Gao P, Song Y, Sun J, Chen X, Zhao J, Xu H, Wang Z. Meta-analysis of the prognostic value of circulating tumor cells detected with the cellsearch system in colorectal cancer. BMC Cancer 2015; 15: 202.

[3] De Bono JS, Scher HI, Montgomery RB, Parker C, Miller MC, Tissing H, Doyle GV, Terstappen LW, Pienta KJ, Raghavan D. Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. Clin Cancer Res 2008; 14: 6302-6309.

[4] Kolostova K, Broul M, Schraml J, Cegan M, Matkowski R, Fiutowski M, Bobek V. Circulating tumor cells in localized prostate cancer: isolation, cultivation in vitro and relationship to T-stage and Gleason score. Anticancer Res 2014; 34: 3641-3646.

[5] Meyer CP, Pantel K, Tennstedt P, Stroelin P, Schlomm T, Heinzer H, Riethdorf S, Steuber T. Limited prognostic value of preoperative circu-lating tumor cells for early biochemical recur-rence in patients with localized prostate can-cer. Urol Oncol 2016; 34: 235.

[6] Nastały P, Ruf C, Becker P, Bednarz-Knoll N, Stoupiec M, Kavsur R, Isbarn H, Matthies C, Wagner W, Höppner D, Fisch M, Bokemeyer C, Ahyai S, Honecker F, Riethdorf S, Pantel K. Cir-culating tumor cells in patients with testicular germ cell tumors. Clin Cancer Res 2014; 20: 3830-3841.

[7] Bluemke K, Bilkenroth U, Meye A, Fuessel S, Lautenschlaeger C, Goebel S, Melchior A, Heynemann H, Fornara P, Taubert H. Detection of circulating tumor cells in peripheral blood of patients with renal cell carcinoma correlates with prognosis. Cancer Epidemiol Biomarkers Prev 2009; 18: 2190-2194.

[8] Rink M, Chun FK, Minner S, Friedrich M, Mauermann O, Heinyer H, Huland H, Fisch M, Pantel K, Riethdorf S. Detection of circulating tumour cells in peripheral blood of patients with advanced non-metastatic bladder cancer. BJU Int 2011; 107: 1668-1675.

[9] Gazzaniga P, de Berardinis E, Raimondi C, Gradilone A, Busetto GM, De Falco E, Nicolazzo C, Giovannone R, Gentile V, Cortesi E, Pantel K. Circulating tumor cells detection has independent prognostic impact in high-risk non-muscle invasive bladder cancer. Int J Cancer 2014; 135: 1978-1982.

[10] Wang HY, Wei J, Zou ZY, Qian XP, Liu BR. Circu-lating tumour cells predict survival in gastric cancer patients: a meta-analysis. Contemp On-col 2015; 19: 451-457.

[11] Khoja L, Backen A, Sloane R, Menasce L, Ry-der D, Krebs M, Board R, Clack G, Hunges A, Blackhall F, Valle JW, Dive C. A pilot study to explore circulating tumour cells in pancreatic cancer as a novel biomarker. Br J Cancer 2012; 106: 508-516.

[12] Tsai WS, Chen JS, Shai HJ, Wu JC, Lai JM, Lu SH, Hung TF, Chiu YC, You JF, Hsiel PS, Yeh CY, Hung HY, Chiang SF, Lin GP, Tang R, Chang YC. Circulating tumor cell count correlates with colorectal neoplasm progression and is a prognostic marker for distant metastasis in non-metastatic patients. Sci Rep 2016; 6: 24517.

[13] Krawczyk N, Banys M, Hartkopf A, Hagen-beck C, Melcher C, Fehm T. Circulating tumour cells in breast cancer. Ecancermedicalscience 2013; 7: 352.

[14] Po JW, Lynch D, de Souza P, Becker TM. Importance and detection of epithelial-to-mesenchymal transition (EMT) phenotype in CTCs. In: Xu Ke, editor. Tumor metastasis. Intech; 2016. pp. 241-256.

[15] Barrière G, Riouallon A, Renaudie J, Tartary M, Rigaud M. Mesenchymal and stemness circulating tumor cells in early breast cancer diagnosis. BMC Cancer 2012; 12: 114.

[16] Ferreira MM, Ramani VC, Jeffrey SS. Circulating tumor cell technologies. Mol Oncol 2016; 10: 374-394.

[17] Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, Reuben JM, Doyle GV, Allard WJ, Terstappen LW, Hayes DF. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med 2004; 351: 781-791.

[18] Kolostova K, Pinkas M, Jakabova A, Pospisilo-va E, Svobodova P, Spicka J, Cegan M, Mat-kowski R, Bobek V. Molecular characterization of circulating tumor cells in ovarian cancer. Am J Cancer Res 2016; 6: 973-980.

[19] Kolostova K, Matkowski R, Gürlich R, Grabows-ki K, Soter K, Lischke R, Schützner J, Bobek V. Detection and cultivation of circulating tumor cells in gastric cancer. Cytotechnology 2016; 68: 1095-1102.

[20] Bobek V, Gurlich R, Eliasova P, Kolostova K. Cir-culating tumor cells in pancreatic cancer pa-tients: enrichment and cultivation. World J Gastroenterol 2014; 20: 17163-17170.

[21] Bidard FC, Michiels S, Mueller V, Pantel K. Abstract S3-01: IMENEO: International MEta-analysis of circulating tumor cell detection in early breast cancer patients treated by NEO adjuvant chemotherapy. Cancer Res 2017; 77: S3-01.

[22] Rack B, Schindlbeck C, Jückstock J, Andergassen U, Hepp PA, Zwingers T, Friedl TW, Lorenz R, Tesch H, Fasching PA, Fehm T, Schneeweiss A, Lichtenegger W, Beckmann MW, Friese K, Pantel K, Janni. Circulating tumor cells predict survival in early average-to-high risk breast cancer patients. J Natl Cancer Inst 2014; 106: dju066.

[23] Scholten L, Terstappen LW, van der Palen J, Mastboom W, Tibbe A, Vermes I, de Groot M. Circulating tumor cells as a possible prognos-

tic tool in newly diagnosed nonmetastatic colorectal cancer. J Clin Oncol 2012; Suppl 4: Abst 395.

[24] Lloyd JM, McIver CM, Stephenson SA, Hewett PJ, Rieger N, Hardingham JE. Identification of early-stage colorectal cancer patients at risk of relapse post-resection by immunobead reverse transcription-PCR analysis of peritoneal lavage fluid for malignant cells. Clin Cancer Res 2006; 12: 417-423.

[25] Lu CY, Tsai HL, Uen YH, Hu HM, Chen CW, Cheng TL, Lin SR, Wang JY. Circulating tumor cells as a surrogate marker for determining clinical outcome to mFOLFOX chemotherapy in patients with stage III colon cancer. Br J Cancer 2013; 108: 791-797.

[26] Zhu WF, Li J, Yu LC, Wu Y, Tang XP, Hu YM, Chen YC. Prognostic value of EpCAM/MUC1 mRNA-positive cells in non-small cell lung cancer patients. Tumor Biol 2014; 35: 1211-1219.

[27] Bidard FC, Fehm T, Ignatiadis M, Smerage JB, Alix-Panabières C, Janni W, Messina C, Paoletti C, Müller V, Hayes DF, Piccart M, Pierga JY. Clinical application of circulating tumor cells in breast cancer: overview of the current inter-ventional trials. Cancer Metastasis Rev 2013; 32: 179-188.

[28] Bidard FC, de Rycke Y, Asselain B, Cottu P, Rodrigues M, Lebofsky R, Pierga JY. Circulating tumor cells T-DM1 phase II trial: assessing the relevance of HER2-amplified circulating tumor cells as a tool to select HER2-negative metastatic breast cancer for treatment with T-DM1. Cancer Res 2014; 73: Abst OT1-1-10.

[29] Fehm T, Müller V, Aktas B, Janni W, Schneeweiss A, Stickeler E, Lattrich C, Löhberg CR, Solomayer E, Rack B, Riethdorf S, Klein C, Schindlbeck C, Brocker K, Kasimir-Bauer S, Wallwiener D, Pantel K. HER2 status of circulating tumor cells in patients with metastatic breast cancer: a prospective, multicenter trial. Breast Cancer Res Treat 2010; 124: 403-412.

[30] Paoletti C, Muniz MC, Thomas DG, Griffith KA, Kidwell KM, Tokudome N, Brown ME, Aung K, Miller MC, Blossom DL, Schott AF, Henry NL, Rae JM, Connelly MC, Chianese DA, Hayes DF. Development of circulating tumor cell-endocrine therapy index in patients with hormone receptor positive breast cancer. Clin Cancer Res 2015; 21: 2487-2498.

[31] Smerage JB, Barlow WE, Hortobagyi GN, Winer EP, Leyland-Jones B, Srkalovic G, Tejwani S, Schott AF, O'Rourke MA, Lew DL, Doyle GV, Gralow JR, Livingston RB, Hayes DF. Circulating tumor cells and response to chemotherapy in metastatic breast cancer: SWOG S0500. J Clin Oncol 2014; 32: 3483-3489.

[32] Liu Y, Liu Q, Wang T, Bian L, Zhang S, Hu H, Li S, Hu Z, Wu S, Liu B, Jiang Z. Circulating tumor cells in HER2-positive metastatic breast can-cer patients: a valuable prognostic and predic-tive biomarker. BMC Cancer 2013; 13: 202.

[33] Antonarakis ES, Lu C, Wang H, Luber B, Nakazawa M, Roeser JC, Chen Y, Mohammad TA, Chen Y, Fedor HL, Lotan TL, Zheng Q, De Marzo AM, Isaacs JT, Isaacs WB, Nadal R, Paller CJ, Denmeade SR, Carducci MA, Eisenberger MA, Luo J. AR-V7 and resistance to enzalutamide and abiraterone in prostate cancer. N Engl J Med 2014; 371: 1028-1038.

[34] Scher HI, Lu D, Schreiber NA, Louw J, Graf RP, Vargas HA, Johnson A, Jendrisak A, Bambury R, Danila D, McLaughlin B, Wahl J, Greene SB, Heller G, Marrinucci D, Fleisher M, Dittamore R. Association of AR-V7 on circulating tumor cells as a treatment-specific biomarker with outcomes and survival in castration-resistant prostate cancer. JAMA Oncol 2016; 2: 1441-1449.

[35] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25: 402-408.

[36] Lucci A, Hall CS, Lodhi AK, Bhattacharyya A, Anderson AE, Xiao L, Bedrosian I, Kuerer HM, Krishnamurthy S. Circulating tumour cells in non-metastatic breast cancer: a prospective study. Lancet Oncol 2012; 13: 688-695.

[37] Yu M, Bardia A, Wittner BS, Stott SL, Smas ME, Ting DT, Isakoff SJ, Ciciliano JC, Wells MN, Shah AM, Concannon KF, Donaldson MC, Seq-uist LV, Brachtel E, Sgroi D, Baselga J, Ramas-wamy S, Toner M, Haber DA, Maheswaran S. Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. Science 2013; 339: 580-584.

[38] von Minckwitz G, Blohmer JU, Costa SD, Denkert C, Eidtmann H, Eiermann W, Gerber B, Hanusch C, Hilfrich J, Huober J, Jackisch C, Kaufmann M, Kümmel S, Paepke S, Schneeweiss A, Untch M, Zahm DM, Mehta K, Loibl S. Response-guided neoadjuvant chemotherapy for breast cancer. J Clin Oncol 2013; 31: 3623-3630.

[39] Toi M, Lee SJ, Lee ES, Ohtani S, Im YM, Im SA, Park BW, Kim SB, Yanagita Y, Takao S, Ohno S, Aogi K, Iwata H, Kim A, Sasano H, Yokota I, Ohashi Y, Masuda N. A phase III trial of adjuvant capecitabine in breast cancer patients with HER2-negative pathologic residual invasive disease after neoadjuvant chemotherapy (CREATE-X, JBCRG-04). Cance Res 2016; 76: Abst S1-07.

[40] von Minckwitz G, Reimer T, Potenberg J, et al. The phase III ICE study: adjuvant ibandronate with or without capecitabine in elderly patients with moderate or high risk early breast cancer. Cancer Res 2015; 75: Abst S3-04.

[41] Colleoni M, Gray KP, Gelber S, Láng I, Thürlimann B, Gianni L, Abdi EA, Gomez HL, Linderholm BK, Puglisi F, Tondini C, Kralidis E, Eniu A, Cagossi K, Rauch D, Chirgwin J, Gelber RD, Regan MM, Coates AS, Price KN, Viale G, Goldhirsch A. Low-dose oral cyclophosphamide and methotrexate maintenance for hormone receptor-negative early breast cancer: international breast cancer study group trial 22-00. J Clin Oncol 2016; 34: 3400-3408.

[42] Lehmann BD, Bauer JA, Chen X, Sanders ME, Chakravarthy AB, Shyr Y, Pietenpol JA. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. J Clin Invest 2011; 121: 2750-2767.

[43] Lehmann BD, Jovanović B, Chen X, Estrada MV, Johnson KN, Shyr Y, Moses HL, Sanders ME, Pietenpol JA. Refinement of triple-negative breast cancer molecular subtypes: implica-tions for neoadjuvant chemotherapy selection. PLoS One 2016; 11: e0157368.

[44] Coumans FA, Siesling S, Terstappen LW. Detection of cancer before distant metastasis. BMC Cancer 2013; 13: 283.

[45] Aceto N, Bardia A, Miyamoto DT, Donaldson MC, Wittner BS, Spencer JA, Yu M, Pely A, Eng-strom A, Zhu H, Brannigan BW, Kapur R, Stott SL, Shioda T, Ramaswamy S, Ting DT, Lin CP, Toner M, Haber DA, Maheswaran S. CTC clus-ters are oligoclonal precursors of breast can-cer metastases. Cell 2014; 158: 1110-1122.

[46] Tie J, Wang Y, Tomasetti C, Li L, Springer S, Kinde I, Silliman N, Tacey M, Wong HL, Christie M, Kosmider S, Skinner I, Wong R, Steel M, Tran B, Desai J, Jones I, Haydon A, Hayes T, Price TJ, Strausberg RL, Diaz LA Jr, Papadopou-los N, Kinzler KW, Vogelstein B, Gibbs P. Circu-lating tumor DNA analysis detects minimal re-sidual disease and predicts recurrence in patients with stage II colon cancer. Sci Transl Med 2016; 8: 34692.

[47] Kasimir-Bauer S, Bittner AK, König L, Reiter K, Keller T, Kimmig R, Hoffmann O. Does primary neoadjuvant systemic therapy eradicate minimal residual disease? Analysis of disseminat-ed and circulating tumor cells before and after therapy. Breast Cancer Res 2016; 8: 1-15.

[48] Munzone E, Nolé F, Goldhirsch A, Botteri E, Esposito A, Zorzino L, Curigliano G, Minchella I, Adamoli L, Cassatella MC, Casadio C, Sandri MT. Changes of HER2 status in circulating tumor cells compared with the primary tumor during treatment for advanced breast cancer. Clin Breast Cancer 2010; 10: 392-397.

[49] Flores LM, Kindelberger DW, Ligon AH, Capelletti M, Fiorentino M, Loda M, Cibas ES, Jänne PA, Krop IE. Improving the yield of circulating tumour cells facilitates molecular characterisation and recognition of discordant HER2 amplification in breast cancer. Br J Cancer 2010; 102: 1495-1502.

[50] Jordan NV, Bardia A, Wittner BS, Benes C, Ligorio M, Zheng Y, Yu M, Sundaresan TK, Licausi JA, Desai R, O'Keefe RM, Ebright RY, Boukhali M, Sil S, Onozato ML, Iafrate AJ, Kapur R, Sgroi D, Ting DT, Toner M, Ramaswamy S, Haas W, Maheswaran S, Haber DA. HER2 expression identifies dynamic functional states within circulating breast cancer cells. Nature 2016; 537: 102-106.

[51] Weitz J, Koch M, Kienle P, Schrödel A, Willeke F, Benner A, Lehnert T, Herfarth C, von Knebel Doeberitz M. Detection of hematogenic tumor cell dissemination in patients undergoing resection of liver metastases of colorectal cancer. Ann Surg 2000; 232: 66-72.

[52] Koch M, Kienle P, Hinz U, Antolovic D, Schmidt J, Herfarth C, von Knebel Doeberitz M, Weitz J. Detection of hematogenous tumor cell dissemination predicts tumor relapse in patients undergoing surgical resection of colorectal liver metastases. Ann Surg 2005; 241: 199-205.

[53] Papavasiliou P, Fisher T, Kuhn J, Nemunaitis J, Lamont J. Circulating tumor cells in patients undergoing surgery for hepatic metastases from colorectal cancer. Proc (Bayl Univ Med Cent) 2010; 23: 11-14.

[54] Pilati P, Mocellin S, Bertazza L, Galdi F, Briara-va M, Mammano E, Tessari E, Zavagno G, Nitti D. Prognostic value of putative circulating can-cer stem cells in patients undergoing hepatic resection for colorectal liver metastasis. Ann Surg Oncol 2011; 19: 402-408.

[55] Kemeny NE, Jarnagin WR, Capanu M, Fong Y, Gewirtz AN, Dematteo RP, D'Angelica MI. Randomized phase II trial of adjuvant hepatic arte-rial infusion and systemic chemotherapy with or without bevacizumab in patients with re-sected hepatic metastases from colorectal cancer. J Clin Oncol 2011; 29: 884-889.

[56] Turan N, Benekli M, Koca D, Ustaalioglu BO, Dane F, Ozdemir N, Ulas A, Oztop I, Gumus M, Ozturk MA, Berk V, Kucukoner M, Uner A, Balakan O, Helvaci K, Ozkan S, Yilmaz U, Buyukberber S. Adjuvant systemic chemotherapy with or without bevacizumab in patients with resected liver metastases from colorectal can-cer. Oncology 2013; 84: 14-21.

[57] Snoeren N, Voest EE, Bergman AM, Dalesio O, Verheul HM, Tollenaar RA, van der Sijp JR, Schouten SB, Rinkes IH, van Hillegersberg R. A randomized two arm phase III study in patients post radical resection of liver metastases of colorectal cancer to investigate bevacizumab in combination with capecitabine plus oxalipla-tin (CAPOX) vs. CAPOX alone as adjuvant treat-ment. BMC Cancer 2010; 10: 545.

[58] Primrose J, Falk S, Finch-Jones M, Valle J, O'Reilly D, Siriwardena A, Hrnbuckle J, Peter-son M, Rees M, Iveson T, Hickish T, Butler R, Stanton L, Dixon E, Little L, Bowers M, Pugh S, Garden OJ, Cunningham D, Maughan T, Bridge-water J. Systemic chemotherapy with or with-out cetuximab in patients with resectable colorectal liver metastasis: the new EPOC ran-domised controlled trial. Lancet Oncol 2014; 15: 601-611.

[59] Baldus SE, Schaefer KL, Engers R, Hartleb D, Stoecklein NH, Gabbert HE. Prevalence and heterogeneity of KRAS, BRAF, and PIK3CA mutations in primary colorectal adenocarcinomas and their corresponding metastases. Clin Cancer Res 2010; 16: 790-799.

[60] Knijn N, Mekenkamp LJ, Klomp M, Vink-Börger ME, Tol J, Teerenstra S, Meijer JW, Tebar M, Riemersma S, van Krieken JH, Punt CJ, Nagtegaal ID. KRAS mutation analysis: a comparison between primary tumours and matched liver metastases in 305 colorectal cancer pa-tients. Br J Cancer 2011; 104: 1020-1026.

[61] Baas JM, Krens LL, Guchelaar HJ, Morreau H, Gelderblom H. Concordance of predictive markers for EGFR inhibitors in primary tumors and metastases in colorectal cancer: a review. Oncologist 2011; 16: 1239-1249.

[62] Yen LC, Yeh YS, Chen CW, Wang HM, Tsai HL, Lu CY, Chang YT, Chu KS, Lin SR, Wang JY. De-tection of KRAS oncogene in peripheral blood as a predictor of the response to cetuximab plus chemotherapy in patients with metastatic colorectal cancer. Clin Cancer Res 2009; 15: 4508-4513.

[63] Mostert B, Jiang Y, Sieuwerts AM, Wang H, Bolt-de Vries J, Biermann K, Kraan J, Lalmahomed Z, van Galen A, de Weerd V, van der Spoel P, Ramírez-Moreno R, Verhoef C, Ijzermans JN, Wang Y, Gratama JW, Foekens JA, Sleijfer S, Martens JW. KRAS and BRAF mutation status in circulating colorectal tumor cells and their correlation with primary and metastatic tumor tissue. Int J Cancer 2013; 133: 130-141.

[64] Kopetz S, Overman MJ, Chen K, Lucio-Eterovic AK, Kee BK, Fogelman DR, Dasari A, Singh Raghav KP, Sanchez E, Phillips J, Shureiqi I, Garrett CR, Wolff RA, Patel K, Aldape KD, Luthra R, Routbort M, Mari DM, Meric-Bernstam F, Eng C. Mutation and copy number discordance in primary versus metastatic colorectal cancer (mCRC). J Clin Oncol 2014; 32: 5s.

[65] Matsusaka S, Ning Y, Yang D, Zhang W, Hanna DL, Cao S, Suenaga M, Okazaki S, Berger MD, Lenz HJ. Epidermal growth factor receptor mRNA expression in circulating tumor cells as a potential mechanism of molecular escape from regorafenib therapy. J Clin Oncol 2016 (Suppl); Abst 11517.

[66] Kalikaki A, Politaki H, Souglakos J, Apostolaki S, Papadimitraki E, Georgoulia N, Tzardi M, Mavroudis D, Georgoulias V, Voutsina A. KRAS genotypic changes of circulating tumor cells during treatment of patients with metastatic colorectal cancer. PLoS One 2014; 9: e104902.

[67] Lyberopoulou A, Aravantinos G, Efstathopoulos EP, Nikiteas N, Bouziotis P, Isaakidou A, Pa-palois A, Marinos E, Gazouli M. Mutational analysis of circulating tumor cells from colo-rectal cancer patients and correlation with pri-mary tumor tissue. PLoS One 2015; 10: e0123902.

[68] Spindler KL, Pallisgaard N, Andersen RF, Jakobsen A. Changes in mutational status during third-line treatment for metastatic colorectal cancer-results of consecutive measurement of cell free DNA, KRAS and BRAF in the plasma. Int J Cancer 2014; 135: 2215-2222.

[69] Pailler E, Adam J, Barthélémy A, Oulhen M, Auger N, Valent A, Borget I, Planchard D, Taylor M, André F, Soria JC, Vielh P, Besse B, Farace F. Detection of circulating tumor cells harboring a unique ALK rearrangement in ALK-positive non-small-cell lung cancer. J Clin Oncol 2013; 31: 2273-2281.

[70] Dietel M, Jöhrens K, Laffert MV, Hummel M, Bläker H, Pfitzner BM, Lehmann A, Denkert C, Darb-Esfahani S, Lenze D, Heppner FL, Koch A, Sers C, Klauschen F, Anagnostopoulos I. A 2015 update on predictive molecular pathology and its role in targeted cancer therapy: a review focussing on clinical relevance. Cancer Gene Ther 2015; 22: 417-430.

[71] Löffler H, Pfarr N, Kriegsmann M, Endris V, Hielscher T, Lohneis P, Folprecht G, Stenzinger A, Dietel M, Weichert W, Krämer A. Molecular driver alterations and their clinical relevance in cancer of unknown primary site. Oncotarget 2016; 7: 44322-44329.

[72] Ross JS, Wang K, Gay L, Otto GA, White E, Iwanik K, Palmer G, Yelensky R, Lipson DM, Chmielecki J, Erlich RL, Rankin AN, Ali SM, El-vin JA, Morosini D, Miller VA, Stephens PJ. Comprehensive genomic profiling of carcinoma of unknown primary site: new routes to targeted therapies. JAMA Oncol 2015; 1: 40-49.

[73] Matthew EM, Zhou L, Yang Z, Dicker DT, Holder SL, Lim B, Harouaka R, Zheng SY, Drabick JJ, Lamparella NE, Truica CI, El-Deiry WS. A multiplexed marker-based algorithm for diagnosis of carcinoma of unknown primary using circu-lating tumor cells. Oncotarget 2016; 7: 3662-3676.

[74] Degeling K. The health economic impact of cir-culating tumour cells analysis in metastatic castration resistant prostate cancer treat-ment. Master Thesis 2015.

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Molecular characterization and heterogeneity of circulating tumor cells in breast cancer

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Running title: Heterogeneity in CTC in breast cancer
Key words: CTCs, circulating tumor cells, breast cancer, cultivation, in vitro, MetaCell, gene expression

Abstract

This study analyses peripheral blood samples from breast cancer (BC) patients. CTCs from peripheral blood were enriched by size-based separation and were then cultivated *in vitro*. The primary aim of this study was to demonstrate the antigen independent CTC-separation method with high CTCs recovery. Subsequently CTCs enriched at several times during the treatment were molecularly characterized.

Patients diagnosed with different stages of BC (N=165) were included into the study. All patients were candidates for surgery, surgical diagnostics or were undergoing chemotherapy. In parallel, 20 patients were monitored regularly and beside CTC-presence, CTCs character was examined by qPCR, especially HER2 and ESR status was the focus.

The CTC positivity in the tested cohort was 76%. There was no significant difference between tested groups, but the highest CTCs occurrence was found in the group undergoing surgery and similarly in the group before start of neoadjuvant treatment. In opposite, the lowest CTC- frequencies have been observed in the menopausal patient group (56%), ESR+ patient group (60%) and DCIS group (44,4%). It is important to comment on CTCs-presence after neoadjuvant therapy (NACT) was completed 77,7% of cases. It can be assumed, that in these patients therapy was working as expected. On the other site, patients under hormonal treatment were CTC- positive only in 52% of case.

Interestingly, HER2 and ESR status of CTCs differs from the status of primary tumor. In 50% of patients HER2 status changed from HER2+ to HER2-, but also from HER2-to HER2+ (33%). ESR status on CTCs changed only in one direction from ESR+ to ESR- Based on the data obtained in the presented study we may conclude, that BC is a heterogenous disease, but CTCs may be detected independently on the disease characteristics in 76% of patients at any time point of disease period. This relative high CTC-occurrence in BC should be considered in planning of long- period patient monitoring.

Introduction

Breast cancer (BC) is one of the most common female tumor diagnosis, worldwide. Enumeration of circulating tumor cells (CTCs) has showed prognostic role in various stages of this disease. Hormone receptors (estrogen and progesterone) and HER2 status of primary BC tumor have been established during standard clinical biopsies and are of crucial importance in treatment choice. Real-time tumor monitoring through CTCs enumerations could be important indicator of individual cancer development (1).

CTCs as biomarkers can offer several valuable information about patients' tumor but it all depends on their reliable detection, separation and characterization. Although occurrence of CTCs in patients' peripheral blood is often very low, enrichment methods can be introduced for CTCs separation before their characterization. They are usually based on surface protein expression, size, density, electric charges or deformability of CTCs.

This study analyses peripheral blood samples from patients with BC. CTCs from peripheral blood were enriched by size-based separation and were then cultivated *in vitro*. The primary aim of this study was to demonstrate the antigen independent high sensitive separation method and a possibility of molecular characterization of CTCs enriched at several time during the treatment.

Materials and Methods

Patients: To date 167 patients with diagnosed BC have been enrolled in the study in accordance with the Declaration of Helsinki. All patients were candidates for surgery, surgical diagnostics or with planned or applied chemotherapy. Based on informed consent, clinical data were collected from all participating patients. Basic cytopathological data are reported in **Table 1.** For each patient, approximately 2 x 8 mL of venous blood was drawn from the antecubital veins and placed into S-Monovette tubes (Sarstedt AG & Co., Numbrecht, Germany) containing 1.6 mg EDTA/mL blood as an anticoagulant. The samples were processed at room temperature using an isolation procedure completed within 24 hours after the blood draw.

Table 1. Basic cytopathological characteristics of patients

Stage	N	%
0	3	2
IA	45	30
IIA	64	42,7
IIB	20	13,3
IIIA	13	8,7
IIIB	1	0,67
IIIC	4	2,67
Histopathological features		%
benign	2	1,7

Tumor size	N	%
T1	63	61,1
T2	36	34,9
T3	4	3,8
Nodal involvement	**N**	
N0	56	56,5
N1	37	37,3
N2	6	6
	99	
Grading	**N**	

DCIS	9	7,6
LCIS	1	0,85
IDC	31	26,27
ILC	14	11,86
NST	45	38,14
mixed	16	13,6
Menopausal status		%
premenopausal	65	39,39
menopausal	18	10,9
postmenopausal	82	49,7

G1	**7**	11,8
G2	24	40,6
G3	28	47,4
HR and HER2 status		%
HR+ HER2+	16	11,7
HR- HER2+	7	5,1
HR+ HER2-	91	66,4
HR- HER2-	23	16,8

CTCs enrichment and culture: A size-based separation method for viable CTC enrichment from peripheral blood has recently been introduced (MetaCell®, MetaCell s.r.o., Ostrava, Czech Republic) (2-6). The size-based enrichment process is based on the filtration of peripheral blood through a porous polycarbonate membrane (with pores of 8 µm diameter). The minimum and maximum volume of the filtered peripheral blood may be adjusted up to 50 mL with fluid. The standard 8 mL peripheral blood sample from patients suffering from OC was transferred into the filtration tube. Gradual transfer of the blood in several steps is preferred to prevent blood clotting on the membrane filter. The peripheral blood flow is supported by capillary action of the absorbent touching the membrane filter. The filtered CTCs were observed immediately after filtration on the membrane. The control and presence of filtered CTCs immediately after isolation eliminates false negative results of the examination. The membrane filter is kept in a plastic ring that is transferred into the 6-well cultivation plate, 4 mL RPMI media is added to the filter top and CTCs are cultured on the membrane in vitro under standard cell-culture conditions (37°C, 5% atmospheric CO_2) and observed by inverted microscope. The CTCs were grown in FBS-enriched RPMI medium (10%) for a minimum of 14 days on the membrane. Alternatively, the enriched CTCs fraction can be transferred from the membrane and cultured directly on any plastic surface or a microscopic slide, or the separation membrane may be translocated on a microscopic slide. Microscopic slide is preferred if immunohistochemistry/immunofluorescence analysis is planned. If an intermediate CTCs-analysis is awaited, the CTCs-fraction is transferred in PBS (1.5 mL) to a cytospin slide. The slide is then dried for 24 hours and analyzed by histochemistry (May-Grünwald staining) and/or by automated immunohistochemistry protocols (Ventana, Benchmark Ultra, Roche) using standard differential diagnostic antibodies in the pathological evaluation process.

Cytomorphological analysis: The stained fixed cells captured on the membrane were examined using light microscopy in two steps: (i) screening at x20 magnification to locate the cells; (ii) observation at x40/x60 magnification for detailed cytomorphological analysis. Isolated cells and/or clusters of cells of interest (immunostained or not) were selected, digitized, and the images were then examined by an experienced researcher and/or pathologist. CTCs were defined as cells with the following characteristics: (i) with a nuclear size ≥10 µm); (ii) irregular nuclear contour; (iii) visible cytoplasm, cells size over 15 µm; (iv)

prominent nucleoli; (v) high nuclear-cytoplasmic ratio; (vi) proliferation, (vii) actively invading cells creating 2D or 3D cell groups.

Gene expression analysis (GEA): The key purpose of GEA is to compare gene expression of tumor-associated markers in the CTC-enriched fractions to that in the whole blood (white blood cells). To confirm the origin of the captured cells on the separation membrane, gene expression analysis can be performed. Gene expression analysis (GEA) allows up to 20 tumor-associated markers in RNA from different cell fractions to be tested within a single quantitative polymerase chain reaction (qPCR) run. Differential diagnostics markers for qPCR test are chosen in accordance with the expected diagnosis.

RNA is isolated from the whole blood and CTC-enriched fraction on the membrane. The CTC enriched fraction of cells grown on the separation membrane *in vitro* (the so-called "membrane fraction") was used for RNA isolation. .

Finally, the CTC-gene expression analysis allows identification of the relative amount of tumor-associated (TA) markers in the whole blood and in CTC-enriched fractions. If the tumor-associated genes are highly expressed in the CTC fraction, a subsequent analysis of chemoresistance-associated (CA) genes is performed. Molecular analysis allows identification of which type of the chemotherapeutic agents may be of use in tumor therapy and assigned as personalized cancer therapy based on CTC.

The cells captured on the membrane are lysed by RLT-buffer with beta-mercapto-ethanol (Qiagen). RNA is then isolated using the RNeasy Mini Kit (Qiagen). The RNA from the whole blood is isolated with a modified procedure and the quality / concentration of RNA is measured by NanoDrop (ThermoScientific). As there are only up to a few hundred cells on the membrane, the median concentration of RNA is quite low (5-10ng/µl). High Capacity cDNA Reverse Transcription Kit (Life Technologies) was used for cDNA production. Gene expression analysis was performed using Taqman chemistry with Taqman MGB-probes for all the tested genes (Life Technologies).

The following genes associated with tumorigenic character and therapeutic potential in ovarian cancer were chosen for the multimarker GEA panel: ACTIN, CD45, CD68, EPCAM, MUC1, KRT18, KRT19, ESR, HER2, CD24, CD44 Additionally, genes associated with chemoresistance were tested (MRP1-10, MDR1, ERCC1).

Statistical analysis: All analyses were performed using clinicopathological information transformed into variables 0 and 1 if applicable for tested characteristics. Chi-squared test, t-tests, cluster analysis and correlation analysis of qPCR data were outperformed using GeneX (MultiD, SE) and GraphPadPrism vs. 5 (Graphpad, US). P-value of less than 0.05 was considered as statistically significant.

Results

The main focus of the study was to detect circulating tumor cells (CTCs) in BC patients by a new methodological approach, which is based on size-dependent separation of CTCs and subsequent cytomorphological evaluation. Cytomorphological evaluation using vital fluorescence microscopy approach (**Fig. 1**) enables further use of the viable captured cells for RNA/DNA analysis.

Patients diagnosed with different stages of breast cancer (BC) (n= 167) were included in the study. The patients were divided based on clinicopathological criteria and CTC-presence was tested. Summary on obtained CTC-positivity data is presented in **Table 2.**

The CTC positivity in tested cohort was 76 %. There was no significant difference between tested subgroups groups, identifying a possible CTC- presence predicting factor significantly, but the highest CTCs occurrence was found in the group undergoing surgery (86,6%) and similarly in the group before start of neoadjuvant and adjuvant treatment (82,3%).

It is important to comment on relatively high CTCs-presence even if neoadjuvant therapy was completed (77,7%). It can be assumed, that in these patients therapy was working as well as expected.

Fig.1. CTCs isolated from a patient with breast cancer, captured on the separation membrane (vital fluorescent staining - NucBlue® and Celltracker®). Bar represents 10µm.

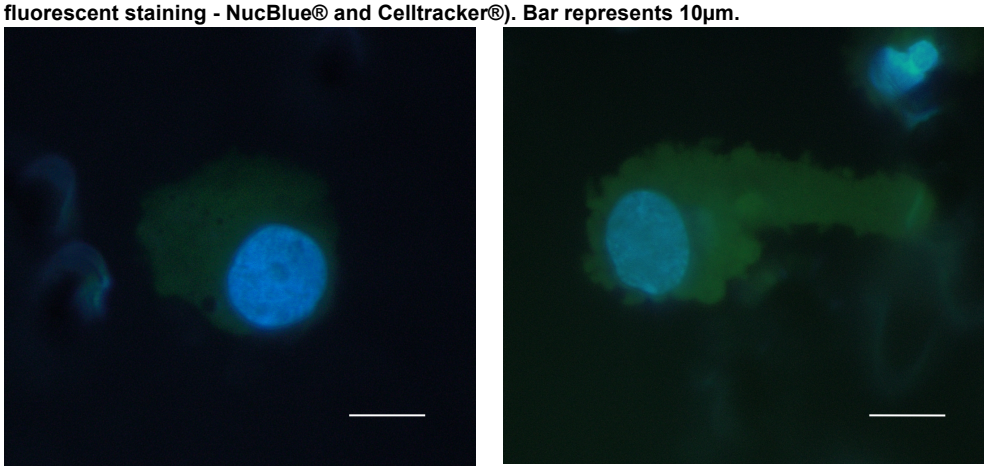


Table 2. CTC positivity detected in different BC-patients subgroups.

CTC Positivity	**CTC+**	**%**
CTC+	**119**	**72,1**
CTC-	**46**	**27,9**
Stage		%
0	3	100
IA	31	68,9
IIA	47	73,4
IIB	16	80
IIIA	10	76,9
IIIB	0	0
IIIC	4	100
Histopathological features		%
DCIS	4	**44,4**
LCIS	1	100
IDC	22	70,9

Tumor size	**N**	**%**
T1	63	74,6
T2	36	88,8
T3	4	75
Nodal involvement		%
N0	23	82
N1	17	78
N2	2	66
Grading		%
G1	**5**	70
G2	14	58
G3	23	82
HR and HER2 status		%
HR+ HER2+	13	81,3

ILC	13	92,9
NST	33	73,3
mixed	9	56,3
Menopausal status		%
Premenopausal	51	78,4
Menopausal	10	**55,5**
Postmenopausal	58	70,7

HR- HER2+	7	100
HR+ HER2-	64	**70,3**
HR- HER2-	22	95,7
Therapy		%
Before therapy	28	82,3
During HT	9	**52,9**
After NACT	7	77,7
Before surgery	39	86,6

There were no significant differences in CTC frequencies observed based on stage definitions. Considering the histopathological character of the primary tumor, the lowest CTC-positivity has been observed in DCIS (44,4%). Relatively low CTC frequency has been observed in menopausal patient group (55,5).

As next, it can be concluded, that in tumors with ESR – expression (ESR+) and without PGR expression (PGR-) CTCs were detected only in 60% (9/15) of tested cases, in ESR+/PGR+ CTC-positivity is 73% (68/93). On the other hand in patients with ESR- negative tumors CTCs were detected in 96,7%, almost all of the tested patients (30/31). In that connection, it has to be mentioned, that during therapy only 52,9 % (9/17) of tested patients exhibited CTCs. Nevertheless, menopausal stage has to be considered if ESR/PGR expression is evaluated. The correlation of the menopausal status and ESR/PGR expression is shown on **Figure 2**, where it can be seen, that hormonal receptor positive tumors are exhibiting the lowest CTC- detection frequencies in comparison to the HR- groups.

Similarly, even if not statistically significant, it can be seen, that HR+ tumors/ HER2 -, independently on the menopausal stage do show the lowest CTC- frequency rates (**see Figure 3).**

In parallel, 20 patients were monitored regularly during disease course and beside CTC-presence also CTCs character was examined by qPCR, especially HER2 and ESR status was the focus. In total, 43 qPCR analysis were evaluated. Therapeutically the most relevant findings are following: HER2 and ESR status of CTCs may differ from the status of primary tumor.

The most frequent changes were seen in the TNBC group (N=12). In these groups 27 samplings were evaluated. There was HER2 presence detected on CTCs in 4 cases. Which means, that the change occurred in 15% of tested samples, but that these 4 changes can be ascribed to 4 different patients. The change was relevant for 4 out of 12 patients (33,3%), what is already a significant number. Similarly, in 50% of patients HER2 status changed from HER2+ to HER2- (3/6).

ESR status on CTCs changed only in one direction from ESR+ to ESR- (3/3). These patients primary tumors were diagnosed as ESR+/PGR+/ ER2-. This group of patient will most probably exhibit very frequent changes.

Fig. 2. CTC positivity in relation to menopausal stage and primary tumor HR - expression.

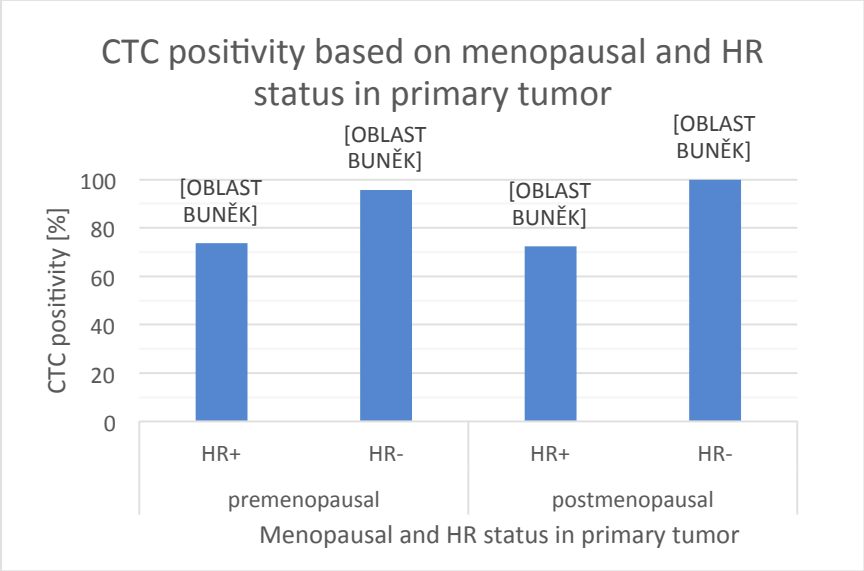
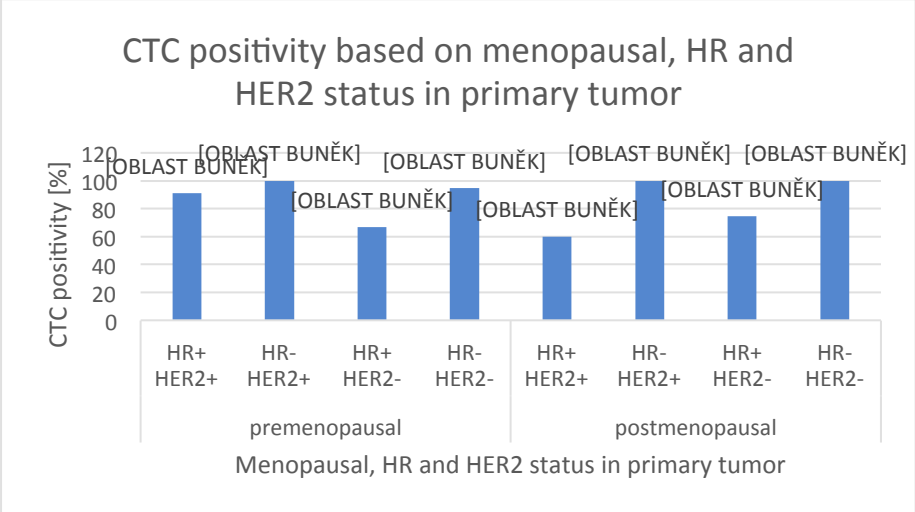


Fig. 3 CTC positivity in relation to menopausal stage and primary tumor HR and HER2- expression.



Taken together, due to relatively high numbers of CTC positivity in different patients groups, we may conclude that certain CTCs number is always present in patients blood. The cells have to be under selection pressure of treatment uninterruptedly. As soon as the selection pressure is stopped, new gene expression profile is displayed by CTCs.

Based on the data obtained in the presented study we may conclude, that BC is a heterogenous disease, but CTCs may be detected independently on the disease characteristics in 76% (119/165) of patients at any time point of disease period. This relative high CTC-occurrence in BC should be considered in planning of long- period patient monitoring.

Discussion

Treatment decisions in BC are based on the characteristics of the primary tumor without considering character of minimal residual disease or metastasis. However, tumors are evolving entities and genetic heterogeneity has been detected comparing the primary tumor with subsequent recurrences and metastases and analyzing different regions of the same tumor or (7). It has been hypothesized that the success of personalized treatments greatly depends on the capability to capture and monitor tumor heterogeneity over time and to consequently modulate therapies (8).

Detection and characterization of CTCs can contribute to the understanding of the disease and improve therapy monitoring as well as personalized treatment options. The key step is sensitive isolation and detection of CTCs. To date, various approaches have been also used to visually identify CTCs; however, the techniques employed to perform cell enrichment, immunohistochemical detection, and image analysis are complicated (9,10). Moreover, epithelial markers are currently used to detect CTCs; tumor cells, however, may lose their epithelial features during metastasis/dissemination or may not express these markers because of their heterogeneity (11). So, part of CTC in EMT (epithelial-mesenchymal transition) could be lost by the common CTC-enrichment strategies relying on epithelial markers (12). According to recent findings, more invasive CTC may lose their epithelial antigens by an EMT process (13) and EMT has been increasingly recognized as a key mechanism of cancer drug resistance (14).

We have used a simple method, without any complicated processing steps, for detecting viable human CTCs in the peripheral blood, by using physical features of CTC-size. We believe that viable CTCs may be a less invasive, repeatable biomarker for monitoring tumor responses.

In our study more than 76 % patients were CTC positive. This result provides evidence that BC cells migrate and disseminate from morphologically very early lesions. Hosseini et al demonstrated that metastatic dissemination often occurs early during tumor formation (15). Dissemination cancer cells detected in patients before the manifestation of breast-cancer metastasis contain fewer genetic abnormalities than primary tumors and indicate that dissemination occurs during the early stages of tumor growth (16-19).

As was demonstrated by the SWOG S0500 trial, the simple enumeration of CTC is not sufficient to guide therapy (20). There are increasing evidences that cancer evolves over time because of its genomic instability and under the selection pressure of systemic treatments. These changes can be responsible for the appearance of drug-resistant clones. In studies of metastatic breast cancer (MBC), a discrepancy was observed between metastases or CTC and the primary tumors in terms HER2, estrogen and progesterone receptor

expression (21,22). The loss of progesterone or estrogen hormone receptor expression in CTC was described in 40% of receptor-positive MBC, a gain in hormone receptor expression was detected in only 8% of triple negative MBC (21).

The clinical use of new CTC detection technique and the molecular characterization of isolated CTCs may lead to the development of personalized anticancer strategy in near future.

References

1. Banys-Paluchowski M, Krawczyk N and Fehm T (2016) Potential Role of Circulating Tumor Cell Detection and Monitoring in Breast Cancer: A Review of Current Evidence. Front. Oncol. 6:255.
2. Kolostova K, Matkowski R, Jędryka M, Soter K, Cegan M, Pinkas M, Jakabova A, Pavlasek J, Spicka J, Bobek V The added value of circulating tumor cells examination in ovarian cancer staging. Am J Cancer Res 2015;5:3363-75
3. Kolostova K, Spicka J, Matkowski R, Bobek V. Isolation, primary culture, morphological and molecular characterization of circulating tumor cells in gynecological cancers. Am J Transl Res 20157:1203-13
4. Kolostova K, Pinkas M, Jakabova A, Pospisilova E, Svobodova P, Spicka J, Cegan M, Matkowski R, Bobek V. Molecular characterization of circulating tumor cells in ovarian cancer. Am J Cancer Res. 2016 May 1;6(5):973-980.
5. Kolostova K, Cegan M, Bobek V. Circulating tumor cells in patients with urothelial tumors: Enrichment and in vitro culture. CUAJ-Canadian Urological Association Journal, Can Urol Assoc J 2014;8(9-10):
6. Kolostova K, Zhang Y, Hoffman RM, Bobek V.: In vitro culture and characterization of human lung cancer circulating tumor cells isolated by size exclusion from an orthotopic nude-mouse model expressing red fluorescent protein. J of Fluorescence, 2014 24(5):1531-6
7. Siu, L.L. Accounting for tumor heterogeneity in the development of predictive biomarkers. Clin. Adv. Hematol. Oncol. 2013, 11, 312–313.
8. Gagan, J.; van Allen, E.M. Next-generation sequencing to guide cancer therapy. Genome Med. 2015, 7, 80.
9. Eliasova P, Kolostova K, Kobierzycki C, Bobek V.Clinical studies monitoring circulating and disseminated tumor cells in gastrointestinal cancers. Folia Histochem Cytobiol. 2013;51(4):265-77.
10. Liberko M, Kolostova K, Bobek V. Essentials of circulating tumor cells for clinical research and practice. Crit Rev Hematol Oncol, 2013 88(2):338-56.,
11. Willipinski-Stapelfeldt, B., et al. 2005. Changes in cytoskeletal protein composition indicative of an epithelial-mesenchymal transition in human micrometastatic and primary breast carcinoma cells. Clin. Cancer Res. 11:8006–8014.
12. Mego, M.; De Giorgi, U.; Dawood, S.; Wang, X.; Valero, V.; Andreopoulou, E.; Handy, B.; Ueno, N.T.; Reuben, J.M.; Cristofanilli, M. Characterization of metastatic breast cancer patients with nondetectable circulating tumor cells. Int. J. Cancer 2011, 129, 417–423.
13. Forte, V.A.; Barrak, D.K.; Elhodaky, M.; Tung, L.; Snow, A.; Lang, J.E. The potential for liquid biopsies in the precision medical treatment of breast cancer. Cancer Biol. Med. 2016, 13, 19–40.
14. Du, B.; Shim, J.S. Targeting Epithelial-Mesenchymal Transition (EMT) to Overcome Drug Resistance in Cancer. Molecules 2016, 21, 7.

15. Hosseini, H. et al. Early dissemination seeds metastasis in breast cancer. Nature http://dx.doi.org/10.1038/nature20785 (2016).
16. Schardt, J. A. et al. Genomic analysis of single cytokeratin-positive cells from bone marrow reveals early mutational events in breast cancer. Cancer Cell 8, 227–239 (2005).
17. Schmidt-Kittler, O. et al. From latent disseminated cells to overt metastasis: genetic analysis of systemic breast cancer progression. Proc. Natl Acad. Sci. USA 100, 7737–7742 (2003).
18. Hüsemann, Y. et al. Systemic spread is an early step in breast cancer. Cancer Cell 13, 58–68 (2008).
19. Sänger, N. et al. Disseminated tumor cells in the bone marrow of patients with ductal carcinoma in situ. Int. J. Cancer 129, 2522–2526 (2011).
20. Smerage, J.B.; Barlow, W.E.; Hortobagyi, G.N.; Winer, E.P.; Leyland-Jones, B.; Srkalovic, G.; Tejwani, S.; Schott, A.F.; O'Rourke, M.A.; Lew, D.L.; et al. Circulating tumor cells and response to chemotherapy in metastatic breast cancer: SWOG S0500. J. Clin. Oncol. 2014, 32, 3483–3489.
21. Aktas, B.; Kasimir-Bauer, S.; Muller, V.; Janni, W.; Fehm, T.; Wallwiener, D.; Pantel, K.; Tewes, M.; DETECT Study Group. Comparison of the HER2, estrogen and progesterone receptor expression profile of primary tumor, metastases and circulating tumor cells in metastatic breast cancer patients. BMC Cancer 2016, 16, 522.
22. Hayes, D.F.; Cristofanilli, M.; Budd, G.T.; Ellis, M.J.; Stopeck, A.; Miller, M.C.; Matera, J.; Allard, W.J.; Doyle, G.V.; Terstappen, L.W. 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. 2006, 12, 4218–4224.