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Circulating tumor cells in breast cancer patients

Cirkulující nádorové buňky u pacientek s karcinomem prsu

Doctoral Thesis

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Herewith I declare that I have worked on the final thesis independently and that I have properly cited and quoted all the sources and literature used. I also declare that the work has not been used to obtain another or the same title.

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ABSTRACT

Circulating tumor cells (CTCs) represent a systemic phase of the localised cancer disease. They can be distinguished and enriched from the peripheral blood and so from the surrounding leukocytes by either physical properties (e.g., density and size) or biological properties (e.g., expression of epithelial proteins such as EpCAM or cytokeratins) and are usually further characterized by immunostaining or RT-PCR assays.

Selecting patients with the risk of disease relaps at the time of diagnosis is crucial for clinicians in deciding who should, and who should not, receive adjuvant chemotherapy. We know that CTCs are strong prognostic factor in patients with metastatic as well as localized breast cancer (BC). It is also known that the prognostic power of circulating tumor cells in women with BC is independent from the standard prognostic indicators. Testing of CTCs known recently as ”liquid biopsy" could be informative not only as predictor of the disease relapse, but also as the predictor of therapy effectiveness.

The clinical use of CTCs must be strictly encouraged by clinical trials results. Monitoring of CTCs in time could zoom in the mechanism of therapy resistance and/or may provide the identification of new druggable targets.

The purpose of my work was therefore to assess the CTCs positivity rate and subsequently CTCs-characteristics in BC patients during different types of therapy phases, e.g. during neoadjuvant, adjuvant and palliative treatment. The aim of our study was mainly the characterisation of CTCs during neoadjuvant chemotherapy (NACT) by examination of tumor-associated genes and genes associated with chemoresistance by the gene expression analysis.

It was shown that tumor volume regression could be monitored by the CTCs chemoresistance profile but not with the CTCs-presence only. The data published by our group support the unique impact of CTCs-character during monitored time sequences. In summary, CTC-character does not correlate to the

clinicopathological characteristic of the primary tumor disease and change dynamically in time.

Finally, we tried to implement CTCs testing into the clinical practice in department of Oncology (General Faculty Hospital in Prague). CTCs-examination is indicated only as a complementary test. The potential clinical applications of the CTCs-testing are summarized in our recent publications, which are a very important part of my dissertation work

ABSTRAKT

Cirkulující nádorové buňky (CTCs) představují systémovou fázi lokalizované maligní nemoci. Jejich identifikace a odlišení od okolních krevních elementů, zejména leukocytů je možné pomocí fyzikálních (např. hustota či velikost) a/nebo biologických vlastností CTCs (např. exprese epitelových znaků jako jsou EpCAM nebo cytokeratiny) a je dále obvykle doplněna o typizaci pomocí barvení či RT-PCR.

Odlišení nemocných s vysokým rizikem relapsu nemoci je klíčovým bodem pro indikaci adjuvantní chemoterapie. CTCs jsou silným prognostickým markerem jak u primárního, tak metastatického karcinomu prsu (BC). Víme také, že cirkulující (diseminované) nádorové buňky jsou markerem nezávislým na standardních prognostických parametrech. Testování CTCs, neboli tzv. tekutá biopsie může být přínosná nejen pro predikci recidivy nemoci, ale také pro predikci léčebné odpovědi.

Klinické využití CTCs musí být doloženo klinickými studiemi. Monitorace CTCs v čase může přiblížit mechanizmy rezistence nemoci a pomoci odhalit terče pro potencionální terapeutické cílení.

Cílem mojí práce byla jednak detekce a stanovení úrovně CTCs pozitivity u nemocných s BC v různých fázích terapie (neo/adjuvantní či paliativní), jednak charakterizace CTCs pomocí tumor-asociovaných genů a genů spojených s

chemorezistencí, a to zejména u nemocných s BC podstupujících neoadjuvantní chemoterapii (NACT).

Regrese nádorového objemu je provázena změnou chemorezistence CTCs, samotná monitorace počtu CTCs nestačí. Data, které jsme publikovali, podporují význam typizace CTCs v průběhu léčby nemoci. Vlastnosti CTCs nekorelují s klinicko-patologickými parametry a dynamicky se mění v čase.

Závěrem, CTCs vyšetření se nám podařilo implementovat částečně do běžné klinické praxe. Vyšetření CTCs je indikováno jako doplňková metoda. Možné využití CTCs v klinické praxi je shrnuto v našich aktuálních publikacích, které jsou také součástí dizertační práce.

TABLE OF CONTENTS

1. INTRODUCTION

An extension of people's live periods and lifestyle changes are reasons why we meet with the term "cancer" more often. Nowadays every third person falls sick to cancer, cancer has became a civilization disease. The development of malignancy is a multistep process and the generalization may be closely linked to the development of primary tumor. Characteristics of the primary disease and cancer cells interactions in context with the surrounding non-tumorous microenvironment determines its behavior in later stages. They determine whether tumor cells will behave more indolent or aggressive.

The key organ for the tumor disease spread is blood. Minimal residual disease (MRD) is the microscopic disease at the level of blood and/or bone marrow. It can have the appearance of CTCs, circulating DNA fragments (cfDNA-ctDNA) or other fragments of tumor cells, circulating exosomes or disseminated tumor cells (DTCs) detected mainly in bone marrow. In all the text below mostly CTCs as the one type of MRD will be discussed. DTCs are discussed in some chapters as an examination completing patient prognosis if relevant.

Viability of CTCs is very limited and the process of their extravasation and transfer into the stroma of another organs is extremely energy demanding. Their fate could be terminated by death, on the other hand tumor cells can fall into the dormancy. It is unclear at what stage / stages of the disease the behavior of cancer cells change and so whether cells future destination is primary molecularly predefined or may change over time. The process of epithelial-mesenchymal transition (EMT) might contribute to the aggressiveness of tumor cells giving them invasive ability. It might be the basis of their dormancy on the other site. It is possible that signals of the immune system and other cells of the tumor microenviroment can affect the behavior of cancer cells. It is also possible that tumor cells can influence stromal cells in that way they are supporting the cancer spread.

Cancer dissemination process is so complex, dynamic and multi-directional, that the fight with cancer might be considered pointless. Fortunately, advances in science are enormous and many therapeutic targets leading to better outcome of our patients with BC were found.

MRD is a rational therapeutic target to prevent the development of metastases. Targeting CTCs could potentially prolong survival of cancer patients. Before this happens, the key is recognizing their function in the metastatic cascade. Detection of CTCs, enumeration and better profiling of individual cells are key strategies. It is important to realize that CTCs may have characteristics of stem cells (SCs), having weapons which the nature itself has created to the struggle for self-preservation.

Cell signaling pathways affecting regulation of EMT are attractive therapeutic targets for anti-metastatic therapies. On the other hand EMT can alter surface marker expression, leading to decreased efficacy of anti-metastatic therapies targeting specific epithelial surface markers. To find and to destroy a cell which is the cause of life and death at the same timet is certainly a courageous fight.

1.1 Minimal residual disease in breast cancer - clinical practice amendments

CTCs/DTCs provide the link between the primary tumor and metastatic sites. MRD is a widely- accepted theory clarifying disease relapse after the primary tumor surgery and longstanding remission. At about 20 - 30 % of chemotherapy treated primary BC (PBC) patients relapse with metastatic disease (Albain K. et al., 2012).

The likelihood of CTCs being released into the blood is probably associated with the aggressivity of the primary disease. The standard pathological examination of the primary tumor is inadequate for the risk of disease relaps prediction. The removal of primary tumor mass may not be sufficient to ensure that the patient is cured. But the examination of MRD is not the standard part of the clinical practice. Their monitoring could predict the tumor relapse long before the secondary tumors formation. Metastases are lesions in bones or visceral organs that result from overcoming the blood barrier by tumor cells. The prognosis of such a disease is bad and results both from heterogeneity of tumor cells and the function of the organ affected by malignancy.

CTCs/DTCs may provide useful prognostic and predictive information to guide treatment decision. The prognostic role of CTCs has been already declared by many studies. To answer the question whether the presence of CTCs could predict the therapy effectiveness we need to ask:

1. Does the presence (number) of CTCs differ in patients responding and non-responding to the therapy?
2. Could the early change of therapy in non-responders (no decrease of CTCs number) improve patients outcome?
3. Does characteristics of CTCs differ in responders/non-responders?
4. How to obtain relevant information about CTCs character (which method has to be used)?
5. Could the CTCs- navigated therapy improve the outcome of BC patients?
6. If not, what is the reason? Does the dynamic behaviour of CTCs have an

impact to the therapy effectiveness?

7. How to treat PBC and MBC patients more effectivelly? Are they more important characteristics of the primary tumor or CTCs characteristics in the therapy indication of PBC/MBC patients?

Answers to these questions we will try to find in the following text.

1.1.1 Breast cancer

BC is the most frequent cancer in women worldwide (1.67 million new cancer cases diagnosed in 2012, i.e 25 % of all cancers (http://globocan.iarc.fr). The incidence of BC in the Czech Republic was 130/100.000 habitants and year in 2012 (www.svod.cz). The improvement in the BC treatment and earlier detection are reasons for the decreasing mortality in recent years (35/100.000). However, BC is still the leading cause of cancer-related death in European women.

The prognosis is very different trough the cohort of BC patients and it depends on many risk factors like the stage, histology, patient age and other clinico-pathological parameters at the time of diagnosis.
The TNM staging system includes the number and maximal diameter of removed tumors, the number of removed lymphatic nodes and number of positive them (they can contain isolated tumor cells, micrometastatis or macrometastasis).
The final pathological diagnosis includes the histological type of the tumor, grade, immunohistochemical (IHC) evaluation of oestrogen (ER) and progesterone receptors (PR) and receptors for epidermal growth factor type 2 (HER2). HER2 gene amplification status may be determined directly by using in situ hybridisation (fluorescent / chromogenic / silver in situ hybridisation) or only for tumors with an ambiguous (2+) IHC score. Ki-67 is a nuclear protein that is associated with cellular proliferation and has the prognostic importance. Other important information is related to resection margins, peritumoral vascular or lymphovascular invasion and perineural spread of tumor cells.

The new era of molecular diagnostics helps us better understand the tumor biology and offers new information about the cancer cells population and

pathological pathways in which they are connected to. Two seminal papers using microarray gene expression profiling, one by Sorlie (Sorlie T. et al., 2011) and the other by van't Veer (van't Veer LJ. et al., 2002) clearly demostrated the association of gene expression profiles and prognosis in PBC patients. In 2011 was adopted the **new molecular classification** based on the recognition of intrinsic biological subtypes within the BC spectrum (Goldhirsch A. et al., 2011).

Luminal A group includes tumors that are ER positive (ER+) and PR positive (PR+), but negative for HER2 (HER2-). Luminal A tumors benefit from hormonal therapy but not from chemotherapy and have the best prognosis. **Luminal B** group includes tumors that are ER+, but can be PR negative (PR-) and/or **HER2+** or have higher Ki67 as the one of possible risk factors of disease recurrence. BC of this type benefit from chemotherapy adding to hormonal therapy, HER2+ cancers are treated with anti-HER2 antibodies. HER2+ group (HER2-enriched) includes tumors that are ER negative (ER-) and PR-, but HER2+. HER2+ BC are likely to benefit from chemotherapy and treatment targeted to HER2. **Triple negative breast cancer (TNBC)** includes a wide spectrum of tumors that are ER-, PR- and HER2-. Recently we differentiate sex subtypes of TNBC: basal-like type 1 (BL-1), basal-like type 2 (BL-2), mesenchymal (M), mesenchymal/stem- like (ML), immunomodulatory (IM) and luminal/androgen-receptor (LAR) positive type (Lehmann BD. et al., 2011). The only option how to treat TNBC is still the systemic chemotherapy.

Luminal B type is prognostically worse than luminal A so the use of chemotherapy can be needed. To distinguish luminal A and B subtype the right level of ER, PR and Ki67 examined by IHC is required (Viale G., 2015). The precise distinction of luminal A and B type is possible only by molecular-genetic testing. Prognostic information using gene expression tools such a MammaPrint, EndoPredict or Oncotype DX can be helpful in this point of view (Bielčiková Z., Petruželka L., 2016). These tests are able to predict the risk of the disease recurrence and to guide the adjuvant therapy decisions. In patients with high risk of disease relapse we indicate adjuvant chemotherapy, on the other hand the low risk recurrence score we expect in luminal A type BC which is treated with hormonal therapy alone. Oncotype DX analyzes the expression of 21 genes within

a tumor to determine a recurrence score that corresponds to a specific likelihood of BC recurrence within 10 years of the initial diagnosis, as well as response to the adjuvant treatment. It may be very useful in patients with ER-, lymph node negative BC (Paik S. et al., 2006, Sparano JA., et al., 2015) but as it was shown in several trials the node positive disease with limited nodal involvement (1-3 positive lymph node) may also have a good prognosis (Mamounas EP. et al., 2012). This means that the tumor biology is as important, if not more important as the disease stage (Gluz O. et al., 2012).

The state of the art in BC treatment does not incorporate tumor biology information into the therapy decision yet. Correctly indicated treatment to the right patient is as important as non-indication of "unnecessary" treatment to the patient who would not profit from it. Unfortunately, standard molecular examination of BC is not possible in daily clinical practice. The prognostic relevance of the pathologic complete remission (pCR) in patients undergoing NACT is connected only to aggressive subtypes of BC (von Minckwitz G. et al., 2012). The other challenge is to choose the best treatment combination to reach pCR. The necessity of predictive markers is obvious.

The most relevant clinical endpoints in a group of M0 patients (no overt metastasis) are disease free survival (DFS) and overall survival (OS) so the time from BC surgery to any disease relapse or death. In metastatic (M1) patients the treatment efficacy results from progression free survival (PFS), tumor response rate (TRR) and OS. PFS is the time from initiation of the treatment to the tumor progression and TRR is the rate of patients experiencing a complete or partial response of their tumor diameter.
Although the M1 disease is considered incurable, thanks to modern treatment becomes a chronic disease in many cases.

1.1.2 Prognostic and predictive value of CTCs/DTCs in breast cancer patients

1.1.2.1 Prognostic importance of CTCs in metastatic breast cancer (MBC):

The first data about prognostic value of CTCs in MBC were validated on

CellSearch system (Cristofanilli M. et al., 2004). Patiets with CTCs count ≥ 5 per 7.5 mL of whole blood had a significantly worse prognosis measured by PFS and OS. Additional studies in MBC using the same cut-off have reported consistent data (Jayes DF. et al., 2006, Dawood S. et al., 2008, Zhang L. et al., 2012).

The prognostic value of CTCs detection has been also investigated with regards to molecular subtypes of BC. According to Giordano (Giordano A. et al., 2012) CTCs have no predictive impact on OS in patients with HER2+ MBC treated with anti-HER2 targeted therapy, in contrast to all other subtypes of BC. Typization of CTCs was not conducted in this study, so only the count of CTCs was compared with OS.

On the other hand, Pierga (Pierga J. et al., 2012) reported strong prognostic impact of CTCs in all subtypes of MBC including HER2+ subtype. He also showed that CTCs count didn't correlate with tumor markers.

In a large prospective multicentre study (Wallwiener M. et al., 2013) the prognostic significance of HER2+ CTCs vs. HER2- CTCs were evaluated. HER2 status of CTCs was determined by immunofluorescence (CellSearch®). Patients with HER2+ CTCs had significantly shorter median PFS than those with HER2- CTCs.

CTCs detection rate and their prognostic significance is different by comparing CellSearch and AdnaTest results. Müller (Müller V. et al., 2012), compared directly these two methods and tested 221 MBC patients. Detection rate for CTCs positivity was 50 % by using CellSearch and 40 % by using AdnaTest. Only CellSearch data had prognostic relevance.

Contrary, in metaanalysis (Zhang L. et al. 2012) of 22 MBC studies included both CellSearch and PCR-based methods evaluated as a whole, prognostic impact of CTCs detection was demonstrated. CELLection™ Dynabeads® were used for CTCs isolation in Gradilone study (Gradilone A. et al., 2011),) expression levels of the four marker genes p1B, PS2, CK19 and EGP2 were measured in Weigelt study (Weigelt B. et al., 2003), in Tewes study (Tewes M. et al., 2009) CTCs

were detected with AdnaTest and in Reinholz study (Reinholz MM. et al., 2011) the amount of CK19 and mammaglobin were counted.

In conclusion: The presence of CTCs has the significant impact on MBC patients prognosis regardless BC subtypes and detection technique. CTCs have the prognostic power in regard to the time of their evaluation (before 1. line therapy/before new line therapy/after one cycle of therapy/during follow up). Change in CTCs number at baseline and after 3 cycles of therapy is associated with the treatment response. The cut-off ≥ 5 cells per 7.5 ml of blood was prospectively identified in a training set of MBC patients and confirmed in a validation set by using CellSearch system. Using AdnaTest, CTCs positivity is defined by the detection of one or more of the 3 markers (GA733.2, MUC-1 and Her2). The chosen cut-off value for positivity was 0.15 ng/µL.

1.1.2.2 Prognostic importance of CTCs in early breast cancer (PBC):

A number of studies including the metaanalysis of individual data from 3.173 patients with M0 BC (Janni WJ. et al., 2016) demonstrated the prognostic value of CTCs detected in PBC patients (see data below).

In the neoadjuvant setting the CTCs detection rate is about 22 - 23 % before the start of chemotherapy (Riethdorf S. et al., 2010, Bidard FC. et al., 2012, Pierga JY. et al., 2008) and about 10 - 17 % after treatment (Riethdorf S. et al., 2010, Pierga JY. et al., 2008). Pierga also showed the prognostic value of CTCs before and after NACT, CTCs positive women had shorter DFS. Lavrov (Lavrov AV. et al., 2014) detected CTCs in 38 % of patients with early TNBC and 42 % of locally advanced TNBC before NACT. By using multi-cytokeratin-specific antibody, Serrano (Serrano MJ. et al., 2012) detected CTCs in 70 % of patients before NACT and 54 % after that and Camara (Camamra O. et al., 2007) even in 83 % of patients before therapy. Prognostic significance of CTCs presented in blood before and after NACT was significant also in other studies (Pierga JY. et al., 2008, Bidard FC. et al., 2010).

In adjuvant setting 19 - 43 % of patients were CTCs positive in bellow published studies. Prognostic impact of CTCs was determined before therapy (Xenidis N. et al., 2006), detection of a single CTC prior treatment was prognostic for reduced DFS and OS furthermore in the prospective SUCCESS trial (Rack B. et al., 2014). Prognostic power of CTCs was also declared in other trials before and after adjuvant chemotherapy (Xenidis N. et al., 2013), before surgery (Franken B. et al., 2012), before and during adjuvant chemotherapy (Pachmann K. et al., 2008). Xenidis (Xenidis N. et al., 2007) reported that the presence of CTCs in peripheral blood of hormone receptor positive PBC is correlated to tamoxifen resistance and worse DFS and OS.

Looking for DTCs in solid organs is technically difficult, DTCs are almost exclusively detected in bone marrow. In the analysis of pooled data from several prospective studies, the detection of DTCs in bone marrow in stage I-III PBC was associated with a significantly higher risk of recurrence and disease-specific death (Braun S. et al., 2005). Persistence of DTCs after therapy predicts a higher risk of relapse in BC patients (Hall C. et al., 2012, Janni W. et al, 2011).

The german group (Kasimir-Bauer S, et al., 2016) analyzed patients with PBC before and after NACT for the presence of DTCs in the bone marrow and CTCs in the blood, including stem cell–like CTCs to prove the effectiveness of treatment on these cells. No significant correlations were found for DTCs or any CTCs before and after therapy with regard to PFS and OS. Interestingly, 72 % of CTCs present after therapy were positive for ERCC1 (marker of chemoresistance to cisplatin) that might indicate a worse outcome of that patients in the future. CTCs were eradicated more effectively than DTCs.

In conclusion: The presence of CTCs has significant impact on PBC patients prognosis regardless BC subtypes, detection technique and the time of their evaluation. Detection of DTCs not correlate with the CTCs presence.

1.1.2.3 Prognostic value of DTCs:

DTCs are similarly to CTCs more prevalent in patients with MBC in comparison to PBC: 73 - 79 % vs. 17 - 21% (Janni W et al., 2000).

First data comparing the presence of CTCs in peripheral blood and DTCs in bone marrow showed the detection rate of CTCs in 31 % and DTCs in 27 % of 92 patients with PBC (Krishnamurthy S, et al., 2010). Both CTCs and DTCs occurred simultaneously in only 7.9 % of patients. Also in other studies was observed a weak concordance between CTCs and DTCs (Fehm T, et al., 2009). On the other hand, Schindlbeck investigated the presence of DTCs and CTCs by simultaneous examinations in 202 BC patients at different stages of the disease. They found a significant congruence 73% between DTCs and CTCs positivity (Schindlbeck C, et al., 2013).

The lack of correlation between CTCs and DTCs raises the possibility of independent modes of dissemination to the different homing sites. Some authors point out that CTCs are short-living in comparison to DTCs so the CTCs would only carry the information similar to the primary tumor and acutely influenced by therapy while DTCs aggregate information about their origin, about the treatment they were influenced by and also about the target organ microenviroment.

DTCs in thebone marrow of BC patients are an independent significant predictor for poor prognosis (Braun S. et al., 2005). Several studies have also indicated that the presence of DTCs in BM after adjuvant therapy is a predictor of poor prognosis (Wiedswang G. et al., 2004, Janni W. et al., 2000).

The identification of patients at increased risk for recurrence after completion of adjuvant chemotherapy is an application of high clinical relevance, since these patients might benefit from an additional 'second-line' treatment, for example, bisphosphonates. It is considered that bisphosphonates may alter the DTCs microenvironment in the bone and so target tumor cells and have antitumor activity (Aft R, et al., 2010). Emerging evidence suggests that zoledronic acid has antitumor and antimetastatic properties, including the inhibition of angiogenesis, tumor-cell invasion, and adhesion in bone; the induction of apoptosis; antitumor synergy with cytotoxic chemotherapy; and immunomodulatory effects (EBCTCG, 2015).

In Kasimir-Bauer study (Kasimir-Bauer S et al., 2016) no prognostic significance was found for DTCs. It seems thet the early clodronate administration can improve prognosis of BC patients.

The prognostic relevance of HER2 expression on DTCs in BM was also documented (Braun S. et al., 2001) and suggests that additional patients could benefit from HER2-directed therapies.

1.1.2.4 Predictive value of CTCs:

Predictive significance of CTCs is the key point for clinical application of CTCs-testing. Here I present the current knowledge about CTCs to discuss questions asked in the introduction of the chapter 1.1:

1. **Does the presence (number) of CTCs differ in patients responding and non-responding to the therapy?**

To properly answer the question No. 1 several metaanalyses were evaluated with a strong focus on methodology used for CTC-separation.

For the bellow pointed data derived from studies using CellSearch system only, the answer is YES . It indicates that CTCs levels < 5 CTCs or ≥ 5 CTCs / 7.5 ml have prognostic importance:

- The baseline detection of CTCs identifies a cohort of patients with a disease intrinsically highly-resistant to systemic therapies and rapidly progressing (Cristofanilli M. et al., 2004).
- A baseline detection of < 5 CTCs indicate an intrinsically indolent disease with sensitivity to systemic therapies (Cristofanilli M. et al., 2004).
- A pretreatment level ≥ 5 CTCs / 7.5 ml was associated with an increased baseline number of metastatic sites compared with < 5 CTCs/7.5 ml. At the time of treatment failure, patients with ≥ 5 CTCs / 7.5 ml more frequently developed new metastatic lesions, have shorter time to develop new / visceral metastases (Giuliano M. et al. 2014).
- Various first-line treatment modalities may have differing capabilities in reducing the number of CTCs (Giuliano M. et al., 2011).
- Patients with persistent CTCs after therapy had worse prognosis than those with decreasing number of CTCs (< 5 CTCs) (Liu M. et al., 2009, Nolé F.

et al., 2008, Martín M. et al., 2013).

- An assessment of CTCs is an earlier and more reproducible indication of disease status than current imaging methods (Budd G. et al., 2006).

According to the meta-analysis of 50 studies (more than 6700 patients) monitoring effect of neo/adjuvant, palliative or combined therapy to the overall CTC-positive rate, CTCs count significantly decreased after therapies (Yan WT. et al., 2017), the answer is YES.

- Compared to pre-therapy, CTCs-positive rates were significantly decreased after treatment in HER2+ patients and HER2- patients, but not in the TNBC patients. These results indicate that different molecular subtypes of BC affect the efficacy of therapeutics on reducing CTCs.
- The CTCs-reduced patients had a longer overall survival period compared to the CTCs-unchanged or -elevated patients (mean difference = 11.61 months).

According to the meta-analysis of neoadjuvant studies (Fei F. et al., 2014) number of CTCs does not correlate with the treatment response,but there is still a lack of data, the answer is NO.

- There is no association between the decrease of CTCs number and pCR after NACT. A decrease in the CTCs count after NACT in locally advanced BC patients did not indicate that they had an improved response to NACT.

2. **Does the early change of therapy in non-responders (no decrease of CTCs number) better patients outcome? NO.**

- SWOG s0500 trial did not support the assumption of clinical benefit of early chemotherapy change in patients with MBC and persistent CTCs after the first cycle of therapy (Smerage JB. et al., 29014).
- **A possible reason: the characterisation of CTCs was not performed in this study.** A possible therapy: patients with persistent CTCs after the

first-line regimen should NOT be treated with standard/empiric chemotherapy and/or patients should by treated with CTC-navigated therapy.

3. Does characteristics of CTCs differ in responders/non-responders? WE HAVE LITTLE DATA.

The answer is that WE HAVE LITTLE DATA, but it seems that CTCs with stem- cells and or EMT characteristics are related to non-respoding patients. Also MBC patients not-responding to first line aromatase inhibitors therapy had different molecular characterisation of CTCs than responders.

- According to Kasimir-Bauer results (Kasimir-Bauer S. et al., 2016) in BC undergoing NACT the number of CTCs decreased during the therapy from 24 % to 20 %, but 72 % of the residual cells were characterized as ERCC1-positive, indicating therapy-resistant tumor cell populations. The fact that ERCC1-positive CTCs were present after these therapies might indicate that these cells survived treatment. Moreover 51 % of CTCs before a 20 % after the NACT had SCs-like characteristics and 47 % and 14 % of CTCs before and after therapy expressed at least one marker connected to EMT. Although no significant correlations were observed for response and SCs-like CTCs before therapy, logistic regression identified a significant relationship between SCs-like CTCs and the group of complete responders vs. no remission.
- Aktas et al. published results of 226 blood samples from 39 patients with MBC. CTCs positivity they detected in 31 % of samples. The most of CTCs-positive samples expressed minimally one of EMT or SCs-markers. The aggressive phenotype of CTCs was detected mainly in the group of non-responding patients (Aktas B, et al., 2009).
- Yu et al. described groups of responders and non-responders (patients with BC undergoing chemotherapy and/or targeted therapy) and proved that patients who responded to therapy showed a decrease in CTCs numbers and/or a proportional decrease in mesenchymal CTCs compared with epithelial CTCs in the posttreatment sample. In contrast, five patients who

had progressive disease while on therapy showed an increased number of mesenchymal CTCs in the posttreatment sample (Yu M. et al., 2013).

- An 8-gene CTCs predictor was identified in MBC patients which discriminates good and poor outcome to first-line aromatase inhibitors. Among 45 patients treated with aromatase inhibitors, those who did not reponse (defined as disease progression or death < 9 month) had different molecular characterisation of CTCs than responders (Reijm EA. et al., 2016).

4. How to obtain relevant information about CTCs character (which method has to be used)? WE DON'T KNOW YET.

Several studies demonstrated the feasibility of genome- wide characterization of CTCs (Lang JE. et al., 2015, Hannemann J. et al., 2011) and also provided evidence of their malignant origin (Magbanua MJ. et al., 2013). For example, microarrays and next generation sequencing analysis have been succesfully used to analyze global gene expression and DNA copy number aberrations in CTCs. But the only proper methodology has not been identified yet.

5. Does the CTCs-navigated therapy better the outcome of BC patients? NOT YET.

Studies indicate the potential clinical significance of monitoring biomarkers in CTCs during treatment or at disease progression. The use of CTC-based biomarkers to guide therapy, however, has not been incorporated into practice due to the need for further technical and clinical validation.

- The use of anti-HER2 therapy in patients with MBC prolonged PFS in those with HER2+ CTCs. Moreover, among patients with HER2+ CTCs, those treated with anti-HER2 therapy had again better PFS than patients not treated with targeted therapy (Liu Y. et al., 2013).
- Several studies have shown that HER2-targeting drugs have a substantial effect in decreasing CTCs count (Pierga JY. et al., 2012, Giuliano M. et al.,

2011) but there are also studies with a converse result showing that anti-HER2 therapy had a limited effect on the number of HER2-overexpressing CTCs (Riethdorf S. et al., 2010).

- Decreased count of CTCs after anti-HER2 therapy also have no positive effect on OS/PFS among studies.
- Ongoing clinical trials should answer the question of CTCs character significance mainly in BC patients with discordant ER and/or HER2 status among primary BC and CTCs (see next chapter).

6. If not, what is the reason? Does the dynamic behaviour of CTCs have an impact on the therapy effectiveness? PROBABLY YES.

- As written above (Liu Y. et al., 2013), targeting HER2 has an impact to PFS prolongation; the autor proposed a CTCs HER2+ criterion, defined as > 30 % of CTCs over-expressing HER2. Among patients with HER2+ CTCs (>30 % of CTC), those treated with anti-HER2 therapy had prolonged PFS, vice versa patients with < 30 % of CTCs (assessed as HER2-) had no benefit from the targeted therapy. Among patients with HER2- CTCs (< 30% of CTCs), anti- HER2 therapy did not significantly improved PFS.
- According to previous mentioned article (Yu M. et al., 2013) rare primary tumor cells simultaneously expressed mesenchymal and epithelial markers, but mesenchymal cells were highly enriched in CTCs. Serial CTCs monitoring in 11 patients suggested an association of mesenchymal CTCs with disease progression. It seems that CTCs represent aggressive tumor clones delivered from the primary tumor into the blood.

7. How to treat PBC and MBC patients more effectivelly? Are they more important characters of the primary tumor or CTCs in the therapy indication? WE HAVE LITTLE DATA YET.

Concordance/discordance between primary tumor/CTCs and/or metastasis in molecular characterisation is the key point to predict therapy effectiveness.

- The utility of using anti-HER2 therapy in patients with HER2- primary tumors and HER2+ CTCs was tested in a phase II study in patients with MBC given lapatinib (Pestrin M. et al., 2012). Among the 7 of 96 patients who had HER2+ CTCs treated with lapatinib, no objective tumor responses were seen.
- The premise of HER2-targeted therapy based on CTCs expression alone is under investigation in the larger ongoing DEFECT III trial, which is randomizing patients with initially HER2- MBC and HER2+ CTCs to standard therapy with or without lapatinib. More data about ongoing studies see next section.

1.1.2.5 Concordance/discordance of the primary tumor and CTCs/DTCs:

CTCs- navigated therapy could be the way how to identify the suitable therapy at the right time.

Aktas et al. analyzed CTCs with AdnaTest and showed high discordance in ER, PR and HER2 status expression in MBC patients (Aktas B. et al., 2011). The overall detection rate for CTCs was 45 % (87/193 patients) with the expression rates of 48 % for HER2 (42/87 patients), 19 % for ER (17/87 patients) and 10 % for PR (9/87 patients), respectively. Comparisons with the primary tumor were only performed in CTCs-positive patients (n=87). In 48/62 (77 %) of patients with ER+ tumors, CTCs were ER- and 46/53 (87 %), patients with PR+ tumors did not express PR on CTCs. Primary tumors and CTCs displayed a concordant ER and PR status in only 41 % (p=0.260) and 45 % (p=0.274) of cases, respectively. Fehm et al. found discordance rates between primary tumors and CTCs in PBC for ER and PR to be 71 % and 75 %, respectively (Fehm T. et al., 2009).

Discrepancy between the receptor status of primary tumor and distant metastases could lead to inappropriate choice of systemic hormonal treatment. In a study a total of 233 distant BC metastases from different sites (76 skin, 63 liver, 43 lung, 44 brain and 7 gastro-intestinal) were IHC stained for ER, PR and HER2, and expression was compared to that of the primary tumor. Using a 10 % threshold,

receptor conversion by IHC for ER, PR occurred in 10 % and 30 % of patients, respectively. Conversion was observed mainly from positive in the primary tumor to negative in the metastases for ER and PR, while HER2 conversion occurred equally both ways in 5 %. PR conversion occurred significantly more often in liver, brain and gastro-intestinal metastases. (Hoefnagel LDC. et al., 2010).

Discordance in HER2 status between primary tumor and CTCs reports are variable, in order of 15–35 % in MBC (Fehm T. et al., 2010, Munzone E. et al., 2010, Flores LM et al., 2010). As well as the varying methods of CTCs enrichment and HER2 detection utilized, the definition of HER2 positivity is widely ranging across studies. HER2 discordance has also been reported in PBC patients, more often in terms of de novo expression of HER2 (50 % of patients with HER2- primary tumors had HER2+ CTCs) on CTCs than vice versa (33 % of patients with HER2+ primary tumors had HER2- CTCs) (Wülfing P. et al., 2006).

Solomayer detected cytokeratin (CK)-positive DTCs in 34 % of patients with PBC. DTCs with HER2+ were found in 20 (43 %) of these patients. Concordance rate of HER2 status between primary tumor and DTCs was 62 %. In 12/20 (60 %) patients with HER2- tumors HER2+ DTC were detected (Solomayer EF. et al., 2006). Krishnamurthy et al. did similar study in patients with stage I-IV BC. Data from 95 patients with PBC and BM from 78 patients were published. The overall rate of discordance in HER2 status was 15 % between primary tumor and CTCs and 28.2 % between primary tumor and DTCs (Krishnamurthy S. et al., 2013). Patients with HER2+ CTCs have been reported to have worse prognosis in comparison with patients with HER2- CTCs or any detectable CTCs (Hayashi N. et al., 2012).

CTCs are very often expressing HER2 or are triple negative. The acquisition of genomic aberrations is associated with tumor relapse or disease progression. HER2 status may change during the course of the disease so it arises the question of using trastuzumab or other HER2- blockers in therapy of HER2+ CTCs. Preliminary data demonstrated efficiency of anti-HER2 therapy in patients with HER2+ CTCs (Meng S. et al., 2004). Meng used fluorescence in situ

hybridazation as a very sensitive blood test to determine HER2 status in CTCs. Another possibility is the using of PCR-based method such as AdnaTest Brest Cancer. The most autors use a cutoff level of at least one HER2+ CTC per 7.5 ml of blood in primary setting or at least one CTC with IHC score of 3+ from a minimally 5 detectable CTCs in metastatic setting. There is no standardized method for determining HER2 status in CTCs yet.

The clinical implications of evaluating HER2 status in CTCs and DTCs in BC needs to be established in prospective clinical trials; the overview was published by Bidard (Bidard FC. et al., 2013). All trials have been designed using the CellSearch System. They should answer the question of predictive role of CTCs, which could guide the treatment decisions of clinicians.

STIC CTC METABREAST phase III trial (Bidard F. et al., 2012) should answer the question of CTCs-driven decision of first line hormonal therapy vs. chemotherapy in patients with hormone sensitive/HER2- MBC.
CircCe01 (Bidard FC. et al., 2013) is a single arm two-step phase II multicenter study with an adaptive design. Patients with HER2- measurable MBC with non HER2-amplified CTCs or no CTCs will be excluded from the study. Patients with HER2-amplified CTCs will be treated by T-DM1, in either cohort “L” (low count: 1 to 4 HER2-amplified CTCs) or in cohort “H” (high count: 5 or more HER2-amplified CTCs). Tumor response (per RECIST criteria) is the main objective of the trial.

TREAT CTC study and DETECT III study are based on BC patients with HER2- primary tumor but HER2+ CTCs in blood.
TREAT CTC study will screen non-metastatic patients after completing neo-/adjuvant therapy and surgery and randomize women with HER2+ CTCs between the trastuzumab arm and observation arm.
DETECT III study will compare standard therapy alone vs standard therapy plus lapatinib in patients with MBC and HER2+ CTCs.
Both studies should be completed in 2018.

In conclusion: The prognostic value of CTCs has a stable position in tumor disease management. The poorest prognostic group, which is defined by positive CTCs count at baseline and after therapy, corresponds to two distinct settings: tumor chemoresistance that is restricted to the type of chemotherapy used and tumor chemoresistance that is not drug dependent. A CTC-driven early switch of chemotherapy may be of benefit in first setting but not in the second.

The predictive ability of CTCs has predominantly been assessed in relation to HER2, with variable and inconclusive results. Limited data exist for other biomarkers, such as the ER. In addition to the need to define and validate the most accurate and reproducible method for CTCs molecular analysis, the clinical relevance of biomarkers, including gain of HER2 on CTCs after HER2- primary breast cancer, remains uncertain.

1.1.3 Therapeutic targeting of cancer stem cells (CSCs)

We still believe that the only therapeutic approach killing both SCs and diferentiated cells is potentialy curative (Alaoui-Jamali MA, et. al., 2011). CSCs are the key cells that drive tumor growth and that must therefore be eliminated in order to achieve the cure. Some properties of CSCs, such as quiescence, resistance to apoptosis (Fuchs D. et al., 2009, Riccioni R. et al., 2010), expression of drug-resistance transporters may make them difficult to elimate using conventional cytotoxic drugs that kill the bulk tumor cells. Furthermore, the acquisition of EMT features has been associated with chemoresistance, which could give rise to recurrence and metastasis after standard chemotherapeutic treatment (Iwatsuki M. et al., 2010).

Molecular pathways for survival and response to injury may be fundamentally different in these cells compared to non-tumorigenic cells. If CSCs are genetically diverse, they could represent a moving therapeutic target (Faltas B. et al., 2011).
Current cancer therapy is mainly based on reduction of tumor mass as much as

possible, usually by combining surgery and relatively unspecific treatment such as chemotherapy or radiotherapy, which target rapidly proliferating cells.

Dr. Wicha et al. found that potent regulators of CSCs are cytokines, in particular, IL-6 and IL-8. IL-8 binds to the cytokine receptor CXCR1, and this stimulates self-renewal of CSCs through the Wnt pathway (Ginestier Ch. et al., 2010).
Repertaxin is an inhibitor of CXCR1 that potently blocks the chemotherapy-induced increase in CSCs. A phase I clinical trial is evaluating the strategy of adding repertaxin to paclitaxel, and early results suggest relative lack of toxicity.
The IL-6 inhibitor tocilizumab also appears promising as a means of reversing resistance to trastuzumab in the HER2-overexpressing population. HER2 is believed to be an important driver of breast CSCs (Korkaya H, et a., 2012). In experiments with docetaxel found that while the taxane killed cancer cells, the dying cells actually stimulated the production of CSCs trough the release of interleukins.

Additional experiments showed that the overexpression of mir-93 inhibits the growth of established tumor xenografts. Wicha's team defined a set of micro-RNAs that are over- or underexpressed in SCs, one of which they considered particularly interesting: micro-RNA-93 (mir-93), which is located in the *MCM7* gene and is believed to function as a tumor suppressor. They found that low mir-93 expression is associated with tumor-initiating capacity, while its overexpression diminishes the presence of CSCs. Resistance to cytotoxic chemotherapy - in this experiment, docetaxel - was also abrogated in the presence of mir-93 (Liu S. et al., 2012).

New therapeutic strategies are based on the presence of known molecular target. First promising molecule was tested in 2006; NOTCH inhibitor MK-0752 and others are expected (Vantictumab). Currently, there are multiple potential anti-CSCs agents in pre-clinical and clinical trials, including Hedge-hog, NOTCH, HER2/AKT (Korkaya H. et al., 2012, Diessner J. et al., 2014, Zhu Y. et al., 2012, Liu Y. et al., 2014).

1.2 Metastatic pathway

1.2.1 From undirectional pathway through seed and soil theory to disseminated tumor cells and parallel model of tumor dissemination

Cancer progression is commonly segregated into the proces of primary tumor growth and secondary metastasis. The term of MRD was incorporated as an intermediate step after the long time of scientific research. The oldest concept of cells circulating in the blood was later supplemented with the term so-called disseminated cells, cells which, after release from the primary tumor and the transient phase in the blood circulation, are seeded into the bone marrow where they can persist for many years. Recently, viable circulating cells have been supplemented with an existence of non-viable part of tumor cells in the form of cell free DNA (cf-DNA), circulating tumor DNA (ct-DNA), circulating exosomes or circulating micro-RNA (miRNA).

Historically, accepted theories of metastatic pathway focus on **undirectional pathway from a primary tumor to metastasis**. This theories rely on rapid growth of primary tumor cells, their genomic aberrancies and influence to microenviroment and finnaly migration to the blood and lymphatic vessels and travelling to distant organs where they form metastases (Weinberg A., 1991, Hanahan D., Weinberg A., 2000). It was also believed that the primary tumor must reach a critical mass to be able to develop metastases.

The discovery of first oncogenes and tumor suppressor gene RB1 in the 80s and 90s of the 20th century were the basis of so-called somatic mutational theory based on the idea of monoclonal beginning of carcinogenesis - tumor formation from a single renegade cells (Weinberg A., 1998). By contrast, the tissue organization eld theory (Sonnenschein C., Soto AM., 1998, Soto AM., Sonneschein C., 2004) considers loss of physiological structure and function by a tissue as key events in tumor development. According to this theory, tumors arise at a tissue rather than at a cellular level (Sonnenschein C., Soto AM., 2005). It is based on a

presumption that proliferation status, rather than quiescence, is the default position of cells in multicellular organisms.

The background for tumor dissemination is the theory called **„tumor self seeding“**. It is an ability of cancer cells to migrate not only to regional and distant sites of the organ but also back to their original source, so migrate into the primary tumor. The cells leaving a tumor mass were called „**circulating tumor cells“** and their presence was recognized in the 1800th (Ashworth TR., 1869). The tumor self seeding was first discovered by Stephen Paget in 1889 (Paget S., 1889). By analysing 735 historical cases of fatal BC, he found that metastases are formed in the liver far more often than in any other organ. "When a plant goes to seed, its seeds are carried in all directions," he wrote. "But they can only live and grow if they fall on congenial soil." He presumed that sites of secondary growths are not a matter of chance, and that some organs provide a more fertile environment than others for the growth of certain metastases. Seed and soil hypothesis was revived fully in 1980 (Hart IR., Fidler IJ., 1980) and later on by Norton and Massague in 2006 (Norton S., Massague J., 2006) who tested their hypothesis in a mouse model. They showed that cells from primary tumors can attract circulating metastatic tumor cells, and they identified several proteins that likely encourage this migration.

They also found that the return of metastatic cells promoted primary tumor growth by releasing proteins that change the tumor’s microenvironment, including blood vessels and immune cells. Tumor-derived inflammatory cytokines, such as IL-6 and IL-8, act as CTCs attractants. The self-seeding CTCs also express matrix metalloproteinases (MMP1/collagenase-1), the actin cytoskeleton component fascin-1, and CXCL1, which promote accelerated tumor growth, angiogenesis, and the recruitment of myeloid cells into the stroma (Kim MJ. et al., 2009).

We can distinguish tumors, which are good or poor self-seeders. In case of good self-seeder CTCs are attracted back to the primary tumor acting as a sponge and contributing to an enlarging locally advanced breast cancer. The effect of self-seeding to the growth of primary tumor is dependet on the size of tumor and the size of CTCs population, the aggressiveness of the CTCs, tumor microenviroment

and other factors. Poor self-seeders but an efficient distant seeders can be seen in cases of small aggressive tumors which are leading to metastatic disease or local recurrence after excision of primary tumor.

CTCs can be more or less aggressive, but in general they are potentially metastasis-initiating cells. The process of self-seeding does select for cancer cells populations that are more aggressive than the bulk population of the primary tumor. Moreover, self-seeding is actively driven by the ability of CTCs to sense attraction signals from the tumor and to extravasate in response to such signals. Although it was shown that only 1 % of micro-metastases progress in macro-metastases (Wittekind C., Neid M., 2005), secondary tumor masses can be the source of new CTCs.

The recent explanation of tumor cell targeted migration in the blood stream is based on discovery of **exosomes.** Cancer cells can release molecules into the bloodstream, which are enclosed in this microscopic exosomal bubbles (Peinando H. et al., 2012). Tumor exosomes carry a cocktail of molecules, including proteins and DNA. When they come into contact with a cell they can fuse with it, delivering their contents and changing the cell behaviour. Different exosomes are equipped with specific combinations of integrin molecules on their surface what is the key how exosomes identify their preferred settlement location (Hoshino A. et al., 2015). It is assumed that one exosome can contain whole genome of the primary tumor cell, on the other hand exosomes can be probably produced by CTCs and carry information of more or less aggressive metastatic cell.

As written above, there are several lines of evidence which indicate that tumor cells can leave the primary site very early during tumor progression so the secondary **tumor mass may grow in parallel with the primary tumor** (Klein Ch., 2009). This model was supported by molecular genetic analyses of primary tumor cells and DTCs. It was shown that precursors of metastasis are derived from the most advanced clone within the primary tumor (Schmidtt-Kittler O. et al., 2003). The presence of micrometastasis in the bone marrow at the time of diagnosis of tumor disease is associated with a poor prognosis (Braun S. et al., 2005) which indicate higher incidence of tumor dissemination.

Parralel dissemination of CTCs/DTCs is the reason why some patients with only primary tumor without involvement of lymphatic nodes and after resection of primary tumor are dying because of development of systemic disease. On the other hand, some patients with extensive disease do not develop systemic metastases. The DTCs can remain dormant for varying periods of time, which could be mediated by cytostatic $CD8^+$ T cells and predicts that immune responses favor dormancy of DTCs (Eyles J. et al., 2010). Contrary, inflammation enhances cancer progression by facilitating EMT and entry into the circulation (Rhim AD. et al., 2012).

The presence of DTCs was also detected in bone marrow of patients of nonsinvasive breast cancer (Sänger N. et al., 2011). But cells of ductal carcinoma in situ (DCIS) seem to be less immunogenic, which might result in a diverging way to evade immunosurveillance (Gruber IV. et al., 2016). So it seem that inflammation and immune response are important mediators of tumor disease spread.

In conclusion: Two papers shed light on the mechanism of early dissemination and describe early disseminated cancer cells (DCCs) which are more metastasis- competent than cells that leave the tumor at later stages (Hosseini H. et al., 2016, Harper KL. et al., 2016). We know that the release of substantial quantities of CTCs occurs early in tumors under 3 mm at diameter (Coumans FA. et al., 2013). Parallel model of tumor dissemination and the possible inhibiting effect of immune system and tumor microenviroment are the two main components of complex tumor spread process.

1.2.2 Tumor microenviroment

The role of the tumor stroma and tumor-stromal interactions is discussed more and more frequently. The microenviroment of tumor cells "stem cell niche" regulates CSCs and by the process of EMT plays the role in reverting non-tumorigenic cells into CSCs and in the progression of the primary tumor, leading to dissemination and invasion of tumor cells. The location of secondary tumors

also seems to be regulated by the microenviroment (Borovski T., De Soula E., et al. 2011).

The niche is a complex of different stromal cells, such a mesenchymal and immune cells, a vascular network, soluble factors, and extracellular matrix components. The stem cell niche plays an essential role in maintaining stem cells or preventing tumorigenesis by providing primarily inhibitory signals for both proliferation and differentiation in adult somatic tissues. However, the niche also provides transient signals for SCs division to support ongoing tissue regeneration. Loss of the niche can lead to the loss of SCs, indicating the reliance of SCs on niche signals. Deregulation of the niche can lead to uncontrolled proliferation of SCs resulting in tumors (Li L., Neaves WB., 2016).

The tumor microenviroment is also important for metastasis formation through the process of EMT and for the localization of secondary tumors by forming the premetastatic niche (Psaila B., Lyden D., 2009). In response to growth factors secreted by the primary tumor (VEGF, PDGF, TGF-β) inflammatory chemokines and serum amyloid A are upregulated in premetastatic sites leading to clustering of bone marrow-derived haematopoietic progenitor cells. These cells secrete a variety of premetastatic factors (TNF-α, MMP, TGF) and activated fibroblasts secrete fibronectin, an important adhesion protein in the niche. Metastatic cells engraft the niche to populate micrometastases. The site-specific expression of adhesion integrins on activated endothelial cells may enhance metastatic cells adhesion and extravasation at these sites. Recruitment of endothelial progenitor cells to the early metastatic niche mediates the angiogenic switch and enables progression to macrometastases.

Over recent years, there has been an appreciable increase in our understanding of the crosstalk between tumor cells and stroma on the systemic, cellular, and molecular level (Langley RR., Fidler IJ., 2011). There were isolated cells that preferentially infiltrate specific organs and the gene expression analysis of these cells zoom in the molecular basis of metastatic spread (Bos PD. et al., 2009, Minn AJ.,et al., 2007, Lu X. et al., 2009).

In conclusion:

The five concepts to understand the tumor microenviroment by AACR (American Association of Cancer Reasearch) dated to 2017:

1. its dynamic evolution is reflecting tissue remodeling, metabolic alterations in the tumor, and changes in the recruitment of stromal cells,

2. how is tumor microenviroment and immune system which is its part educated by tumor cells,

3. pathways of communication between stromal and tumor cells via exosomes and cell fusion,

4. immunomodulatory roles of the lymphatic system which can promote immune tolerance and distant metastasis, and

5. contribution of the intestinal microbiota in inflammation-associated carcinogenesis (Schwartz MA. et al., 2012).

1.2.3 Tumor cell dormancy

The tumor microenviroment is also dictating the tumor cell dormancy. The mechanism of the cell dormancy is largely unknown but it is supposed, that it concerned about survival strategy. The tumor cell dormancy explains the long time of clinical remission and disease relapses years after the diagnosis was done. The bone marrow may contain dormant cancer cells, so the assessment of the environment that shapes the metastatic niche in the bone marrow might be even more informative (Uhr JW., Pantel K., 2011).

It has been shown that approximately 70 % of BC cells settled to bones in still metastasis-free patients corresponded to SC phenotype. These cells are sometimes capable of initiating bone metastases, sometimes not. Zhang et al. (Zhang X. et al., 2009) reported that a gene-expression signature of c-Src activation is associated with late-onset bone metastasis in breast cancer. c-Src mediates AKT regulation and cancer cell survival responses to CXCL12 and TRAIL, factors which are distinctively expressed in the bone metastasis microenvironment.

Dormant cells are nonproductive: “growth-suppressed or sleeping” by certain organ microenviroments. Bragado (Bragado P. et al., 2012) published three

potential scenarios contributing to the tumor cell dormancy. The first possibility supposes the activation of inhibitory signals from target organ microenviroment, which can activate dormancy in DTCs. The second scenario shows the possibility that the gene signature of primary tumor prime new DTCs to enter dormancy. So the gene signature of the primary tumor carries information about metastasis formation timing, self-seeding and microenviroment modulation. The third scenario hypothesizes that pre-malignant cells undergo EMT to form early DTCs, which aren't probably fit to initiate metastatic cascade so thus undergo dormancy. The microenviroment or epigenetic mechanism can reverse the growth-restrictive signals and allow early DTCs to growth and accumulate additional genetic alterations that eventually produce cells fit to initiate metastasis. The above mentioned and recently published data about the significance of early DCCs support the third hypothesis.

Cheng et al. (Cheng Q. et al., 2014) published their findings suggesting that EMT-related genes in the tumor epithelium are related to both stromal activation and escape from disease dormancy. They identified a late recurrence gene signature in the primary tumor suggesting that intrinsic features of the tumor regulates the transition of DTCs into a dormant phenotype with the ability to outgrowth as recurrent disease.

DTCs carry information about their origin (i.e. primary tumor microenviroment), about how treatment influenced their adaptation and how the targed organ microenviroment influenced their adaptation and/or selection. Due to the microenviroment influence, DTCs can rest in inactive cell cycle phases and survive the systemic treatment or can overcome dormancy control. It is considered that bisphosphonates may alter the DTCs microenvironment in the bone and so target tumor cells and have antitumor activity (Aft R. et al., 2010).

CTCs, because they are short- living in circulation, carry only information similar to the primary tumor and acutely influenced by the therapy. CTCs and DTCs represent the tool for studying the metastatic process in its initial stage. Thus comparison of CTCs and DTCs gene profile could provide crucial information about whether they provide similar or different information about dormancy

phases and subsequent progression to overt disease.

In conclusion: Tumor cells dormancy is the key strategy for metastasis formation after the long term of disease remission. The main role in this process is probably played by DCCs. The reactivation of dormant cells is done by microenviroment signals or epigenetic mechanisms.

1.2.4 Cancer stem cells (CSCs)

Cancer is known to result from the accumulation of multiple genetic mutations in a single target cell, sometimes over a period of many years. Because SCs are the only long-living cells in many tissues, they are the natural candidates in which early transforming mutations may accumulate.

They are three key observations, which define the existence of a CSCs population (Dalebra P. et al., 2007):

a) Tumors grown from tumorigenic cells contain mixed populations of tumorigenic and non-tumorigenic cancer cells, thus recreating the full phenotypic heterogeneity of the parent tumor.

It was shown that normal mammary SCs and breast CSCs exist side-by-side; population of $CD44^+$ tumor and normal cells are similar to each other. In a normal breast tissue there is a small population of SCs, which both self-renew and, by the process of asymmetric cell division, give rise to an intensively proliferating progenitors- transit amplifying cells (TA) which are the source of terminally differentiated cells (TDC) in breast.

b) They have the tumorigenic potential.

The process of CSCs arising can be double-dealing (Cariati M., Purushotham AD., 2008). In the first way, the oncogenic mutation affects the normal SCs or cells in the niche: the microenviroment has a crucial role in the control of SCs maintenance and self-renewal. In the second way it is effect on TA cells leading to the loss of SCs dependence on niche inhibitory signaling. The process of EMT

can also change the cell phenotype. This phenomenoma is connected to the process of tumor dissemination in which cells (probably SCs also) obtain the phenotype of invasiveness and motility, so characteristics of cells which are able to circulate in blood, overcome barriers and colonize second organs. Along with differentiation-dedifferentiation plasticity of SCs in conjunction with microenviromental changes, the tumor is extreme plastic and dynamic mass explaining the prognostic role of tumor cells with high tumorigenic activity (Liu R. et al., 2007). The model of CSCs also explains the reason of similarity for the primary tumor and metastasis (Weigelt B. et al., 2003).

c) They are characterized by a distinctive profile of surface markers.

Al-Hajj (Al-Hajj M. et al., 2003) isolated a $CD44^+/CD24^-$ subpopulation of BC cells in 2003. It was for the first time when someone distinguished the tumorigenic cells from the nontumorigenic cancer cells based on cell surface markers expression. $CD44^+/CD24^-$ cells represent 11 - 35 % of total cancer cells. SC phenotype was also confirmed in DTCs (Balic M., et al. 2006).

The CD44 antigen is a cell-surface glycoprotein involved in cell–cell interactions, cell adhesion and migration. In humans, CD44 is encoded by CD44 gene on chromosome 11. CD44 is expressed in a large number of mammalian cell types. CD44 is a receptor for hyaluronic acid and can also interact with other ligands, such as osteopontin, collagens, and MMP. CD44 gene transcription is at least partly activated by β-catenin and Wnt signalling (also linked to tumor development). CD44 protein participates in a wide variety of cellular functions including lymphocyte activation, recirculation and homing, hematopoiesis, and tumor metastasis. Variations in CD44 are reported as cell surface markers for some breast and prostate CSCs. It is involved in cell proliferation, cell differentiation, cell migration, angiogenesis, presentation of cytokines, chemokines, and growth factors to the corresponding receptors, and docking of proteases at the cell membrane, as well as in signaling for cell survival. In the relation to SC phenotype no specific marker has been identified yet.

In connection to BC stemness we can consider also ALDH1 or ABCB1/ABCG2, from signal pathways NOTCH, Wnt-β-Catenin, HedgeHog or JAK-STAT pathway (Hatina J. et al., 2013). Characteristics of SCs like resistance to DNA damage, expression of genes of Bcl-2 family or detoxifying enzymes like ALDH (Ginestier C. et al., 2007), and up-regulation of cell surface transporters of the ATP-binding cassette family (ABC) which are responsible for the efflux of cytotoxic compounds (Doyle LA. et al., 1998) are the reason of limited effect of the conventional anti-mitotic therapy.

ALDHs are a group of enzymes that catalyse the oxidation (dehydrogenation) of aldehydes. ALDH1 can be used for identification of SCs (Douville J. et al., 2009). CXCR1 or IL8RA gene encodes Interleukin 8 receptor, G-protein, which binds to interleukin 8 (IL8). IL8 is upregulated in breast cancer and is associated with poor prognosis. CXCR1 and CXCR2 are important in regulating breast CSCs activity (Singh JK. et al., 2013).

FoxO-3 is a transcription factor, a member of family FoxO. The Pi3K-Akt-FoxO signaling pathway plays a central role in diverse processes including cellular energy storage, growth and survival. It was showed its function in cell cycle regulation, apoptosis, cellular senescence and cellular autophagy in the process of tumorigenesis (Zhang Y. et al., 2011). Akt pathway has an important role in SCs renewal (Ma S. et al., 2008). Akt signaling is often connected to high expression of HER2.

HER2 overexpression drives mammary carcinogenesis, tumor growth and invasion through its effects on normal and malignant mammary stem/ progenitor cells (Korkaya H. et al., 2008). This was demonstrated by *in vitro* mammosphere assays and the expression of SC marker ALDH. So the clinical ability of trastuzumab may relate to its ability to target the CSCs in HER2 amplified tumors. Resistance to trastuzumab could by explain by the development of secondary mutations such as deletion of tumor suppressor PTEN (Saal LH. et al., 2007) or others, which can increase the activity of PI3K/AKT pathway (Berns K. et al., 2007).

1.2.5 Epithelial- to mesenchymal transition (EMT)

Cells undergoing the process of EMT acquire mesenchymal-like phenotype including invasiveness, motility and increased resistance to apoptosis. Due to the process of EMT cells lose cell-to-cell interactions, cell polarity, survive in the bloodstream and get the motility by modifying cytoskeleton. Non-tumorigenic cells can revert into CSCs by this process (Owens TW, Naylor MJ., 2013).

EMT can be induced by variety of signals and factors including Notch, Hedgehog, TGF-β, receptor tyrosinkinases or Wnt (Bonnomet A. et al., 2010). The subsequent signaling involves MAPK, PI3K, NF-kB, Smads, β- katenin, GLI-1, ROS and Notch. In the next step of signaling pathways there are transcription factors and co-activators including Snail, SIP1, delta EF-1, Twist, FOXC2, NFkB and so on. In the final step there are downregulated genes connected to epithelial markers and upregulated genes connected to mesenchymal markers.

Cells invasiveness is associated with the reorganization of many types of intercellular junctions, proteolytic degradation of extracellular matrix (ECM) or cytoskeleton modifications (Thiery JP. et al., 2009). Many cytokeratins are downregulated (E-catherin, occludin, cytokeratin, desmopakin, claudins etc.) and others are upregulated (vimentin, N-catherin, MMPs, fibronectin, collagen etc.). Tumor cells derived by EMT are interacting with endothelial cells by induction of N-catherin and VE-catherin expression. Adherence of tumor cells to the ECM is mainly mediated by integrins. Degradation of the ECM is predominantely mediated by MMPs and urokinase plasminogen activator (uPA) system.

Pro-angiogenic role of EMT is connected to the enhaced expression of proteases, VEGF-A or IL-8. Angiogenic switch is favoring the growth of new vessels.

The markers contributing the resistance of apoptosis are TGF-β, Snail or Twist. CTCs with mesenchymal properties could also extravasate and grow in distant organs.

In the process of metastasis formation, a reverse process appears to occur. It is the process of menechymal-to-epitelial transition (MET), which re-activate certain epithelial properties of tumor cells important in tissue construction so we can see the morphological similarities between primary tumour and metastatic lesions (Chaffer CL. et al., 2007).

It was proven that SCs and EMT associated markers are frequently overexpressed in CTCs of MBC patients (Aktas B. et al., 2009). In this study 226 patients with MBC were evaluated and in a CTCs positive group (31 %) the expression rate of EMT and SCs markers were as follows: 42 % (TWIST), 62 % (Akt2), 58 % (PI3K), and 69 % (ALDH1). SC phenotype of DTCs detected in bone marrow of BC patients was associated with CD44+/CD24+ expression.

More than 60 % of CTCs express NOTCH1 (mainly HER2+ CTCs), a gene associated with self-renewing cancer-initiated cells (Reuben JM. et al., 2010). Furthermore, the acquisition of EMT features has been associated with chemoresistance.

In conclusion: The processes of CSCs arising and tumor cells dissemination are both mediated by the EMT. The whole process of tumor disease evolution and spread requires the close cooperations of tumor cells and tumor niche mediators.

1.3 Detection of minimal residual disease – Liquid Biopsy

A liquid biopsy consists of taking a blood sample for detection of biomarkers of cancer, which might be used for initial diagnosis, determination of prognosis, elucidation of cancer predisposition, and predicting response to (targeted) therapy (Alix-Panabières C., Pantel K., 2013, Alix-Panabières C., Pantel K., 2016).
As reported recently several fractions of a liquid biopsy can be analyzed, including CTCs, exosomes, and free or complexed nucleic acids (Hoffman P., Popper HH., 2016). How the different circulating elements will be extracted and analyzed depends on the clinical situation and the questions to be answered by the liquid biopsy.

CTCs are very rare with the frequency of one cell in 100 million to one in a billion of blood cells (Yu M. et al., 2011). The process of CTCs examination can be divided into the step process of **CTCs enrichment and the step of CTCs detection**.

The principle of the CTCs-enrichment methods is to increase the likehood of CTCs detection. Positive selection is the approach how to select desired cells using their characteristics, the principle of negative selection relies on eliminating undesired blood cells in order to retain tumor cells.

The principle of CTCs detection relies on staining or molecular analysis of CTCs. CTCs can be marked with molecules that target mostly cell membranes of CTCs (e.g. antibodies or aptamers) or molecules that target DNA of CTCs (i.e. DNA primers, DNA probes). The proper step of CTCs detection is done via microscopy, cytometry, reverse-transcription polymerase chain reaction (RT-PCR), quantitative RT-PCR (qPCR), fluorescent in situ hybridization (FISH), or other methods.
Most of CTCs-enrichment procedures rely on combinations of bellow mentioned methodologies. The most frequently used methods are summarized in the table No. 1.

Table No. 1: CTC detection methods – commercialized protocols

CTC detection method	General description of the method	Commercial tests
Immunomagnetic separation	CTCs are selected from a blood with the use of immunological marking and magnetic forces. Detection of cytokeratins and/or EpCAM protein is used. The problem could be non-specific binding of magnetic beads to the blood cells.	CellSearch AdnaTest Mag Sweeper EasySep™
Size-based separation	Size-based separation (or filtration) detects CTCs without being dependent on EpCAM expression of cells. The problem could be the lower sensitivity and specificity. CTCs can be "too" small and pass through the membrane. Bigger pores could cause of contamination of CTCs with white blood cells.	ISET™ ScreenCell™ CTC Membrane Microfilter CTC Chip Metacell® Cellsieve™ Parsortix™
Microchip microfluidic techniques	They capture viable CTCs in devices that exploit physical and/or biological differences between CTCs and other blood cells. CTCs can be further analyzed by cellular, microscopic, or molecular techniques. Dong et al. (Dong Z. et al., 2013) identified 18 different techniques how to detect CTCs by using microfluidic systems.	CEE™ Microfluids HB-Chip ClearCell GEDI
Density gradient centrifugation	It is based on physical properties of CTCs. Cells migrate differentially during centrifugation according to the density. The result is the separation of different cell types into distinct layers.	Ficoll-Hypaque Lymphoprep™ OncoQuick®
Dielectrophoresis field flow fractions (DEP-FFF)	They allow CTCs detection independent of EpCAM expression. These assays analyze morphological and electrical dissimilarities of different cells in blood.	ApoStream® Depparray®
Functionalized structured medical wire (FSMW)	FSMW is a wire that is used for detection of CTCs *in vivo*, which can analyze the bigger amount of the patients' blood volume compared to other previously mentioned techniques.	GILUPI CellCollector™
Immunocytochemistry (ICC) image analysis	It is a method without the EpCAM enrichment. The automated device provides images of CTCs in high definiton (HD-CTC).	HD-CTC

EpCAM- Epithelial Cell Adhesion Molecule

The optimal enrichment scheme doesn't exist due to the extreme heterogenity of CTCs and their rarity in blood. Into other limitations of CTCs detection belong: the volume of blood that can be collected and analyzed and finally the enrichment step by itself, which might be associated with loss of cells others than those with preselected markers on their surface. It is generally accepted that CTCs should be

EpCAM positive, CK-positive, and CD45 (marker of white blood cells) negative. However, some CTCs might be EpCAM/CK-negative in case they went through the process of EMT. Moreover, CTCs and primary tumor cells can differ (Chang HJ. et al., 2011) as well as CTCs can change over time (Wang LH. et al., 2010).

The research activity in the CTCs technology field has almost tripled over the last 10 years. Previously mentioned technical limitations generated inconsistent results from clinical studies performed with the use of different CTCs methods. The rare occurrence of CTCs in non-metastatic disease is the reason why the threshold for CTCs-positivity is different in primary disease in comparison to metastatic malignancy. Therefore the prognostic significance of CTCs, demonstrated by using of various approaches, has not the same impact and need standardization to allow implementation of CTCs technology into the clinical practice.

CTCs have been detected in a majority of epithelial cancers. Available literature about the prevalence of CTCs, detected in BC patients, is summarised in Krawczyk review; CTCs positivity rate reaches 2 - 55 % in primary tumors depending on detection methods, in metastatic setting CTCs positivity is higher, 23 - 75 % (Krawczyk N. et al., 2013). The ability to release tumor cells into the blood is discordant in a variety of different tumors (see table No. 2).

Table No. 2: CTC detection rates differ between tumor types

Source	**Number of patients**	**Malignant disease**	**Stage of the disease**	**CTC positive patients [%]**
Nastaly P. et al., 2014	143	testes	I-III	17.5
Bluemke K. et al., 2009	154	kidney	I-IV	53
Rink M. et al., 2011	5 50	bladder	IV NMA	100 30
Gazzaniga P. et al., 2014	102	bladder	T1G3	20 - 30
De Bono JS. et al., 2008	231	CRCP	IV	57
Kolostova K. et al., 2014	55	PC	T1-T3	20
Wang HY. et al., 2015	774	stomach	III-IV	54
Meyer CP. et. al., 2016	152	pancreas	T1-T3	11
Khoja L. et al., 2012	54	pancreas	IV	40 - 90
Huang X. et al., 2015 Tsai WS. et al., 2016	NA 15	CRC	IV	40 - 85*

*NMA - advanced, non-metastatic disease, CRPC - castrate- resistant prostate cancer, NA - not available, * by the type of methodology*

1.3.1 Non-affinity based enrichment technologies

CTCs enrichment strategies based on technologies which can distinguish CTCs among the surrounding hematopoietic cells according to their physical (density, size, electric charges, deformability) and biological (viability) characteristics, are called non-affinity based.

Size-based separation:

In case of size and deformity differencies, diameter of CTCs is about 12-25 μm, red blood cells are about 5-7 μm big and white blood cells 7-15 μm. Size-based separation (or filtration) of CTCs from the blood sample can detect CTCs, without being dependent on the EpCAM expression. On the other hand the size-based separation shows low sensitivity and specificity. The reason for this is that the small CTCs may be too small and pass through the filter and vice versa some large white blood cells might get caught by the filter and contaminate the CTCs population. Technogies working with this strategy are for example:

- ISET (Isolation by Size of Tumor/Trophoblastic Cells)- isolation by size of epithelial tumor cells using a polycarbonate filter (Paterlini-Brechot P., Benali NL., 2007).
- ScreenCell (Desitter I. et al., 2011) - allows isolation of live cells able to grow in the culture.
- CTC Membrane Microfilter (Zheng S. et al., 2011) is the three-dimensional microfilter device that can enrich viable CTCs.
- CTC Chip - microfluidic platform capable of efficient and selective separation of viable CTCs from peripheral whole blood samples, mediated by the interaction of target CTCs with antibody (EpCAM)-coated microposts under precisely controlled laminar flow conditions, and without requisite pre-labelling or processing of samples (Nagrath S. et al., 2007).
- Metacell® (s.r.o., Czech Republic)- peripheral blood is filtered through membrane (8 μm pores), membrane with captured cells is transferred into cultivation plate in the next step. Captured cells are cultured *in vitro* under standard conditions (37^{o}C, 5% CO_2) for 3-5 days and then nucleus and

cytoplasm of viable cells are stained and identified according to usual cytopathologic criteria for CTCs (Kolostova K. et al., 2014, Kolostova K. et. al., 2016, Bobek V. et al., 2014).

- CellSieve™ (Adams D., et al. 2014) - Photolithographic fabrication allows for the ability to precisely control the size, shape and pore distribution of the membrane's pores for in depth systematic testing of CTCs isolation. This system provides the capability to compare various membrane patterns and aid in providing a universal standard for the capture of CTCs using any membrane material.
- Parsortix (Xu L. et al., 2015) - an epitope independent, size and compressibility-based platform for CTCs isolation. The Parsortix system utilizes microfluidic based particle separation technology, with a disposable cassette containing a separation structure comprising a series of steps across which cells are forced to pass, leading them to a terminal gap of 10 μm. Most blood cells pass across the steps and through the terminal gap and cells whose size and rigidity prevent them from passing through the step structure (e.g. CTCs) are retained in the cassette.

Density Gradient Centrifugation:

Density gradient centrifugation leads to the separation of mononuclear cells from other blood cells. The cells migrate differentially during centrifugation according to the buoyant density. The result is a separation of different cell types e.g. caner cells into distinct layers (Boyum A. et al., 2007). Pitfalls of this technique are as follows: density gradient separation is limited by its poor sensitivity, mainly through the possible loss of tumor cells that either migrate to the plasma layer, or due to the formation of aggregates to the bottom of the gradient; also, whole blood can mix with the density gradient if the centrifugation step is not performed immediately.

Several gradient media types are used to enrich blood cells and/ or cancer cells:

- Ficoll- Hypaque is used for separation of lymphocytes from other formed elements in the blood; the sample is layered onto a Ficoll-sodium metrizoate gradient of specific density; following centrifugation, lympho

cytesare collected from the plasma-Ficoll interface.

- Lymphoprep™ - density gradient medium recommended for the isolation of mononuclear cells from peripheral blood, cord blood, and bone marrow by exploiting differences in cell density. Granulocytes and erythrocytes have a higher density than mononuclear cells and therefore sediment through the Lymphoprep™ layer during centrifugation. Lymphoprep™ can be substituted for Ficoll-Paque™.
- OncoQuick® - prevents mix of density gradient by addition of a porous barrier above the density gradient (Gertler R. et al., 2003). It has been developed to enrich monocyte fraction on cancer cells.

Microchip microfluidic techniques:

They capture live CTCs in devices that exploit physical and/or biological differences between CTCs and other blood cells. CTCs can be further analyzed by cellular, microscopic, or molecular techniques. Sensitivity of microfluidic techniques is higher. Dong (Dong Y. et al., 2013) identified 18 different strategies how to detect CTCs by microfluidic techniques (see table No. 3).

- The DEPArray™ analysis platform utilizes high quality, image-based selection to allow identification and isolation of cells of interest on the DEPArray™ cartridge. The automated system uses a six-channel fluorescent microscope and camera to capture images and identify cells demonstrating the desired fluorescence labeling and morphological characteristics.
- CTC iChip (Sequist LV., et al. 2009, Karabacak NM., et al. 2014) - combines depletion of non-tumor cells using anti-CD45 and anti-CD15 antibodies with hydrodynamic cell-sorting based on cell size, so it is able to isolate CTC without CTC capture.
- Microcrescent chip (Tan SJ., et al. 2010) - isolation of tumor cells using size and deformation.
- MEMS (Zheng S., et al. 2007) - micro electromechanical system, integration of CTC saze- based isolation and on-chip cell lysis for PCR-based genomic analysis.

- Microwall chip (Mohamed H., et al. 2009) - isolation of tumor cells using size and deformation.
- ApoCell (Becker FF., et al. 1995) - separation of CTC on the basis of dielectric differences between cancer cells and normal blood cells.
- HB-Chip (Scott SL., et al. 2010) - contains eight microchannels with patterned herringbones on their upper surface, the internal walls are cooted with antibodies against EpCAM.
- CellOptics® (Vona G., et al. 2000) - a miniaturized microcavity array (MCA) system developed for the highly efficient entrapment of single cells by filtration based on differences in the sizes of cells (Hosokawa M., et al. 2012).

Electrical property-based dielectrophoresis:

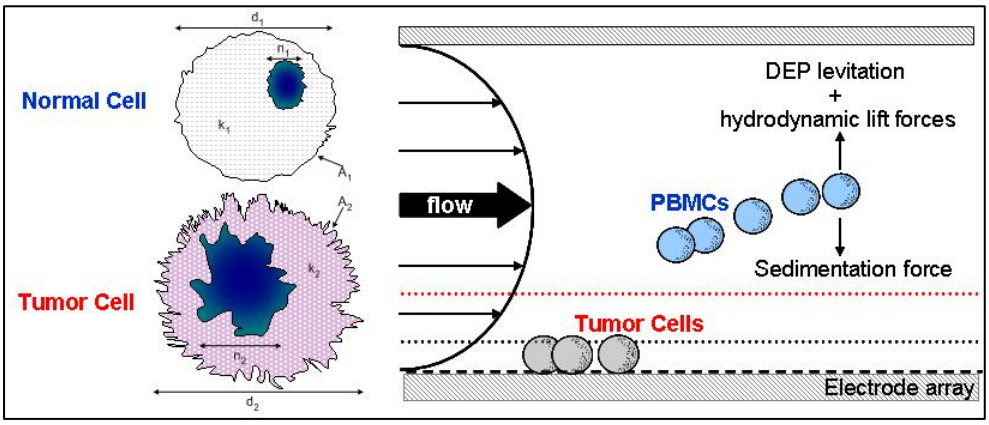
- Dielectrophoresis field flow fractions (DEP-FFF) technique allows detection of CTCs independent of the EpCAM expression. These assays analyze morphological and electrical dissimilarities of different cells to isolate CTCs from blood (Alazzam A. et al., 2000). A particle suspended in a medium of different dielectric characteristics becomes electricaly polarized when subjected to an alternating electrical field. Interaction between this induced polarization and the field gives rise to various electrokinetic effects. Becker (Becker FF. et al., 1995) showed that the dielectric characteristics of cultured BC cels are significantly different from those of blood cells and could be exploited in a dielectric afinity column to remove tumor cells from dilute blood.
- ApoStream® (Gupta V. et al., 2012) - allows isolation and enrichment of viable cells and their molecular characterization (see also Figure No. 1).

Table No. 3: Micrcofluidic based CTC- enrichment technologies

CTC properties used	Strategy	Commercialization
Size filtration	Micromachined device of four successively narrower channels (Mohamed H. et al., 2009)	Clearbridge Biomedics (Singapore)
	Arrays of pillars forming crescent-shaped isolation wells (Tan S. et al., 2009)	
	Microfilter with embedded microelectrodes, capable of on-membrane electrolysis (Zhenkg S. et al. 2007)	
Size streamline sort	Microdevice with multiple asymmetrical channels for pinch flow fractionation and hydrodynamic filtration of cells (Yamada M. et al., 2004)	
	Microchannels using changing channel dimension to sort cells into individual laminar flow streamlines (Carlo DD., 2009)	
	Microdevice with three sequential regions to focus, pinch, and collect cells by contraction and expansion of channel dimensions (Bhagat AAS. et al. 2011)	
Size and particle polarizability	DEP for retention of cells through differential flow (Becker FF. et al., 1995)	ApoCell (Houston, TX)
	Thin DEP microchamber for retention of cells through differential flow (Gascoyne PRC. et al., 2009)	
Binding cell surface markers	Microchip with staggered microposts coated with anti-EpCAM (Davies J. et al., 1994)	CytoScale Diagnostics (Los Angeles, CA)
	Microtube coated internally with halloysite nanotubes presenting selectin (Spitznagel TM., Clark DS., 1993)	
	Silicon nanopillar arrays coated with anti-EpCAM (Hughes AD., King MR., 2010)	
	Microchip with channels in a herringbone design generating microvortexes to enhance contact with anti-EpCAM–coated walls (Wang S.et al., 2009)	
	Anti-EpCAM–coated fluid-permeable membrane sandwiched between two polydimethylsiloxane flow chambers to promote cell rolling and adhesion (Wang S. et al., 2011) Microchip with a combination of solid- and porous-coated micropost arrays to enhance antibody capture at material boundaries (Mittal S. et al., 2012)	
	Microchip with magnetic beads coated with anti-EpCAM that form self assembling pillars (Ephesia) (Chen GD. et al., 2011)	
Binding cell surface markers and electrokinetic manipulation	DEP for migration of cells bound to antibody-coated magnetic nanoparticles (MIRACLE) (Liu C. et al., 2011)	BioFluidica (Baton Rouge, LA)
	High-throughput microsampling unit combining sinusoidal channels coated with anti-EpCAM for capture and electromigration for concentration (Saliba AE. et al., 2010)	
Size and binding cell surface markers	Microchip with microposts coated with anti-EpCAM arranged in a gradient design for affinity capture and size filtration (Dharmasiri U. et al., 2011)	On-Q-ity Inc.

DEP- dielectrophoretic

Figure No. 1: Principles of Apostream microfluidic flow chanel using dielectrophoresis in the CTC- isolation processes. A combination of forces, dominated by the electrophoretic charge, attracts or repels cells to a charged electrode. Differential flow rates relative to distance from the electrode aid in the fractionation of the different cell types (apocell.com).



1.3.2 Affinity-based enrichment technologies

These technologies utilize a unique antigen expression pattern on CTCs, so the identification of antigen(s) expressed by CTCs that are not shared with other normal components circulating in the blood is needed. The most common example is the use of the EpCAM for CTCs capture in epithelial cancer types. Expression of the transmembrane glycoprotein EpCAM occurs in normal epithelium of different organs. It is on interest to know which tumor types express or overexpress these proteins, and in what frequency (Went PTH. et al., 2004).

The historically oldest and most frequent is immunomagnetic isolation (Fehm T. et al., 2005). Antibodies are coupled to magnetic beads, thus the antigen–antibody complex is subsequently isolated from the solution with a magnetic field. EpCAM is the by far most used antigen due to its expression across numerous tumor entities. The problem is that CTCs with no or low EpCAM expression could be overlooked and underestimated (Königsberg R. et al., 2011). The lack of reliable target antigens for cellular capture still represents a significant limitation to the procedure. The sensitivity of CTCs detection can be increased by combining of CK and EpCAM markers (Deng G. et al., 2008).

The heterogeneity of CTCs populations due to the process of EMT (Gorges TM. et al., 2012) and capture antibody efficiency could be other limitations in CTCs

detection rate. A phenotypic change thought to be an important feature in the metastatic process and CTCs may represent viable metastatic precursors. To overcome these limitations new technologies combining panels of targets (Joosse SA. et al., 2012, Mostert B. et al., 2011), combining affinity-based capture with other principles and/or switching from positive capture to negative depletion of non-tumor cells in the sample have been discovered. Negative selection can be achieved by red blood cells lysis and detection of CTCs using fluorescence microscopy or flow cytometry or by immuno-depletion of white blood cells using antigen CD45 (Liu Z. et al., 2011). For more microfludic techniques see capture 1.3.1.

The list of antibody-based microdevices is quite long, e.g. CellSearch, AdnaTest, Micropost chip, MagSweeper, Micropillar chip, MicroGEDI chip, Microvortex chip, Nanopillar chip etc.

CellSearch is the gold standard in the CTCs field and is still the only technology that is cleared by US Food and Drug Administration (FDA) for clinical CTCs testing (Cristofanilli M. et al., 2004). It consists of ferrofluids coated with EpCAM antibodies to immunomagnetically enrich epithelial cells, a mixture of two phycoerythrin-conjugated antibodies that bind to CK 8, 18, and 19, an antibody to CD45 conjugated to allophycocyanin, nuclear dye 4′,6'-diamidino-2-phenylindole (DAPI) to fluorescently label the cells, and buffers to wash, permeabilize, and resuspend the cells. The criteria for an object to be defined as a CTC include round to oval morphology, a visible nucleus (DAPI positive), positive staining for CK, and negative staining for CD45. Results of cell enumeration are always expressed as the number of cells per 7.5 mL of blood (Allard WJ. et al., 2004).

AdnaTest is the second frequent technigue. The aim of the test is not only immunomagnetic-based EpCAM enrichment, but also analysis of labeled cells using multiplex RT-PCR to detect specific tumor biomarkers GA733-2, MUC-1 and HER-2 (see also below Detection of CTCs). The use of magnetic beads conjugated with tumor specific antibodies are confronted in comparison studies (Andreopoulou E. et al., 2012).

The MagSweeper is an automated immunomagnetic separation technology that gently enriches CTCs from blood and eliminates cells that are not bound to magnetic particles coated with anti-EpCAM antibodies. Purified cells can then be individually selected for biochemical analysis (Talasay AH. et al., 2009).

As the CTCs research is expanding, affinity-based CTC-capture platforms are improving and new technologies are emerging. CTC-Chip technology was mentioned above; it utilizes microspots functionalized with highly effective anti-EpCAM antibodies to capture CTCs. The technique is reducing the sample volume, the second generation of the chip enables also the capture of CTCs clusters (Hou JM. et al., 2012) and the third generation of CTC-iChip not only allows for positive affinity-based enumeration of CTCs by magnetic activated cell sorting, but also allows for negative depletion of leucocytes for antigen agnostic CTCs enumeration (Ozkumur E. et al., 2013).

OncoCEE^{TM} (Mayer JA., et al. 2011) is CTCs detection method using a cocktail of capture antibodies, followed by detection with an expanded anti-cytokeratin (CK) cocktail mixture and anti-CD45.

NanoVelcro is another chip to capture CTCs based on their EpCAM expression (Hou S. et al., 2013) and GILUPI CellCollector collects CTCs by a wire with anti-EpCAM antibodies that is inserted into the cubital vein of cancer patients (Saucedo-Zeni N. et al., 2012).

Telomerase-targeted CTCs detection is a method that may target the entire CTCs subpopulation and also circulating CSCs, since elevated telomerase activity is one of the hallmarks for SC identification/characterization (Maurelli R. et al., 2006). To detect telomerase activity the whole blood sample has to be lysed so all intact CTCs are destroyed and no other measurement could be done.

Aptamers are single-stranded RNA or DNA molecules, which are manufactured by the technique of Systematic Evolution of Ligands by Exponential Enrichment (i.e., SELEX). It was created to target cancer cells via extracellular membrane proteins (Cerchia L, de Franciscis V., 2010).

1.3.3 Detection methods of CTCs and their functional characterization

After blood volume reduction and CTCs enrichment the detection of CTCs follows. There are three main principles used for CTCs detection: **cytometric approaches, molecular detection and CTC culture/microscopy.**

1.3.3.1 Cytometric approaches:

Classic immunocytochemistry is the most widely used immunological approach. Among the current EpCAM-based technologies, the gold standard for CTCs detection remains CellSearch as mentioned previolusly. A semi-automated fluorescence-based microscopy system consents a computer-generated recostruction of cellular images. CellSearch works as enrichment and enumeration technique. It employs a multi-step cell preparation approach and a manual image screening process to identify intact or damaged CTCs.

The number of epithelial cells in blood from subjects without known cancer is very low and almost never exceeds 1 cell per 7.5 mL of blood. In contrast, ≥ 2 CTC were detected in 7.5 mL of blood in 36 % of the specimens from patients with various types of carcinomas (Allard WJ. et al., 2004). Hence, identification of CTCs by their morphology can be challenging due to the possible presence of debris/fragments, apoptotic/damaged cells, and tumor-like cells. Considering the heterogeneity of the CTCs population, and the co-captured white blood cells, immunofluorescence is still the gold standard for for automatized CTCs counting. There were also developed protocols to optimize the CellSearch system for characterization of CTCs with respect to userdefined protein markers of interest (Lowes LE. et al., 2012).

Flow cytometry (FCM) is an optical detection system which use lasers to excite fluorescence of molecular labels. Emissions of different colors and intensities are detected with the scan. New technologies offer the possibility of combining flow cytometry with microscope imaging increasing the sensitivity of the examination (Basiji DA. et al., 2007). One of the major strengths of FCM remains its ability to

perform multiple measurements on single cells within a heterogeneous mixture; however, the instruments were not developed to count cells.

Laser Scanning Cytometry uses a variety of fluorochrome-labelled antibodies. The single cell within 1 million cells is traceable (Pozarowski P. et al., 2013). Disadvantage of many systems is that they are not able to distinguish between viable and apoptotic cells. Technologies that allow for viable CTCs capture are driving CTCs culture technology development and ex vivo drug treatment assays (Yu M. et al., 2014).

Some affinity-based platforms that allow for viable CTCs capture are the NanoVelcro chip or Graphene Oxide nanosheets (Yoon HJ. et al., 2013).

EPISPOT (EPithelial ImmunoSPOT) assay is based on detection of proteins secreted from single epithelial cancer cells (Alix-Panabières C., 2012). After depletion of CD45 positive cells, remaining cells in a whole blood are cultured for 24 hours. Proteins are captured by antigens in a membrane, catching cells and subsequently detected by secondary antibodies labeled with fluorochromes. The culture cell medium is enriched with growth factors so only viable cells (CTCs/DTCs) are able to immunospot formation.

1.3.3.2 Molecular detection:

Molecular assays based on the analysis of nucleid acid by PCR have much higher sensitivity and specificity. They are based on the isolation of total RNA from viable CTCs, and subsequent RT-PCR amplification of tumor-specific or epithelial-specific targets (A), or isolation of genomic DNA from CTCs and subsequent detection of mutations (B) or DNA methylation in CTCs (C) (Andergassen U. et al., 2013).

A. RT-PCR-based characterization

B. Mutational testing (FISH, qPCR-based mutational test: High resolution melting, Sanger sequencing, NGS-sequencing, CGH-arrays: comparative genomic hybridization, digital droplet PCR)

C. Epigenetic testing (DNA-methylation tests)

D. Immunohistochemistry

A. PCR-based characterization

The most of advantages of PCR-based methods are:

- they give information about living cells as they are mostly targeting specific mRNA producing only by viable cells,
- a variety of molecular markers can be detected (multimarker testing),
- they offer extreme sensitivity.

The main disadvantages concern:

- pre-analytical issues and CTCs stability during sample shippment and storage,
- the lack of information about CTCs morphology and the number of CTCs,
- a low-level transcription of targeted mRNA in normal cells and presence of pseudogenes could also be limiting factors for RT-PCR-based detection. Therefore, quantitative real-time (qRT)-PCR frequently uses a cut-off value to differentiate between positive and negative findings.

Development of RT-PCR in the meaning of quantitative PCR (qPCR) technology had an enormous impact on cancer diagnostics, since it can provide significant and quantitative information on gene expression in an automated, rapid, versatile and cost-effective way (Bernard P. et al., 2002).

Many different molecular assays based on qRT-PCR were designed for different gene transcripts such as CK-15, CK-19 (Xenidis N. et al., 2009), mammglobin (Ignatiadis M. et al., 2008) or EGFR. Stathopoulou developed an RT-PCR assay for KRT19 mRNA (Stathopoulou A. et al., 2006). Multiplex qRT-PCR uses more markers to higher the detection rate of CTCs (Strati A. et al., 2011).

AdnaTest (Fehm T. et al., 2009) is a commercially available RNA-based CTCs assay which utilizes combination of principles: immunomagnetic separation combined with multiplex RT-PCR (non-quantity) to identify putative transcripts presence (PCR product is present in a pre-defined concentration or above the cut-

off). Following markers are tested: MUC1/HER2/EpCAM. The principal limitation, along with the others related to EpCAM-based methods, is that MUC1 expression has been found on activated T lymphocytes (Agrawal B. et al., 1998). Another aspect to be considered is the fact that the RT-PCR is unable to quantify the tumor cell load, since PCR-products evaluated by the capillary electrophoresis may be the result of one single cell as well as a thousand.

Studies using the commercially available AdnaTest assay evaluated additionaly also the expression of EMT markers and ALDH1 in primary BC patients CTCs were also published (Kasimir-Bauer S. et al., 2012). In this context, re-evaluation of ER/PR/HER2 status by molecular characterization of CTCs is a strategy with potential clinical application (Fehm T. et al., 2009). With the respect to drug resistance against HER2-targeted therapy, mutation analysis of PI3K on CTCs has a potential clinical relevance (Schneck H. et al., 2013).

B. Mutational testing (NGS-sequencing)

Next-generation sequencing (NGS) technologies are extremely powerful and in the combination with reliable single CTC isolation offers a new dimension in the area of CTCs molecular characterization (Peeters DJ. et al., 2013). Several interesting results have been published in the last 2 years reflecting the evolution of CTC genome in comparison to the primary tumors.

C. Epigenetic testing (DNA-methylation tests)

Epigenetic mechanisms silencing tumor suppressor genes are frequently seen in tumor cells. Interestingly, highly methylated gene promoter sequences, e.g. SOX17 promoter, were found in CTCs of EpCAM positive/*CK-19* negative BC patients. Methylation status of CTCs correlated with the primary tumor and cf-DNA, so the connection between the presence of CTCs and cf-DNA in operable BC patients was found (Chimonidou M. et al., 2013).

1.3.3.3 CTC culture / microscopy:

The culture and expansion of CTCs is desirable as a mean of yielding a CTCs population suitable for comprehensive functional characterization and drug testing.

The main premise of successful strategies of *in vitro* cultures is the possibility to obtain viable CTCs. In addition, to the difficulties associated with viable CTCs isolation, the optimal culture conditions for CTCs expansion must also be experimentally defined. Generally saying, methods that best retain cell viability are less effective at enriching CTCs and conversaly efficient CTCs capture methods are less effective at retaining viable cells.

Using antibodies to enrich CTCs, in terms of tumor formation *in vivo* seem to be more appropriate in EpCAM negative CTCs with brain metastatasis signature (Zhang L. et al., 2013). The majority of studies that have shown success in the enrichment and functional characterization of viable CTCs have employed a density gradient-based enrichment method (Choesmel V. et al., 2004). On the other hand, sensitivity of Ficoll-Hypaque to enrich CTCs is limited.

First positive data have been published about this hot topic and using different strategies: combination of gradient-based centrifugation for CTCs enrichment and novel cell culture reagent (TrueCells) by McGregor (Mc Gregor JR. et al., 2012), GEDI device by Kirby (Kirby BJ. et al., 2012), multigene FACS by Zhang in MBC patients (Zhang L. et al., 2013), CTC iChip for isolation of CTCs in ER+ MBC patients (Yu M. et al., 2014). Polymer grafted silicon nanowires (Hou S. et al., 2013) or functionalized graphene oxide nanosheets (Yoon HJ. et al., 2014) on a patterned gold surface are another effective approaches of CTCs isolation. Size-based separation CTCs technologies offer in general a possibility to obtain viable CTCs and grow them as reported by ISET, ScreenCell , Parsortix, MetaCell, but the culture efficiency differs between the listed approaches. To date the most data on the viable CTCs-capture and culture has been reported using MetaCell isolation tube.

Further, the ability to maintain viable CTCs in culture would not only be important in the research setting, but could enhance the clinical utility of CTCs for therapeutic monitoring through analysis of drug sensitivity.

1.3.4 Detection of tumor cell-associated and/or -derived materials/markers

Besides CTCs, the analysis of tumor cell-associated and/or -derived materials/markers may provide complementary information as liquid biopsy (Hong B., Zu Y., 2013). This information can be used as companion diagnostics to improve the stratification of therapies and to obtain insights into therapy-induced selection of cancer cells.

Circulating tumor microemboli (CTM) are tumor cell clusters/aggregates associated with high metastatic potential. They are composed of at least two tumor cells, and occasionally, normal blood cells. It is believed that CTM arise from collective migrated tumor cells and cell clusters intravasated from primary tumor to blood (Friedl P, Wolf K., 2003). The inhibition of VEGF-A could also play a role in this process (Kats-Ugurlu G. et al., 2009). The CTM formation is conneted to faster growing of metastatic tumor and better survival of tumor cells. CTM are also protected from the immunological assault by lymphocytes and natural-killer cells (Hou JM. et al., 2011). Several methods have been used for CTM collection/detection, the enrichment of CTM by ISET seems more reliable; flow cytometry is a readily accessible platform for the detection and enumeration of CTM.

Circulating tumor materials (CTMat) are fragments of dead tumor cells. Because CTCs are continuously shed and destroyed, such CTMat can accumulate (Rao GC. et al., 2011). There is a potential role of CTMat as prognostic and predictive factor (Coumans FA. et al., 2010). It is therefore speculated that the numbers of intact CTCs and CTMats, as well as their ratio to one another, may provide an important tool for the assessment of tumor burden, the proliferative capability of tumor cells, and therapeutic efficacy.

ct-DNA or cf-DNA are critical components of CTMat (Kaiser J., 2010). The level of ct-DNA in patients' blood has been demonstrated correlate with the malignancy status and the therapy response (Schwarzenbach H. et al., 2011).

1.3.5 Conclusion

To review the information above, we can say that no optimal method for detection of CTCs exists yet. CTCs were initially characterized by their morphological features; as non-leukocytic (CD45-negative) nucleated cells (DAPI-positive) that are typically epithelial in origin (i.e. EpCAM or CK-positive).

However, it is known now that the morphology of CTCs can vary by disease and disease stage. In addition they can lose their epithelial antigens in the process of EMT (Wicha MS, Hayes DF., 2011) or change their markers over the course of therapy (Wang LH. et al., 2010). Moreover, molecular and phenotypical alterations may vary over time and finally CTCs population as a unit is composed of subsets of different cells with more or less aggressive characteristics. Multiple metastatic sites are heterogeneous in biomarkers expression in comparison to primary tumor and among themselves. Finally, CTCs may be damaged and fragmented due to the multi-step cell preparation processes, causing inaccurate detection and misinterpretation.

To obtain high sensitivity and specificity of CTCs detection it seems to be necessary to combine the enrichment and detection step. No single approach showed efficient quality. CTCs enrichment is the first part of CTCs isolation from the whole blood. It could be affinity or non-affinity-based. The next step is provided by tumor cells staining using antibodies, or gene labeling using DNA probes and/or primers. Finally, characterization of CTCs is the key proces of CTCs detection. Techniques which could be applied are: cytometry, microscopy, fiber- optics, RT-PCR, FISH, CGH.

The majority of CTC detection methods are designed as bench-top instruments, such as flow cytometers, the CellSearch system, high-definition fluorescence scanning microscopy, fiber-optic array scanning technology (FAST), isolation by size of epithelial tumor cells (ISET), and laser scanning cytometers. Some methods combine bench-top instruments with an additional assay system, such as the processes of Ficoll, OncoQuick, and RT-PCR. Microdevices have attracted plenty of attention with their unique merits on providing rapid, low-cost, simple, and automated immunoassays (Chen J. et al., 2012).

Most microdevices, despite employing only one principle, have still demonstrated better CTCs recovery and enrichment rates than the CellSearch system, and a capability in maintaining cell viability, which is important for additional CTCs characterization and potential function analysis. Until now the only FDA-approved system for detection of CTCs in breast, prostate and colorectal cancer is CellSearch (Riethdorf S. et al., 2007). Although new microfluidic systems offer several advantages including reduced sample volume, faster processing time or high sensitivity, routine use of CTCs for diagnostic or management purposes is not readily accepted. The one problem are lacking data about predictive value of CTCs examination, other problem is the lack of standardization and comparison of different technologies. CTCs culture technology development offers microscopic evaluation of viable cells and their functional characterization.

In the future, identification of specific therapy-related molecular targets on CTCs could offer important information, early on to choose for the correct treatment and moreover explain resistance to established therapies (Lianidou ES. et al., 2013). Multiplex PCR or NGS molecular characterization of CTCs has the potential to expand our knowledge of basic molecular pathways of invasion, migration, and immune surveillance and might contribute even to the identification of metastatic CSCs (Becker TM. et al., 2014). We can probably approach these goals by using new molecular detection technologies.

2. AIMS OF THE THESIS

1. To examine and attest CTCs isolation and detection methods in clinical setting for different tumor types (chapter 3.2, 3.3, 4.1).

2. To monitor minimal residual disease by means of CTC- analysis in patients with BC in different therapeutical subgroups (neoadjuvant, adjuvant, palliative) (chapters 4.1).

3. To define prognostic, predictive and diagnostic utility of CTCs in clinical practice (chapter 4.2 - 4.4).

4. To prepare implementation of CTC examination as of a complementary diagnostic tool into personalized therapy of tumor diseases (chapter 4.5).

Sub-objectives and hypotheses defining the aims in detail (A-E) are listed below:

- A. Hypothesis: The two-step CTC-examination protocol consisting of cytomorphological examination and subsequent qPCR analysis of CTCs enriched by size-based separation is in principle more sensitive if compared to AdnaTest® technology.

  A. Objective: Comparison of CTCs detection rates of AdnaTest® and MetaCell®-qPCR completed test.

  Relevant results are described and discussed in Chapter 4.1.

- B. Hypothesis: The presence and properties of CTCs do not correlate with conventional clinicopathological parameters of the disease, e.g. histological tumor type, grade, tumor size, presence of nodular metastases, age of patients, menopausal status, etc.

  B. Objective: The comparison of CTCs and clinicopathological parameters of disease in BC patients.

  Relevant results are described and discussed in Chapter 4.2.

- C. Hypothesis: The systemic response to the treatment is independent from the local response in patients undergoing neoadjuvant chemotherapy in BC.

  C. Objective: The comparison of primary disease response and CTCs – response in BC patients treated with neoadjuvant chemotherapy.

  Relevant results are described and discussed in Chapter 4.3.

- D. Hypothesis: CTCs have predictive value in BC disease management.

  D. Objective: The comparison of CTCs characteristics among responding and non-responding BC patients undergoing neoadjuvant chemotherapy.

  Relevant results are described and discussed in Chapter 4.4.

- E. Hypothesis: CTCs monitoring could have significant clinical impact in BC therapy personalization.

  E. Objectives: Clinical indications to CTCs examination and the presentation of our original data and current experience.

  Relevant results are described and discussed in Chapter 4.5.

3. PATIENTS AND METHODS

3.1 Patients characteristics

Four different groups of patients were monitored and examined for the presence of CTCs:

Patient group No. 1: Between years 2008 and 2010 the group of 197 patients with PBC or MBC was observed. Patients were treated in two different oncology clinics (General Faculty Hospital and Faculty Hospital Kralovske Vinohrady, Prague).

> ⇒ *the primary aim of the clinical study relevant to this patients group was to asses the CTCs positivity rate in patients undergoing systemic treatment using AdnaTest® and to confirm therapy response by CTCs analysis.*

The eligibility criteria were as following: age ≤18 years; patients with PBC eligible for adjuvant chemotherapy (NACT) or adjuvant chemotherapy (ACT); patients with measurable or evaluable lesions at MBC; predicted life expectancy more than ~ 2 months; no severe uncontrolled comorbidities or medical conditions; no second malignancies.

Patients with MBC had either a relapse of BC diagnosed years before and were to start palliative treatment, or had documented progressive BC before receiving a new endocrine therapy or chemotherapy. Prior adjuvant treatment, radiation or any other treatment of metastatic disease was permitted.

Patients with PBC (N=138) represented 77 % of cases, patients with MBC (N=42) represented 23 %. The average age of our group was 49.1 years; 19 % of patients were aged less than 35 years. Fore more details see tables bellow (Table No. 4).

Table No. 4: Patient group No. 1 - Patients characteristics (N=197)

	N of patients	%
Menopausal status		
premenopausal	79/186	43
postmenopausal	103/186	55
men	4/186	2
Therapy		
neoadjuvant	38/180	21
adjuvant	100/180	56
palliative	42/180	23
Median age	49.1 years	

Table No. 5: Patient group No.1- Disease characteristics (N=197)

	N of patients	%
Tumor size		
T1	77/183	42
T2	69/183	38
T3	21/183	11
T4	16/183	9
Nodal status		
N0	76/187	41
N1	79/187	42
N2	18/187	10
N3	14/187	7
ER status		
ER positive	118/182	65
ER negative	64/182	35
PR status		
PR positive	96/179	54
PR negative	83/179	46
HER2 status		
HER2 positive	42/176	24
HER2 negative	134/176	76
Triple negative	44/176	25
Histology		
IDC	152/180	84.5
ILC	11/180	6
mixed	10/180	5.5
other	7/180	4
Grading		
G1	15/153	10
G2	54/153	35
G3	84/153	55

Patient group No. 2: Between years 2014 and 2016 blood samples from 20 PBC patients treated with NACT in Oncology Clinic of General Faculty Hospital, Prague were collected regularly in relation to the therapy schedule.

> *⇒ the primary aim of the relevant clinical study was the comparison of tumor response to NACT by measuring tumor volume reduction and by the CTC- examination in parallel.*

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). Out of the NACT indicated patients, 16 patients were treated because of localy advanced BC (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 (80 %) carcinomas had poor degree of differentiation, i.e. grade 3 (G3), 3/20 (15 %) carcinomas had middle grade (G2) and 1/20 (5 %) carcinoma had high degree of differentiation (G1).

All patients had very aggressive disease according proliferation parameters (Ki 67) of tumor cells. The value of Ki 67 was in one case 20 %, all other primary tumors exhibited minimally 40 % of Ki67 expression. Alltogether 12/20 (60 %) tumors were oestrogen receptor positive (ER+) and/or progesteron receptor positive (PR+), 6/20 (30 %) patients were human epidermal growth factor receptor type 2 positive (HER2+). 9/20 (45 %) patients were classified as TNBC. Patient no 7 was also classified as TNBC as the postoperative ER and PR were negative (ER-, PR-). According the subtypes classification, 5/20 (25 %) patients were HR+/HER2- (luminal B), 4/20 (20 %) patients were HR+/HER2+ (luminal B, HER2+) and 2/20 (10 %) HR-/HER2+ (HER2- amplified/overexpressed), 9/20 (45 %) were TNBC (ER-, PR- and HER2-).

Clinicopathological features for every patient involved into the study and their individual risks are reported in details (N=20) in tables 6 and 7.

Table No. 6: Patient group No. 2 - 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 No. 7: Patient group No. 2 - Clinicopathological features and therapy regimen for every patient in details

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		

Patient group No. 3: Between the years 2013-2016 in total 154 patients with PBC were included to CTCs testing. Patients were treated in three different oncology clinics (General Faculty Hospital in Prague, Faculty Hospital Kralovske Vinohrady in Prague, Wroclaw Medical University).

⇒ *the primary aim was the comparison of clinicopatholigical parameters and CTCs presence.*

Table No. 8: Patient group No. 3 - Patients characteristics

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
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
HR and HER2 status		%
HR+ HER2+	16	11.7
HR- HER2+	7	5.1
HR+ HER2-	91	66.4
HR- HER2-	23	16.8

Patient group No. 4: Individual patients cases with different oncological diagnosis treated in Oncology Clinic of General Faculty Hostpital in Prague undergoing CTC- examination will be described in chapter 4.5.

> ⇒ *the primary aim was to show possible implementation of CTCs testing into the clinical practice, different applications of CTC- testing are shown.*

We indicated CTCs examination in patients with BC, colorectal cancer (CRC), castrate resistant prostate cancer (CRPC), non-small cell lung cancer (NSCLC) and tumors of unknown primary site (C80) as a complementary test in our clinical practice. In total 100 patients were included.

3.2 CTCs testing methodology

3.2.1 AdnaTest™ – Immunomagnetic CTCs enrichment

CTCs testing by using immunomagnetic-based method AdnaTest™

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. Additionally, to the AdnaTest BreastCancerSelect™ the Adnatest BreastCancerDetect™ kit is used to detect tumor- associated transcripts in the enriched CTC-cells.

For the patient group No.1 of tested BC patients the AdnaTest technology was used. Blood samples and/or bone marrow samples (min 5-8 ml) were incubated with a ready-to-use bead-antibody mixture commercialized as AdnaTest BreastCancerSelect™ according to the manufacturer's instructions. A magnetic particle concentrator attracted the labeled cell and after several washing steps CTCs were enriched.

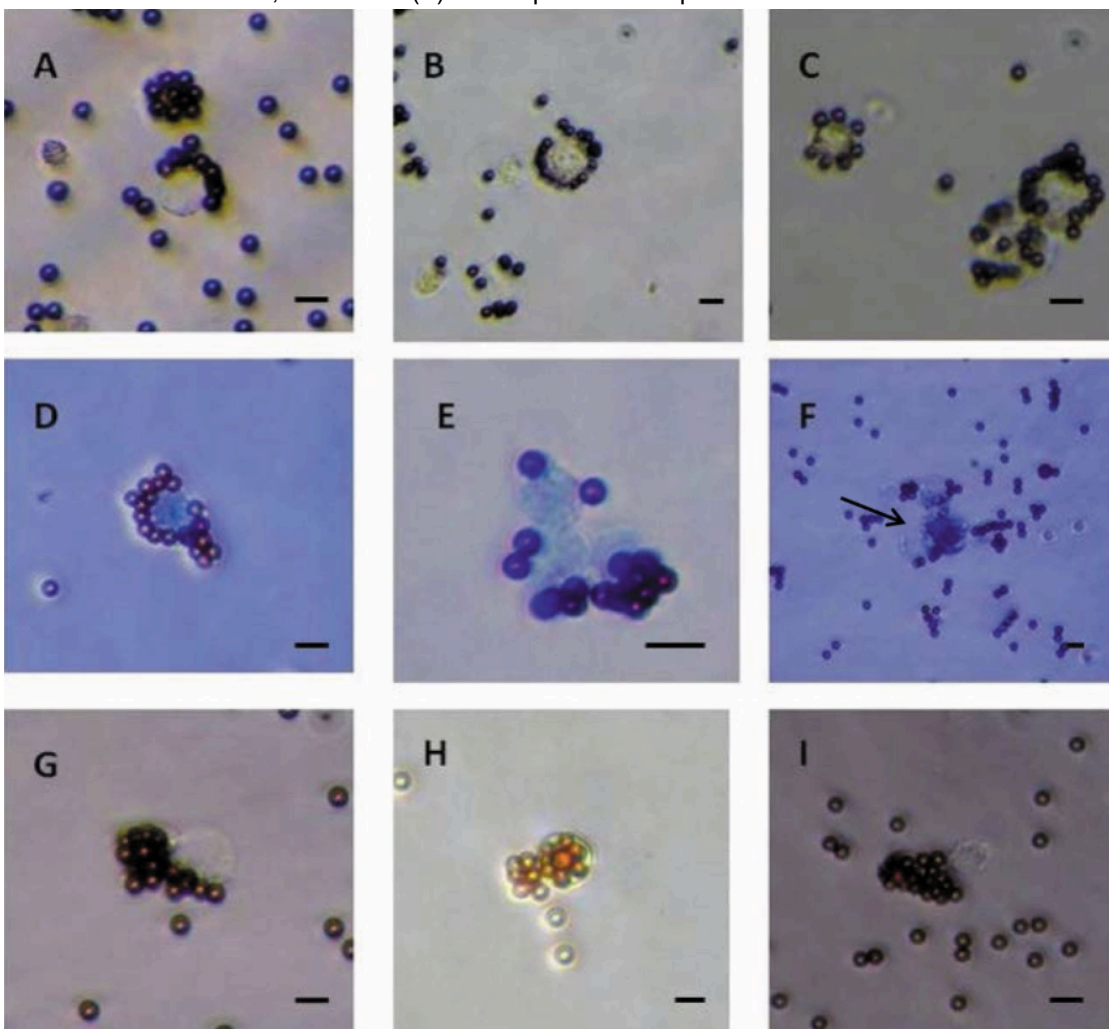
The Adnatest BreastCancerDetect™ is used for the detection of BC-associated genes in enriched CTCs by reverse transcription and PCR. mRNA isolation from lysed, enriched cells was performed according to the manufacturer's instructions with the Dynabeads mRNA DIRECT™ Micro Kit (Dynal Biotech GmbH, Hamburg, Germany) which is included in AdnaTest BreastCancerDetect™. Reverse transcription results in cDNA. Sensiscript Reverse Transcriptase (QIAGEN GmbH, Hilden, Germany) was used for the reverse transcription (recommended for RNA amount 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 analysis 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 following PCR products may be detected - EpCAM: 395 base pairs (bp), MUC1: 293 bp, HER2: 270 bp, and actin: 114 bp. 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 sample was considered positive.

Tumor cell visualization has been introduced into the CTCs-detection process as an innovative step. We have additionally withdrawn 1 ml of the peripheral blood. The blood has been processed following: 10 μl of immumomagnetic beads (Adnatest™) were added and were incubated for 15-30 min. The enriched cells have been evaluated under the inverted microscope (Figure No. 2). We dissolved cells in the PBS and did standard trypan blue staining for viability assessment immediately after isolation (Figure 2D, 2E, 2F).

Statistical analysis: Chi-squared test and Fisher's exact test were used to evaluate the relationship between CTCs positivity and clinicopathological factors. The McNemar test was used to compare the relationship of CTCs 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.

Figure No. 2: Circulating tumor cells as observed under light microscopy after immunomagnetic separation from 1 ml of blood. We expect that cancer cells are envelopped by immunomagnetic beads. The arrows are indicating beads. The cell viability was tested via Trypan blue exclusion method (D-E). In several cases CTCs clusters were detected, see arrow (F). Bar represents 10 μm.



3.2.2 MetaCell® – Size-based CTC enrichment

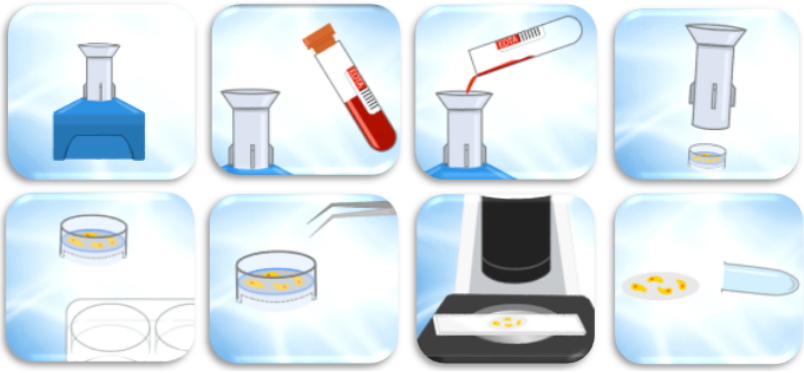
CTCs were examined by using a two-step CTC-enrichment and detection protocol described bellow.

First, cytomorphological evaluation of captured viable cells was evaluated. Second, qPCR analysis of mRNA isolated from captured CTCs –like cells were provided. A set of tumor-associated (TA)-genes and genes associated with the chemoresistance (CA-genes) was analysed. Gene expression of the CTC- enriched fraction was compared to the patients own white blood cell fraction to obtain data on level of CTC- enrichment.

Enrichment and cultivation of CTCs

Minimum of 6 ml of peripheral blood was filtered through membrane (8 µm pores) of the Metacell® (Metacell s.r.o., Czech Republic) device. Immediatelly after filtration process, a 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. This fraction can be assigned as CTC- enriched fraction without *in vitro* culture.

Figure No. 3: Summary on MetaCell isolation procedures



Minimum of 6 ml of peripheral blood was 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 antibiotics (SIGMA-ALDRICH, USA) to avoid contamination were added on the membrane.

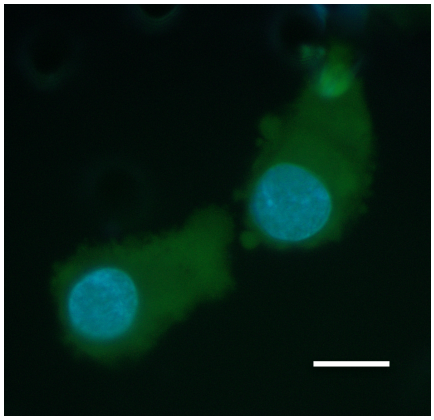
Captured cells were cultured *in vitro* under standard conditions (37°C, 5 % CO_2) for 3-5 days. This fraction can be assigned as CTC-enriched fraction with *in vitro* culture. This fraction is evaluated cytomorphologically.

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 (at magnification x 40) C software, Olympus IX51 fluorescent microscope with built in camera, Olympus U-RFL-T power supply unit) and identified according to usual cytopathological criteria for cancer cells including CTCs: nuclei larger than 10 µm, cell size of ≥ 12 µm, proliferation- mitosis presence, presence of tridimensional cell-sheets, high nuclear/cytoplasmic ratio, prominent nucleoli, irregular nuclei, visible

cytoplasm (see Figure No. 4). 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 for subsequent gene expression analysis.

Figure No. 4: CTCs identified according the cytomorphological analysis by vital fluorescence microscopy in BC- patient undergoing adjuvant treatment. Bar represents 10µm.



Whole peripheral blood processing – leucocyte (WBC) 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.

RNA isolation and reverse transcription

RNeasy Mini Kit (Qiagen, Germany) was used for RNA isolation in samples 1-4: 1. Whole peripheral blood, 2.CTC-enriched cells without *in vitro* culture, 3. CTC-enriched and *in vitro* cultured cells, 4. CTC-enriched cells, cultured and overgrowing the separation membrane (located on the culture flask bottom) mixed with Buffer RLT supplemented by β-mercaptoethanol was 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 manufacturer instructions (Table No. 9).

Table No. 9: 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 manufacturer instructions (Table No. 10).

Table No. 10: Cycling protocol using optimized conditions for High-Capacity RNA-to-cDNA™ Kit

Step	**Temperature (^oC)**	**Time (min)**
Step 1	37	60
Step 2	95	5

Step 3	4	∞

Gene expression analysis

Differences between samples 1-4 were detected by qPCR analysis of TA and/or CA-genes. TaqMan™ Gene Expression Assays (Thermo Fisher Scientific, USA) were used for gene expression analysis in 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 find out their expression level in CTCs (Table No. 11). The connection of CA-genes to cytostatics resistance, see Table No. 12.

Gene expression analysis was measured by COBAS® 480 (Roche s.r.o., Czech Republic). Temperature conditions in were set according to manufacturer instructions of TaqMan® Fast Advanced Master Mix (Thermo Fisher Scientific, USA) (Table No. 13).

Table No. 11: List tested genes and relevant of Hs codes for TaqMan probes used to monitor gene expression

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

Table No. 12: Associations of tested CA-genes to chemoresistance

Resistance to:	Genes associated with chemoresistance:						
antracyclins	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			
vinka-alkaloids	MRP1				MRP7		
multidrug resistance							MDR1
gemcitabin							RRM1

MRP: Multidrug resistance-associated proteins encoded by the adenosine triphosphate (ATP) binding cassette (ABC) transporter genes, RRM1: Ribonucleotide reductase M1 is associated with gemcitabine chemosensitivity in cancer cell

Table No. 13: Thermal-cycling conditions for qPCR analysis.

Step	Cycles	Temperature (^{o}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

Patients' blood collections were classified as CTCs positive by combined cytomorhologicalal microscopic evaluation and by molecular analysis, respectively.

Samples with two or more relatively elevated expression of TA-markers in cultured CTC-fraction compared to whole blood leucocyte fraction were evaluated as CTCs positive based on gene expression analysis.

Statistical analysis

The qPCR data evaluation was based on standard ddCT method (Livak KJ, Schmittgen TD, 2001). qPCR results were analysed by means of GenEx Professional software (MultiD) enabling multifactorial comparisons between involved groups. Relative RNA levels are displayed graphically in clusters. The differences between tested samples were compared by Mean - Whitney testing (significancy level $p < 0,05$ if not set automatically by GenEx).

3.2.3 Short evaluation of AdnaTest and MetaCell CTC- testing protocols according to our experience

Both CTC- enrichment and detection methods have their advantages and disadvantages.

AdnaTest is a two-step process of immunomagnetic CTC-separation and subsequent PCR evaluation of EpCAM, MUC1 and/or HER2 – presence. But PCR as provided in this test is not quantitative. EpCAM, MUC1 and/or HER2 PCR-product presence are most probably all cells with the epithelial origin. Tumor cells with mesenchymal features are most probably lost during the enrichment steps. The sensitivity of this CTC-test is lower by this fact. Visualization of tumor cells is possible but is not the standard part of CTCs detection procedure recommended by the producer. Not to see the cells limits the control on possible leucocyte contamination. Cytomorphological evaluation of CTCs is not a component of this testing.

Metacell enrichment tube works with a prerequisite that all cells bigger than 8 µm are captured by the separation membrane during filtration process. Filtration could be seen as a tricky part of the CTC – isolation protocol, because of pro-thrombic stage in cancer. MetaCell filtration is driven by capillary action which causes a gentle blood flow through the separation membrane, what keeps the captured cells fully viable, both epithelial and mesenchymal. The separated cells can be *in vitro* cultured and analyzed by all the type of downstream molecular analyses like: cytomorphology by vital fluorescence microscopy, immunohistochemistry, gene expression analysis and mutational staining.

Table No. 14: Comparison of AdnaTest and MetaCell devices

	MetaCell	**AdnaTest**
CTC can be characterized by their morphological features	yes	no
CTC visualization	yes	not standard
Detection of epithelial cells	yes	yes
Detection of mesenchymal cells	yes	no
Cell viability after cell-separation step	yes	yes
CTC -immunohistological evaluation	yes	no

4. RESULTS

4.1 CTCs detection by using AdnaTest® and MetaCell®

- A. Hypothesis: The two-step CTC-examination protocol consisting of cytomorphological examination and subsequent qPCR analysis of CTCs enriched by size-based separation is in principle more sensitive if compared to AdnaTest® technology.
- **A. Objective: Comparison of CTCs detection rates of AdnaTest® and MetaCell®-qPCR completed test.**

Summary of relevant results as described in study I (Ušiaková Z., Mikulová V. et al., 2014) ⇒ CTCs presence was evaluated in 197 patients, in total 419 samples were analyzed, 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 samples (72 samples, 17 %) were inconclusive and not evaluated.

Patients with PBC were divided according to the type of therapeutic approach into the neoadjuvant (n=38) and adjuvant (n=100) group. In the adjuvant group, 26 % of patients were CTCs positive before the start of adjuvant treatment and 13 % after therapy. In the neoadjuvant group, 35 % of samples were positive before and 5 % after therapy. Among patients with MBC, CTC were described in 42 %, in at least one sampling before and in 12 % after therapy (Table No. 15).

The DTCs presence has been tested in parallel with CTCs abundance in PBC patients (n=16). Based on the results of AdnaTest 6/16 patients were evaluated as DTC–positive (37.5 %), out of the DTCs-positive patients four CTCs-positive patients were described (66 % of DTCs-positive patients were CTC-positive). Similarly, in the DTCs-negative group four CTCs-positive patients have been found (40 % of the DTCs-negative patients were CTC-positive). Totaly, 25 % of patients were both CTCs and DTCs-positive and 37.5 % of patients were both CTCs and DTCs-negative (Table No. 16).

Table No. 15: CTCs positivity rate in different therapeutical settings.

	Number of patients	CTC positivity before therapy	CTC positivity after therapy
Neoadjuvant treatment	38	35 %	5 %
Adjuvant treatment	100	26 %	13 %
Palliative treatment	42	42 %	12 %

Table No. 16: CTCs positivity in peripheral blood and DTCs positivity in bone marrow in parallel withdrawn samples in PBC patients.

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

Another study including relevant to deliver results to answer the hypothesis A, was the study where MetaCell – qPCR complemented technology was used to test CTC – presence.

Summary of relevant results as described in study II (Bielcikova Z., Jakabova A. et al., 2017 under review process) ⇒ CTCs were detected in 85 % of patients before starting NACT and 72 % after NACT. 100 % of patients were having some CTCs detected after surgery in different time points of follow-up (F-U). In comparison to CTCs positivity before operation (after NACT), detection of CTCs increased (Table No. 17).

Comparison of results from both studies working with two different CTC-analytical technologies is shown in Table No. 18 .

Table No. 17: CTCs positivity rate in different therapeutical settings.

	CTCs-positivity (number of patients)	%
Before NACT	17/20	85
During NACT	15/17	88
Before surgery	13/18	72
After surgery at any time of F-U period	19/19	100

Table No. 18: Comparison of CTC positivity using MetaCell® and AdnaTest®

	MetaCell	Adnatest
	CTC positivity (%)	CTC positivity (%)
Before NACT	85	35
During NACT	88	
Before surgery	72	5
After surgery at any time of F-U period	100	26

Conclusion: As is seen in table No. 18, CTCs detection rates by using MetaCell® are much more higher than the ones if AdnaTest® was used. This does not neccesarily mean that the sensitivity of the MetaCell test is higher.

We also observed discrepancy in CTCs and DTCs status measured by AdnaTest®; (2/6) 34 % of DTCs positive patients were CTCs negative and (6/10) 40 % of DTCs negative PBC patients were CTCs positive.

4.2 CTCs in comparison to clinicopathological features

- B. Hypothesis: The presence and properties of CTCs do not correlate with conventional clinicopathological parameters of the disease, e.g. histological tumor type, grade, tumor size, presence of nodular metastases, age of patients, menopausal status, etc.
- **B. Objective: The comparison of CTCs and clinicopathological parameters of the disease in BC patients.**

We compared clinicopathological characteristics of the primary breast tumor and CTCs in patients with PBC and MBC.

Summary of relevant results as described in study I (Ušiaková Z., Mikulová V. et al., 2014) ⇒ Correlation of CTCs abundance with tumor size, hormonal receptor status, and lymph node involvement was not statistically significant in the adjuvant setting (for more details see original article in chapter 9).

Concordance of HER2 status: HER2+ CTCs were detected in 35 % of patients with HER2- primary tumors. In those with HER2+ primary tumors, the concordance of HER2 expression was 68.2 % (Table No. 19). Considering the fact that also in TNBC cases, 33 % of the detected CTCs expressed HER2, we may expect that one- third of metastases arising in patients with HER2- primary tumors may be HER2+. If DTCs were detected in bone marrow of HER2- patients, they have been HER2+ in 100% cases.

Table No. 19: Concordance of HER2 status in CTCs and primary tumor.

Primary tumor	Patients	CTC-positivity (%)	HER2+ CTCs (%)
HER2+	42	12/42 (28.6)	8/12 (68.2)
HER2 -	134	37/134 (27.6)	13/37 (35)
TNBC	44	12/44 (27)	4/12 (33.3)

plus (+) means positive

Conclusion: correlation of CTCs presence with tumor size, hormonal receptor status, and lymph node involvement was not statistically significant in the adjuvant setting. Among patients with HER2+ primary disease, (4/12) 33 % of CTCs were HER2- and vice versa (14/37) 35 % of HER2- primary disease had HER2+ CTCs. 33 % of TNBC patients had HER2+ CTCs.

Summary of relevant results as described in study II (Bielcikova Z., Jakabova A. et al., 2017 under review process)⇒ The discordancy in ER and HER2 expression in primary tumors and CTCs can be seen in tables No. 20 and No. 21.

The distribution of CTCs during NACT was very discordant in ER+ (only 1/27 CTC-positive samples was ER+), on the other hand, in cases of TNBC and HER2+ primary tumors concordancy were higher (Table No. 20): during NACT 67 % of TNBC patients had triple negative CTCs and 80 % of HER2+ patients had HER2+ CTCs. If we check phenotype of CTCs in any time of patients observation, quite good concordancy is only in TNBC seen (68 % of TNBC patients had ER- and HER2- CTCs).

For more detailed stratification see table No. 21 or figure No. 5. In luminal B tumors, concordancy rate in ER+ was only 4 - 8 %, in HER2- 69 - 74 %. In TNBC, concordancy rate in ER- and HER2- was seen in 100 % and 67 - 68 % of samples. In ER+/HER2+ patients, only 0 - 19 % of samples were ER+, concordance in HER2+ was good during NACT (90 %) but only in 50 % of samples monitored during patients observation. Finally, among two ER- /HER2+ patients, 2/5 samples during NACT were concordant in ER status and 3/5 in HER2 status, from 12 samples in a longer time of patients monitoring 9/12 and 5/12 were concordant in ER and HER2 status.

Table No. 20: 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)

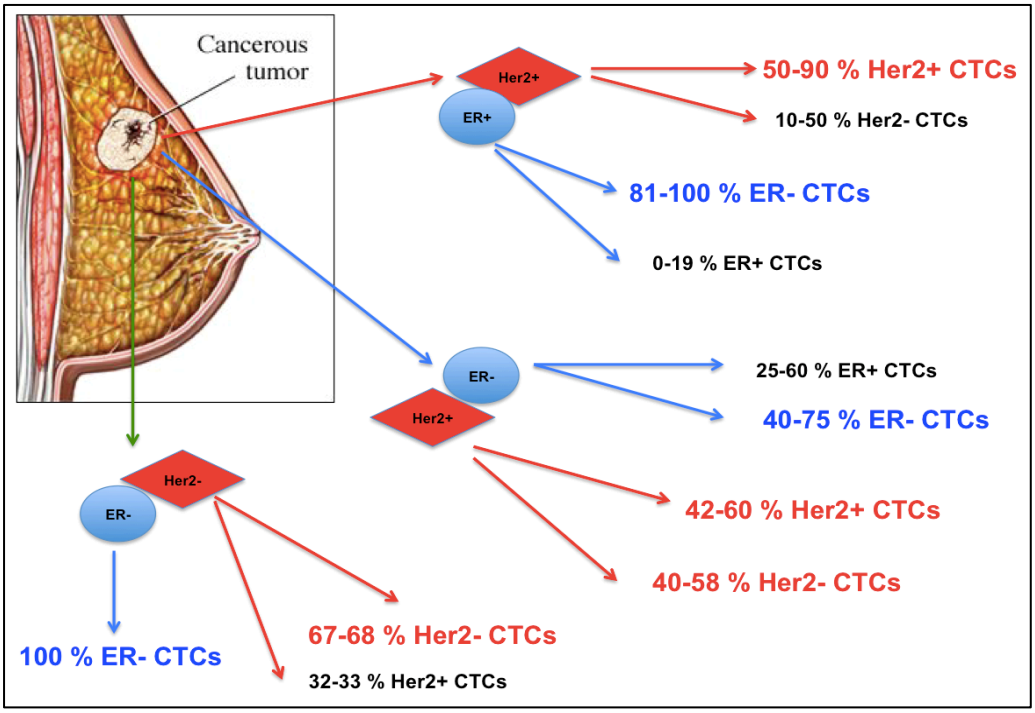
plus (+) means positive

Table No. 21: Concordancy of CTCs in ER and/or HER2 status with different types of PBC

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 %)

plus (+) means positive, minus (-) means negative

Figure No. 5: CTCs retain aggressive properties predominantly (more often after the therapy than during the therapy)



We can conclude, that during NACT we monitored CTCs with a quite high rate of concordancy in TNBC a HER2+ BC patients. The worst concordancy was seen in ER+ PBC independently on HER2 status.

The monitoring of CTCs status during NACT and after surgery shows that majority of CTCs are ER- and HER2- in HER2- primary BC, in HER2+ PBC, CTCs are predominantly ER- and HER2+ during NACT but they are loosing HER2+ status in time after surgery. CTCs retain aggressive properties predominantly.

Summary of relevant results as described in study IV (Jakabova A., Bielcikova Z. et al., 2017 under review process) ⇒ The CTC positivity in all groups was 76 %. There was no significant difference between tested groups if taking different therapy stages into account. The highest CTCs occurrence was found in the group undergoing surgery and similarly in the patient group analyzed before start of NACT. It is important to comment on CTCs-presence even if NACT was completed. It can be assumed, that in these patients therapy was ineffective. 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 from ESR+ to ESR- (50 %).

4.3 Comparison of primary disease response and CTCs response to NACT

- C. Hypothesis: The systemic response to the treatment is independent from the local response in patients undergoing neoadjuvant chemotherapy in BC.
- **C. Objective: The comparison of primary disease response and CTC – response to neoadjuvant treatment in BC patients.**

Summary of relevant results as described in study II (Bielcikova Z., Jakabova A. et al., 2017 under reveiw process) ⇒ The effect of NACT was observed clinically by bimanual palpation of breast and by ultrasound examination roughly done at the same time of blood withdrawal. Regression of the 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 %, as minimal (reponse rate 1) if the degree of regression was less then 50 %. For a not observed tumor regression or even progression a number 0 was used.

Report on CTCs positivity and tumor volume reduction during NACT is shown in Table No. 22. It seems that good treatment response is linked to effect of antracyclines (AC) more often than to (TAX) taxanes (8/20, i.e. 40 % of patients were responding significantly - response rate 3). Effect of TAX was evaluated as significant in 4/17 (23 %) of patients. CTCs positivity was detected in 87.5 % of AC-responders and 75 % of TAX-responders. In non-responders (response rate 0 - 1), 85 - 100 % of CTCs positivity was registered.

We declare that tumor volume reduction is not in connection to CTCs positivity rate. The effect of the whole NACT was accompanied by a non-significant decrease in CTCs positivity (85 % of CTCs-positive patients before NACT and 72 % of CTCs-positive patients before operation).

Table No. 22: Comparison of tumor volume response and CTCs response in patients undergoing NACT

	CTCs+ patients (%)	**Regression of tumor volume**							
		RR 0 (%)	CTCs+ in pts with RR2	RR 1 (%)	CTCs+ in pts with RR1	RR 2 (%)	CTCs+ in pts with RR2	RR 3 (%)	CTCs+ in pts with RR3
Before AC	17/20 (85)								
Before TAX	15/17 (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)**
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)**

plus (+) means positive, RR- response rate, pts- patients

4.4 Predictive value of CTCs

- D. Hypothesis: CTCs have predictive value in BC disease management.
- **D. Objective: The comparison of CTCs characteristics among responding and non-responding BC patients undergoing neoadjuvant chemotherapy.**

Summary of relevant results as described in study II (Bielcikova Z., Jakabova A. et al., 2017 under reveiw process) ⇒ We hypothesised that expression of TA-genes and CA- genes could reflect the effect of NACT and treatment response. The chemoresistance to AC, TAX, 5-fluorouracil, cisplatin, carboplatin and other cytostatics was tested in CTCs. Our patients were treated only by AC and/ or TAX-based therapy.

We observed an elevation in gene expression of CA- genes MRP 1 (association with the chemoresistance to AC), MRP 2 and/or MRP 7 (associated with the chemoresistance to TAX), which could be the basis for cross-resistance causing zero effect of later administered cytostatics.

Results can be also seen in Table No. 23; significant response to therapy is shown in yellow. **Epithelial signs of CTCs** (TA-gene expression) and **minimal CA-**

genes expression during NACT could characterize the good clinical effect of AC or TAX **in responding patients**. Interestingly, CTCs 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), an unique effect of AC (patients no 2-9, no 16, 17 and 19) is documented. The best overall response (response rate 2 - 3) to AC and/ or TAX was more frequently demonstrated in TNBC (7x) and HER2+ patients (5x), less frequently in ER+ patients (3x).

On the other hand, **in non-responders** (response rate 0 - 1) **two or more CA-genes were usually frequently expressed** (shown in green in Table No. 23). As the characteristics of CTCs dynamically changed during the NACT, in few cases we observed expression of only one CA-gene before cytostatic therapy and two or more during or after that. CTCs were **more often CD24/CD44-positive** in non-responding patients.
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.

We may conclude that the bigger is the volume of resistant CTCs present in the captured CTCs-fraction, the worse therapy outcome is observed clinically. Resistant patients have elevated mainly MRP1 during AC therapy and MRP1 and MRP7 genes during TAX therapy. In responders, CTCs were more frequently negative or had epithelial characteristics.

Some more complicated cases with unanticipated therapy effect, not correlating with CTCs characteristics are shown in red in Table No. 23. E.g. in patients no. 2 and no. 5, presence of CTCs during TAX therapy was not detected, yet the response to the therapy was minimal. In patients no 16, 17 and 19 multiresistant CTCs before the AC therapy were detected, despite this fact the patients responded to the treatment very well. Possible reasons are discussed in chapter 5.

We observed the presence of CTCs long **time after the tumor resection**, we also

often detected expression of MRP1 gene as the most frequently seen elevated CA-gene after AC-based therapy. Expression of MRP7 (TAX-associated resistance) was not so frequent.

On the other hand, multi-resistant CTCs (expression of MDR1 or two other MRP genes and additionally ERCC1) detection supports a possible scenario of SCs-like CTCs selection after cytostatic therapy.

Although we didn't assess the SCs markers beside CD24/CD44, mainly in cases of ER+ disease we detected CTCs with epithelial signs more often before/during NACT and CTCs with CD24/CD44 properties after operation. It seems that aggressive subtypes (HER2+ and TNBC) are more frequently CD24/CD44 positive from the beginning of the disease. Fore detailed information see pictures in Annex.

Table No. 23: Response rates evaluated by tumor volume measurements in relation to the CTCs characteristics are presented.

Pts	CTCs+ before NACT	CA-genes	Clinical effect of AC (RR)	CTCs after AC	CTCs after AC	Clinical effect of TAX (RR)	CTCs before surgery	CTCs before surgery	effect of whole NACT (RR)
	TA- genes			TA- genes	CA- genes		TA- genes	CA - genes	
1	EPCAM, KRT19, MUC1	MRP1, 4,5,7	1	EPCAM, KRT19, MUC1, HER2, CD24	MRP1,5,7	0	-	-	1
2	CTC negative	-	3	EPCAM, KRT29, MUC1, HER2	-	1	CTC negative	-	3
3	EPCAM, KRT19	MRP7	2	KRT19, HER2	MRP1, 7, ERCC1	1	EPCA, HER2, KRT19	MRP2, MRP4	2
4	HER2, KRT19	-	3	HER2, KRT19, CD24,CD44, EpCAM MUC1	MRP1,5,7,ERCC1	2	CD24, CD44, KRT19	-	3
5	HER2, KRT19	-	3	CD24, KRT19, HER2	-	0	CTC negative	-	2
6	EPCAM, KRT19, HER2	MRP5	3	CTC negative	-	1	CD24,CD44, HER2, KRT19,MGB, MUC1	MRP5, MRP7, ERCC1	3
7	EPCAM, KRT19	-	2	CD24,CD44, HER2, ESR1, KRT19, MUC1	MRP1	3	CTC negative	-	3
8	CTC negative	-	2	CD24, ESR1, HER2, KRT19, MUC1	-	1	CD24, HER2, KRT19, MGB	MRP1, MRP5, MRP7	3
9	CD24, HER2, KRT19	-	3	-	-	no taxanes	CD24, EPCAM, HER2, KRT19	MRP1, MRP7	2
10	CD24, HER2, KRT19	MRP1,5, MRP7, ERCC1	1	CD24, CD44, HER2, KRT19, MGB, MUC1	MRP1,5,7	0	CD24, CD44, KRT19	MRP1, MRP4, MRP5,	1

								ERCC1	
11	CD24, HER2, KRT19	MRP1, MRP5, MRP7	2	CTC negative	-	3	CD24, KRT18, KRT19	MRP1 MRP7	3
12	CD24, CD44, KRT19, MGB, ESR1	MRP1, MRP4	1	CD24, CD44, KRT19, HER2	MRP1	1	CD24, CD44, KRT18, KRT19	MRP1, MDR1	1
13	CD24, CD44, KRT19, MGB	MRP1, MRP5, MRP7	1	-	-	1	CTC negative	-	3
14	CTC negative	-	1	CD24, CD44, KRT18, KRT19	ERCC1, MRP1	1	-	-	0
15	CD24, KRT18, KRT19	MRP1,M RP4	0	CD24, KRT18, KRT19	MRP1, MRP7	1	CD24, KRT18, KRT19	-	0
16	CD24, CD44, KRT18, KRT19, MUC1, HER2	ERCC1, MDR1, MRP1, MRP5, MRP7	3	CD24, CD44, KRT18, KRT19, MUC1	ERCC1, MRP1, MRP2, MRP5	no taxanes	-	-	3
17	CD24, CD44, KRT18, KRT19	MRP1, MRP4, MRP7	3	CD24, KRT18, KRT19	-	3	CTC negative	-	3
18	CD24, EpCAM, KRT18, KRT19	MRP1	1	CD24, KRT18, KRT19	MRP4	1	CD44, KRT18, KRT19, MUC1	MRP1, MRP2, MRP7	3
19	CD24, CD44, KRT18, KRT19	ERCC1, MRP1	3	CD24, CD44, KRT18, KRT19	MRP1, MRP7	3	KRT18, KRT19	MRP1, MRP2, MRP7	Died
20	CD24, KRT18, KRT19	ERCC1, MDR1, MRP1, MRP4	1	CD24, CD44, KRT18, KRT19, MUC1	MRP1	3	CD24, CD44, KRT18, KRT19	MRP4	3

4.5 Clinical indications to CTCs examination: original data on current experience

- E. Hypothesis: CTCs monitoring could have significant clinical impact in BC therapy personalization.
- **E. Objectives: Clinical indications to CTCs examination: original data of our current experience.**

Summary of relevant results as described in study III (Bielcikova Z., Jakabova A., et al 2017) ⇒ Below presented original data in the form of case reports (1-6) demonstrate our current experience with CTCs examination and their potential use. CTCs examination was indicated only as a complementary test. Questions that lead to the indication of CTC- testing can be bound to several therapeutic points (Table No. 24). Published data are shown in detail in chapter 9. Several of presented case reports are discussed partially below.

Table No. 24: Clinical indications to CTCs examination

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

4.5.1 Prediction of disease response to neoadjuvant chemotherapy (NACT) in a patient with BC (Case report 1)

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

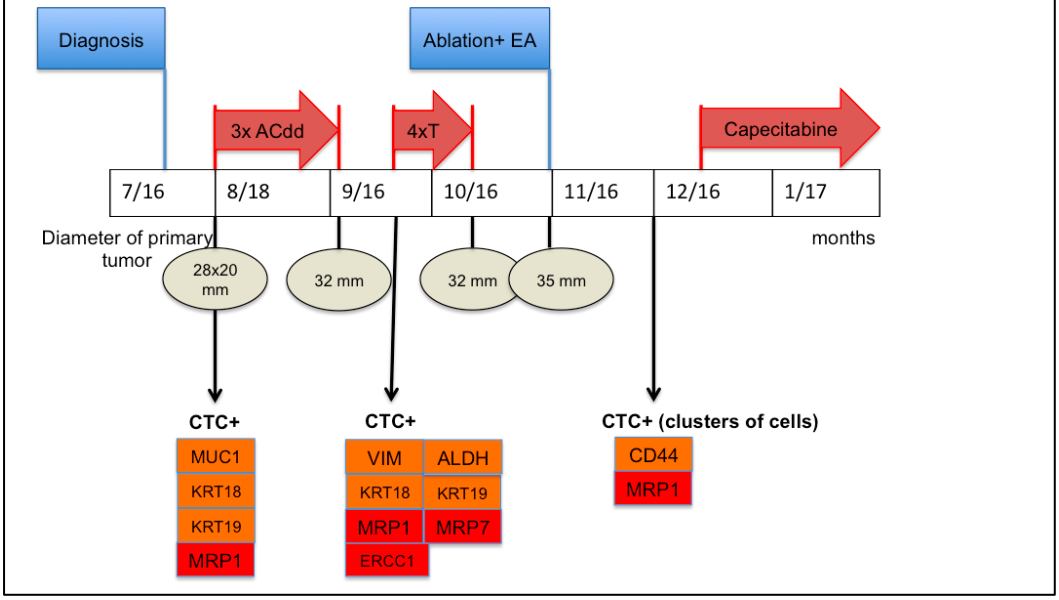
The response to NACT in TNBC patient (34 years old, stage II) has been monitored. Tumor size was 28 mm at the beginning of the NACT; ultrasound examination described several pathological lymph nodes. CTCs were present before NACT had started (Figure No. 6).

CTCs displayed 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 observed by ultrasound examination. CTCs 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 a prediction of TAX chemoresistance, nevertheless patient received 4 cycles of paclitaxel in weekly mode. According to the ultrasound imaging, tumor size remained at 32 mm.

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

Figure No. 6: CTCs monitoring during NACT

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%



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 (vimentin), ALDH (aldehyddehrogenase), markers of epithelial cells: KRT18/19 (keratins), MUC1 (mucin), MRP: markers of chemoresistance

To be discussed: Because of existing anthracyclines resistance (MRP1), the age of patient and adverse outcome of NACT, the patient continues with adjuvant capecitabine therapy (indication is not clear as we have only limited data - for more details see original article in chapter 9 and below presented next case report).

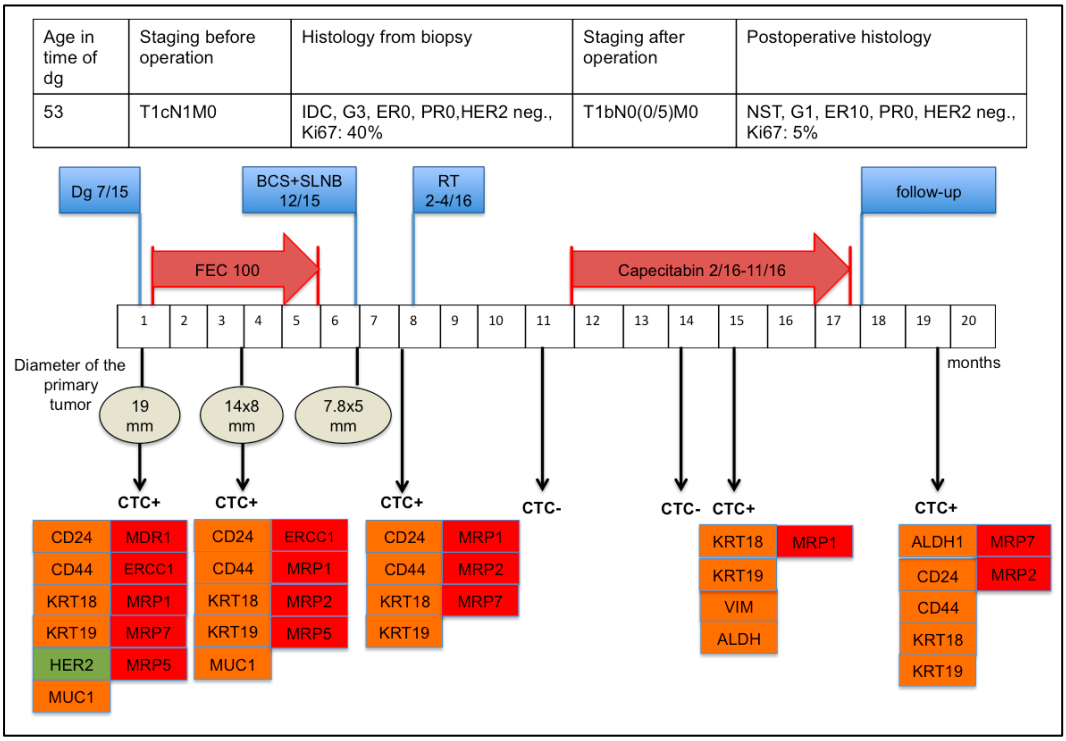
4.5.2 Indication of "additional" adjuvant chemotherapy (ACT) in residual disease in a patient with BC (Case report 2)

Hypothesis: CTCs molecular analysis during ACT may help to predict therapy efficiency and failure.

A case report of TNBC patient (44 years old, stage I) undergoing additional ACT after the completion of NACT is reported (Figure No. 7). ACT indication was based on the CTCs 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 overexpressed in CTCs-enriched fraction (for more details about CTCs during NACT see Figure No. 7).

Figure No. 7: Indication of "additional" adjuvant chemotherapy (ACT) in residual disease in a patient with BC



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 (keratins), HER2: human epidermal growth factor receptor, MUC1 (mucin), MRP: markers of chemoresistance

CTCs persisted postoperatively, as well as their chemoresistant character. Although no CTCs were present after RT, additional ACT with capecitabine was started. The presence of CTCs after the 4th capecitabine cycle was not confirmed. After the 6th capecitabine cycle CTC were detected again, furthermore cells exhibited clustering and overexpressed markers associated with mesenchymal character: vimentin (VIM) and aldehydehydrogenase (ALDH1). We assume that the super-selection of aggressive clone arose during the course of capecitabine therapy. Expression of HER2 was seen only at the beginning of NACT. Because of persistent sensitivity to current treatment we continued up to 8 cycles of

capecitabine therapy. Patient is currently being monitored without therapy and without disease relapse until now.

To be discussed: In the presented case report we can demonstrate the aggressiveness of the tumor defined by persistent CTCs long after completion of primary therapy and possible therapeutic strategy of "watchful waiting" with the administration of systemic therapy apart from the completion of primary treatment. Indication of capecitabine according the Create-X study is not entirely definite. On the other hand, we know that the release of substantial quantities of CTCs occurs early in tumors under 3 mm at diameter (Coumans FA et al., 2013). Clusters of CTCs have under observations in mice, 23-50x higher metastatic potential, their presence thus predicts the ability of cells to establish secondary lesions (Aceto N et al., 2014).

4.5.3 Observation after adjuvant therapy (AT) in a patient with TNBC (Case report 3)

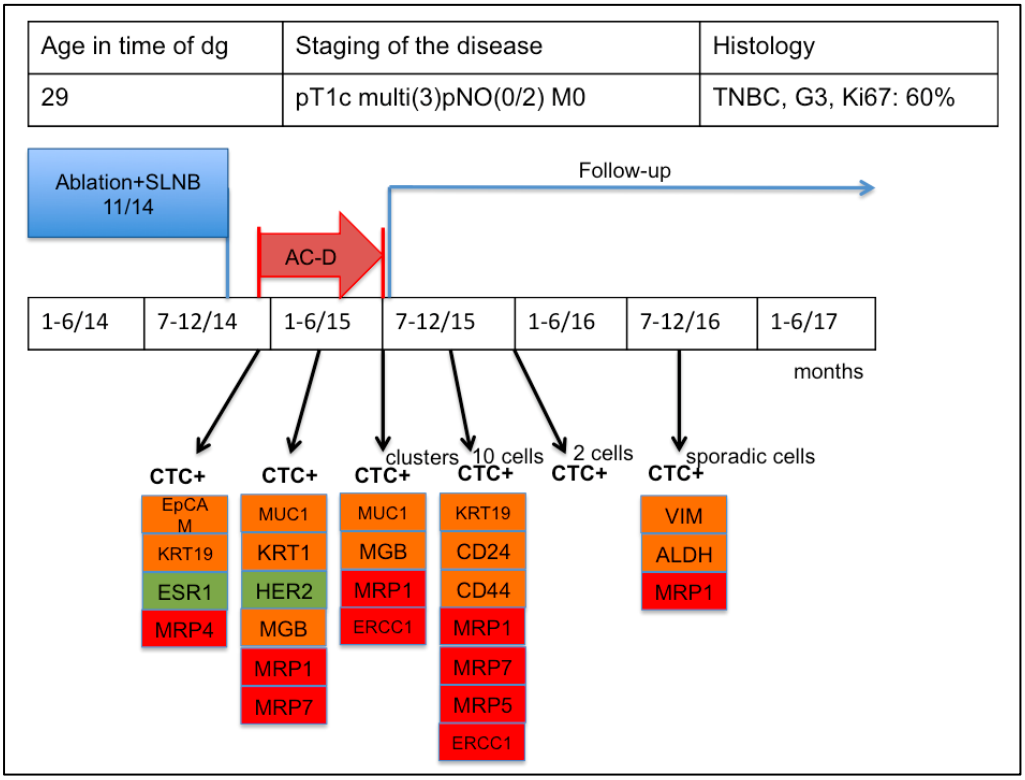
Hypothesis: CTCs molecular analysis after AT may help to predict disease relapse.

We enclose results of postoperative CTCs monitoring for a patient with TNBC (29 years old, stage I). CTCs tests were provided during AT and subsequently in a follow-up period (Figure No. 8).

As it can be seen in the picture the presence of CTCs with epithelial origin was detected during the AT course. Although the primary tumor was TNBC, CTCs overexpressed oestrogen receptor (ESR) and HER2. Before the last docetaxel cycle (07/2015) during AT, we observed clustering of CTCs and ER / HER2 lost. In samples taken 09/2015, 12/2015 and 09/2016 the number of CTCs decreased and characters of cells changed from epithelial to mesenchymal (increased expression of VIM and ALDH). After finishing therapy, CTCs remained resistant to antracyclines (epression of MRP1) for the rest of the time. We also registered elevated ERCC1 expression, which seems to be connected to SCs-like phenotype

of CTCs quite often as published in 2016 by Kasimir-Bauer et al (Kasimir-Bauer S et al., 2016).

Figure No. 8: Monitoring of CTC during adjuvant therapy (AT) and in a follow up period in patient with TNBC



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, MRP: markers of chemoresistance

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

4.5.4 CTCs monitoring after AT and during metastatic disease in a patient with HER2+ BC (Case report 4)

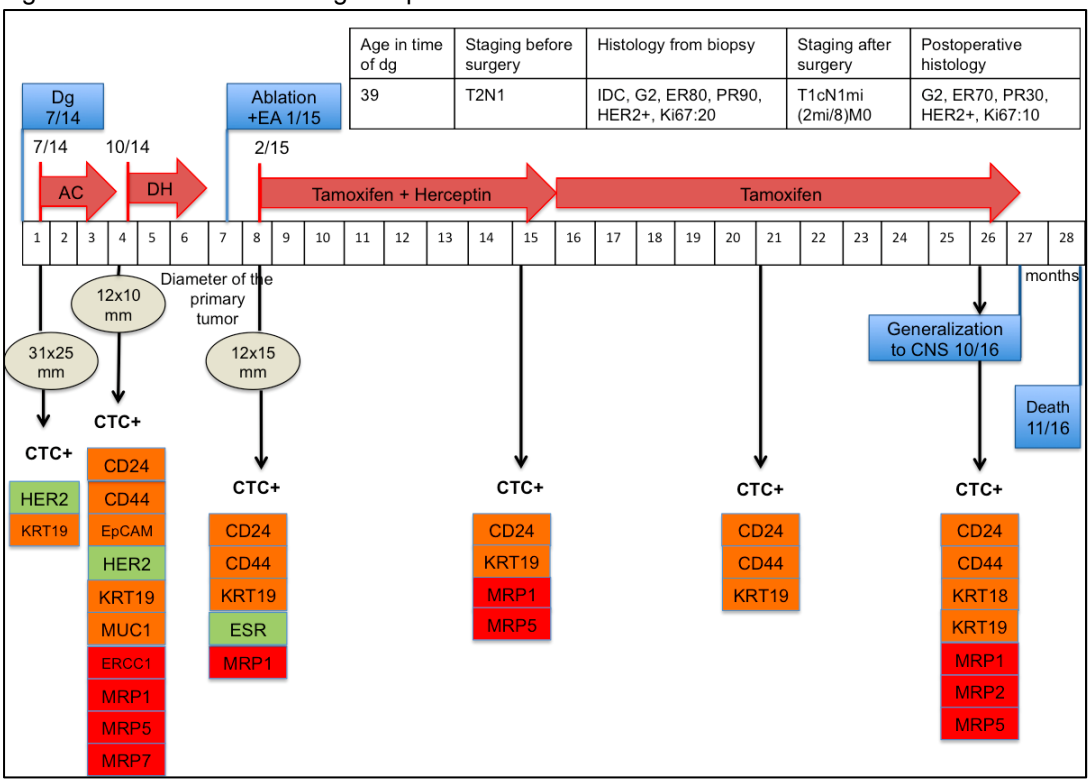
Hypothesis: CTCs molecular characterisation during the metastatic disease follow up period may help to predict therapy indication.

We observed the presence of central nervous system (CNS) metastases in a patient (39 years old, stage II) with HER2-positive locally advanced BC. CNS metastases were detected 11 months after the completion of trastuzumab therapy (Figure No. 9).

Patient started the NACT in 2014, tumor responded to AC-based therapy well but the effect of TAX and trastuzumab was quite poor. CTCs were expressed HER2 at the beginning of the disease therapy, but not later during the TAX-based therapy.

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

Figure No. 9: CTC monitoring in a paitent with HER2+ BC



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, markers of stem cells: CD44/CD24, markers of epithelial cells: KRT18/19 (keratins), EpCAM (Epithelial Cell Adhesion Molecule), MUC1 (mucin), MRP: markers of chemoresistance

Elevation of CTCs count and chemoresistance was documented again before disease progression and CNS metastasis were detected. Expression of KRT18 and CD44 was elevated. Shortly after trastuzumab therapy ending, CTCs expressing HER2 were not present anymore. Patient's death occurred very quickly after diagnosis of brain metastases.

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

4.5.5 CTCs-testing after resection of metastases and early prediction of disease relapse in a patient with metastatic colorectal cancer (CRC)

Hypothesis: CTCs-examination including chemoresistance profile analysis could help in therapy indication in metastatic disease course.

Patient (35 years of age) with CRC was undergoing surgery because of adenocarcinoma of rectum. 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 left liver lobe. Tumor was cured by radiofrequent ablation (RFA) and FOLFOX regimen (Figure No. 10).

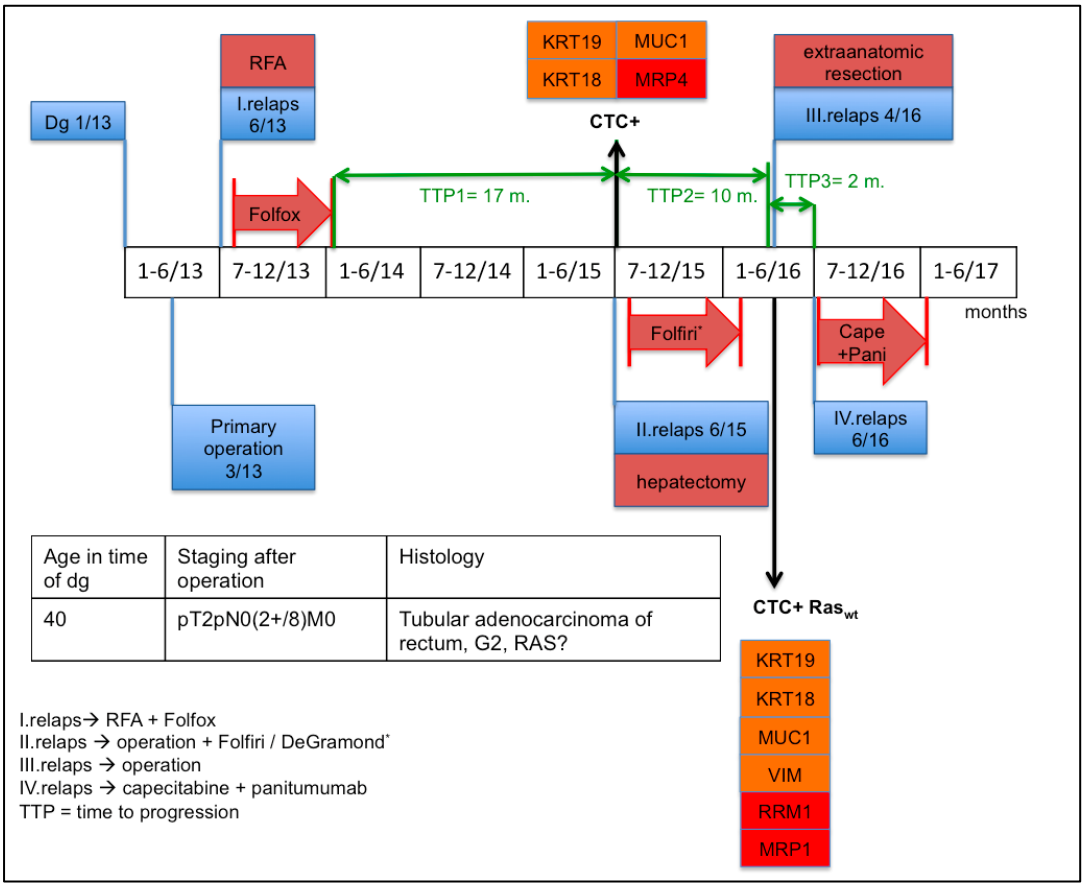
18 months later the second liver relapse appeared. Liver metastasis was resected again. Patient was secured with systemic therapy (FOLFIRI) and blood draw for CTCs examination was indicated. Results were showing CTCs presence by molecular analysis, higher expression of keratins and MUC1 was confirmed, no mesenchymal markers were detected. The relatively small number (units of cells) of CTCs could be reason of quite long time to the next progression (TTP2).

The third liver relapse developed after 10 months in 04/2016 and CTCs test was positive again. Not only liver metastases but also CTCs were tested for the presence of RAS mutations with negative result (RAS_{wt} was confirmed).

Expression of VIM, clustering of CTC and high number of CTC were signs for high disease relapse risk.

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

Figure No. 10: CTC- testing after resection of metastasis 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 (keratins), MUC1 (mucin), VIM (vimentin), MRP: genes of chemoresistance

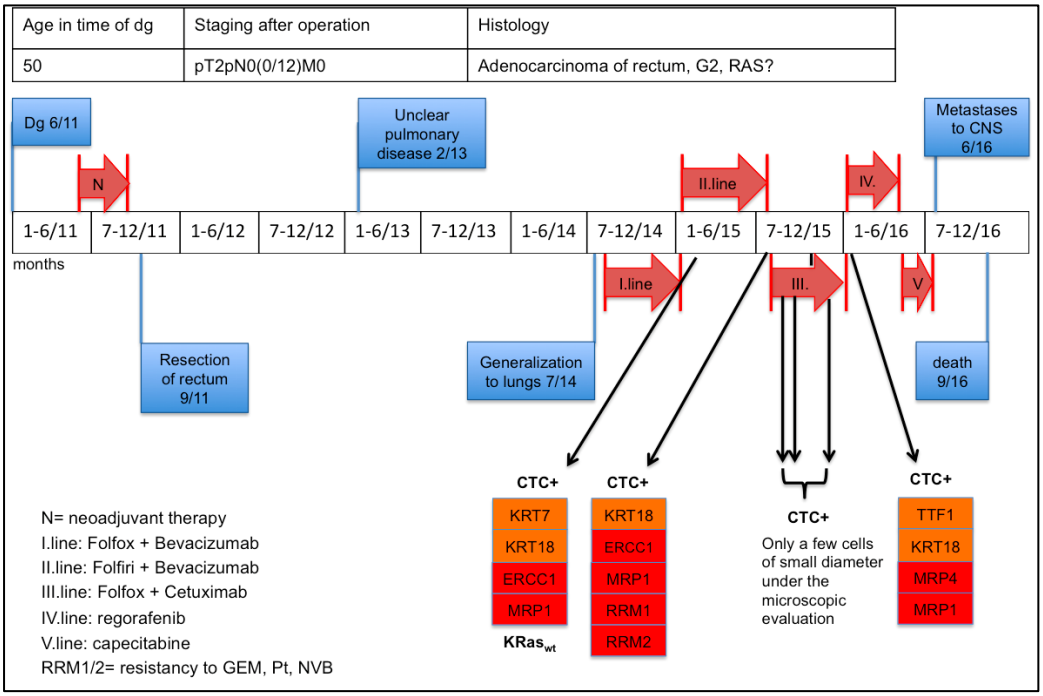
To be discussed: The disease volume after surgical removal of metastases and KRAS status could be controlled by monitoring of CTCs.

4.5.6 Assessment of KRAS mutation status from CTCs in a patient with metastatic rectal adenocarcinoma (case report 6)

Hypothesis: CTCs could present a relevant real-time information source displaying mutational status for genes relevant in therapy indication process.

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 No. 11).

Figure No. 11: Assesment of KRAS mutation status from CTC in a patient with metastatic colorectal cancer



CTC positivity: 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), MRP: genes of chemoresistance

The bulk in the left lung was discovered one year after NACT therapy (02/2013). Patient has been under observation only because of bulk low diameter and the absence of another signs of an active disease. In 07/2014 lung metastases were confirmed by PET/CT and CTCs were detected in blood. 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. CTCs examined after FOLFOX were resistant to oxaliplatin.

Before the initiation of the 3rd line of therapy a biopsy from newly discovered

tumor mass in liver was executed, but tumor cells were not aspirated. Analysis of KRAS was provided based on CTCs-material with the result of $KRAS_{wt}$. Nevertheless, combined FOLFOX and cetuximab therapy failed unfortunately again. Only relatively small cells with several cancerous morphologic features were detected in blood after the therapy completion. CTCs expression profile was not done because of small amount of RNA.

We explain the therapeutic failure of anti-EGFR therapy by tumor heterogeneity and by administration of two previous therapy lines, which might cause 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 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 patient at the stage of generalization, early treatment initiation is critical, but the verification of pulmonary focus (07/2014) could not be done, unfortunately. Liquid biopsy in such a case could replace screening, focused on disease relapse verification. The molecular analysis of CTCs including KRAS status analysis should be more perspective at the beginning of the disease. The effectiveness of anti-EGFR therapy was certainly affected by previous treatment and by chemoresistance of the disease, which was documented by examination of CTCs.

4.5.7 Strategy of using CTCs for the palliative treatment guidance in a patient with NSCLC (case report 7)

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

A 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 mutations in genes EGFR, KRAS, NRAS, BRAF and ALK fusion was also not found in primary tumor. Therapy was conducted from 01 to 05/2016. Examination in 02/2016 showed the presence of CTC with expression of TA-associated genes EpCAM, MUC1, KRT18 and KRT19. CTCs showed resistance to platinum (ERCC1) and cross-resistance to several other antineoplastic agents (MRP1).

CT (computer thomography) scan from 05/2016 showed mild diseffect of the therapy. Control blood test was carried out at the same time, the characterization of CTCs changed partly, and the expression of VIM was newly verified. Disease developed more multi-resistant cells (expression of MRP1, MRP2, MRP4, MRP7, and ERCC1). Based on this result and based on the preserved sensitivity of the disease on derivatives of 5-fluorouracil (MRP5 expression has not been proven) and gemcitabine (expression level of RRM1 or RRM2 was not elevated), we indicated treatment with gemcitabine and capecitabine in the 2nd line.

CT scan at 09/2016 showed slight progression of a one pulmonary node but regression of tumors in other locations. Unresponsive focus was subsequently irradiated and after completion of RT (09-10/2016) we continued with palliative treatment in mentioned scheme till 12/2016. Control CT scan unfortunately revealed further bilateral progression of lung focuses. Despite of this result we declare the effect of second-line treatment lasting for half of year as successful.

To be discussed: CTCs-assisted therapy supplemented by chemoresistance testing may contribute to the better therapeutic effect.

4.5.8 Typing of tumors with unknown primary site (C80) or duplicate tumors (case report 8)

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

A 57-years old patient with duplicate CRC and prostate cancer (PC), CTCs examination was indicated to obtain prognostic information and to identify the type of CTCs. CRC was resected, post-operative stage was pT3pN0(14)M0, microsatelite stable (MSS). PC stage T3bN0-1, GS 3+4 was planned to examine by using cholin- 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 CTCs overexpressed following TA-genes: KRT18, KRT19, VIM, ALDH, VEGF, AMACR. The subset of genes confirmed presence of cells with epithelial origin (keratins), but elevation of SCs markers (VIM, ALDH1) was also demonstrated. Additionally VEGF expression supporting tumor angiogenesis was elevated. The cells were exhibiting morphological features of cells found in patients with CRC, but elevated expression of AMACR gene could be ascribed to the cells of prostate origin. We concluded that probably both cell types from both tumor types were present in patient's blood. Genes associated with chemoresistance to anthracyclines (MRP1) and platinum (ERCC1) were detected.

We also indicated Oncotype DX Colon Cancer (Genomic Health, USA) examination with the result of middle to high risk of disease relapse according molecular print of 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 CTCs examination helped us to distinguish the risk of relapse in two different malignant diseases. Stage II CRC does not always require ACT. Liquid biopsy could predict the need of post-operative therapy in such cases. In comparison to molecular assays targeting primary disease, liquid biopsy offers real-time monitoring of CTCs volume in time.

5. DISCUSSION

The amount of CTCs in blood is very low vice versa the number of CTCs detection techniques is enormeous. The only FDA approved method for the detection of CTCs is CellSearch based on separation of EpCAM positive cells and additional keratines (KRT18, KRT19) testing. Another immunomagnetic separation method detecting tumor cells of an epithelial origin is AdnaTest selecting EpCAM and/or MUC1 positive cells. Detection of CTCs is based on multiplex PCR technology for analysis of three tumor-associated transcripts: HER2, MUC1 and EpCAM.

By using AdnaTest technology **(described in chapter 3.2.1 or original article Ušiaková Z., Mikulová V. et al., 2014)** we detected CTCs in 35 % and 26 % of patients with PBC and in 42 % of patients with MBC. According published data (Krawczyk N. et al., 2013), the presence of CTCs in PBC patients vary between 2 - 55 % by using PCR-based technology and 40 - 80 % in MBC patients what is in correlation with our findings.

Among 100 patients treated with adjuvant therapy we detected CTCs in 26 % of them before and 13 % after the therapy. Published data indicate CTCs positivity in 19 - 43 % of patients in adjuvant setting (Xenidis N. et al., 2013, Franken B. et al., 2012, Pachmann K. et al., 2008, Xenidis N. et al., 2006). Lavrov et al. detected CTCs in 38 % of patients with early TNBC and 42 % of locally advanced TNBC (Lavrov AV. et al., 2014).

In 16 PBC patients we also tested the presence of DTCs in bone marrow **(see chapter 4.1 or original article Ušiaková Z., Mikulová V. et al., 2014)**. Based on results of the AdnaTest, 37.5 % of patients were evaluated as DTCs-positive. In total, 25 % of patients were both CTCs and DTCs-positive and 37.5 % of patients were both CTCs and DTCs-negative so the concordance rate is quite low. On the other hand, our group of patients was very small. In published data, DTCs were detected in 17 - 30 % of PBC patients (Janni W. et al., 2000, Krishnamurthy S. et al., 2010). First data comparing the presence of CTCs in peripheral blood and DTCs in bone marrow showed both CTCs and DTCs simultaneous

occurrence in only 7.9 % of patients (Krishnamurthy S. et al., 2010).

In neoadjuvant studies, the positivity rates for CTCs were reported in a range of 22 -23 % before and 10-17 % after NACT (Pierga JY. et al., 2008, Bidard FC et al., 2012, Riethdorf S. et al., 2010). In our first group of patients monitored by using AdnaTest **(see chapter 4.1 or original data presented in Ušiaková Z., Mikulová V. et al., 2014 article in annexes)** we reported results in the range of mentioned data: 35 % of CTCs- positive patients before and 5 % after NACT.

By using multi-cytokeratin-specific antibody, Serrano et al. detected CTCs in 70 % of patients before NACT and 54 % after that (Serrano MJ. et al., 2012) and Camara even in 83 % of patients before NACT (Camara O. et al., 2007).
Since 2014 we are using MetaCell device **(chapter 3.2.2 or in article Bielcikova Z., Jakabova A. et al., 2017 under review process).** Our data, to be published report CTCs positivity in 85 % of patients before starting NACT and 72 % after NACT **(see chapter 4.1).** One possible reason of a relatively high detection rate of CTCs is involvement of patients with advanced disease due to the clinical stratification: 95 % young premenopausal woman, 75 % HER2+ and 45% TNBC, 100 % of tumors with high Ki67 and 80 % with grade 3, 75 % of patients had locally advanced BC with lymphatic node involvement. Another cause of very high CTCs detection rate is a technique used for CTCs detection.

We have data on prognostic power of CTCs decrease during the systemic therapy. In our group of 20 PBC patients we observed CTCs-positivity in a high number of them before, during and in pre-surgery time (85 % of CTCs-positivity before, 88 % during and 72 % after NACT). The same result of CTCs number decrease was seen also among 197 patients monitored using AdnaTest in adjuvant setting.

In the context of CTCs enumeration during NACT, there are two questions to be asked: The first one relates to the risk reduction in CTCs-negative patients and the disease relapse risk in patients with not decreasing CTCs amount. The second one is related to the dynamic behaviour of CTCs and disease relapse risk in patients being CTCs-negative after NACT and before surgery but CTCs-positive in a follow-up period. Of course, there is no right answer yet to these questions. In

connection to the clinical practice, the most important questions are those related to the risk of disease relapse, true distinction of responders and non-responders and treatment recommendation not only for the high risk group but also for the low risk group as the key how to treat BC less aggressively.

The presented two-step protocol combining MetaCell size-based filtration with both cytomorphological and molecular characteristics of CTCs may identify CTCs in such cases, when they are not detected by other methods (e.g. out of the 116 samples, EpCAM elevated expression has been confirmed in only 16 cases, expression of KRT 18/19 in 90 samples, HER2 in 34, MUC1 in 31 and MMG in 12 samples).
We believe that enrichment step of CTCs filtration and vizualization by using MetaCell device enhaces the sensitivity of CTCs detection process **(see chapter 3.2.3)**. On the other hand, we keep in mind that we miss data comparing both methods in one sample set.
Methods working on combined platform, like microfluidic systems help to overcome the low number of CTCs in blood and by using physical properties of tumor cells these methods increase the probability of CTCs detection despite of their dynamic behaviour (Magnabua MJ, Park JW. et al., 2014). PCR-based methods increase the specificity of CTCs detection by testing of a wide panel of TA-genes including genes connected to EMT process and stemness.

We compared status of HER2, ESR and PGR in CTCs and primary tumor and other clinicopathological characteristics to affirm the hypothesis that the presence and properties of CTCs not correlate with conventional clinicopathological parameters **(chapter 4.2).**

About 1/3 (35 %) of patients tested by AdnaTest had HER2+ CTCs in HER2- PBC **(see chapter 4.2 or original data presented in Ušiaková Z., Mikulová V. et al., 2014 article in annexes)**. As the AdnaTest don't offer the evaluation of ESR expression level in CTCs, we weren't able to compare it with ER-status of primary tumors. The correlations of CTCs presence with the tumor size, hormonal receptor status, and lymph node involvement was not statistically significant.

Discrepancies in concordance status of HER2 were higher by using MetaCell approach **(see chapter 4.2 or original data in Bielcikova Z., Jakabova A et al., 2017 article in annexes)**. The distribution of CTCs during NACT was very discordant in ER+ tumors (only 8 % CTCs-positive samples was ER+), on the other hand, 67 % of TNBC patients had triple negative CTCs and 80 % of HER2+ patients had HER2+ CTCs.
Differencies were more obvious if we compared primary disease and CTCs status in any time of patients observation (before, during or after NACT and in follow up period); 50 - 58 % of HER2+ PBC became HER2- and vice versa, 26 - 32 % of primary HER2- cancers developed HER2+ CTCs. The highest concordancy rate among BC subtypes was seen in TNBC (67 - 100 %). The worst concordancy was seen in the status of ER independently on HER2 status.

Fehm et al. described discordance rates between primary tumors and CTCs to be 71 % for ER status (Fehm T. et al., 2009). Other comparative analysis revealed a wide range of agreement (40 - 70 %) in ER/PR status (Tewes M. et al., 2009, Aktas B. et al., 2011, Somlo G. et al., 2011, Paoletti C. et al., 2015). Discordance rates in HER2 status between primary tumor and CTCs are variable, in the order of 15 – 35 % in MBC (Fehm T. et al., 2010; Munzone E. et al., 2010; Flores LM. et al., 2010). In PBC patients, HER2 discordance has been reported more often in terms of de novo expression of HER2 (50 % of patients with HER2- primary tumors had HER2+ CTCs) on CTCs than vice versa (33 % of patients with HER2+ primary tumors had HER2- CTCs) (Wülfing P. et al., 2006). In another study comparing HER2 status of CTCs versus that of primary and metastatic tumors has shown concordance of 69 and 74 %, respectively (Wallwiener M. et al., 2015).

The monitoring of CTC status during NACT and after surgery shows that majority of CTCs were ER- and HER2- in HER2- PBC, in HER2+ PBC, CTCs were predominantly ER- and HER2+ during NACT but they often lost HER2+ in time after surgery. We conclude that CTCs retain aggressive properties predominantly. Our findings are consistent with Yu et al. (Yu M. et al., 2013) who published that cells of the primary tumor are a mixture of epithelial and mesenchymal cells, while CTCs are predominantly mesenchymal or acquire

mesenchymal properties in non-responding patients.

Although the prognostic significance of CTCs count, the predictive value of CTCs number has not been clearly demonstrated yet. Well-known study SWOG S0500 did not support the assumption of clinical benefit of early chemotherapy change in case of persistent CTCs positivity after the first treatment cycle in patients with MBC (Smerage JB. et al., 2014). According to the meta-analysis of neoadjuvant studies (Fei F. et al., 2014) the number of CTCs does not correlate with the treatment response also. A decrease in the CTCs count after NACT did not indicate that patients had an improved response to NACT. We observed the same result among 20 PBC patients treated neoadjuvantly **(chapter 4.3 or original data in Bielcikova Z., Jakabova A. et al., 2017 article in annexes).** We detected CTCs in 85 - 100 % of non-responding patients and 75 - 100 % of responders. The number of CTCs increased during the NACT in comparison to CTCs count before therapy (85 % before and 88 % CTCs-positive samples during NACT). We expect that tumor cells mobilization occurs by the effect of chemotherapy.

Although the total number of CTCs-positive samples decreased before surgery (72 %), in a follow-up period (after surgery) all patients (100 %) were CTCs-positive one or more times. The presence of CTCs after tumor resection (Ignatiadis M. et al, 2007) or after completion of adjuvant therapy (Rack B. et al., 2014) could lead to the disease relapse. But we have no information about the prognostic impact of increasing number of CTCs after NACT and surgery. In our high-risk group of young patients we observed disease relapse in 4/20 of them. The 1y-OS of our group is 95 % (1/20 died), 2y- OS is 78 % (3/14 died). Median OS (mOS) is 23.5 months. 1y- OS and 2y- OS in HER2+ PBC patients exceeds 95 % (e.g. according HERA-trial), 2y- OS in TNBC is around 89 %. Survival rate in our group of patients is lower. One from dead patients was BRCA-positive, one had TNBC and two patients died because of HER2+ BC. Their survival was shorter than predicted OS according mentioned studies. CTCs-positivity could be the reason of bad prognosis of BC patients.

We distinguished reponders and non-responders according to the tumor volume reduction during NACT a compared it with CTCs characteristics **(see chapter 4.4**

or original article in Bielcikova Z., Jakabova A. et al., 2017). Responders were found mainly in the group of patients with CTCs expressing epithelial markers and CTCs with a minimal CA- genes expression. In non-responders, two or more CA-genes were usually frequently overexpressed in CTCs fraction. In responders (response rate 2 - 3), a unique effect of AC (patients no 2 - 9, no 16,17 and 19) was documented, the best overall response 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, in non-responders (response rate 0 - 1) developing TAX chemoresistance was documented 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 (more details in chapter 9).

From our four dead patients: patients no 1 and 12 were classified as non-responders during NACT (response rate 1), patient no 5 was classified as moderate responder (response rate to AC was 3 but no response to TAX therapy), patient no 19 responded very well (response rate 3), but primary staging (stage III) was unfavourable.

We declared that CTCs characteristics are more important predictors of disease relapse than the CTCs number. We know that patients achieving pCR after NACT have better outcome in ongoing clinical trials. pCR is most often seen in HER2+ BC or TNBC. If we assume aggressive tumor cells in these subtypes, we could pCR explain as the eradication of highly proliferating tumor cells with probably epithelial properties. Mesenchymal cells would not be so sensitive to NACT. Less aggressive epithelial cells in less agressive disease (typically luminal types) do not respond well to NACT. Moreover, pCR doesn't have predictive value in these tumor types. The presence of less proliferating cells or SCs-like cells could explain this aspect. The same reason probably causes disease relapse in smaller part of patients who achieved pCR. In our study, pCR was achieved in 4/9 TNBC; one could predict uniform biological subtypes of these 4 tumors. In two cases response to both AC and TAX were very good, in another two cases worse response to AC but very good effect of TAX was seen. 4/13 (31 %) of samples were CTC-negative. All pCR patients are still alive, without signs of disease relapse, but all four patients are still CTC-positive after surgery. CTCs are mostly

epithelial with minimal chemoresistance in patients no 6 and 17, but CTCs of patients no 7 and 20 have more aggressive characteristics (see pictures in chapter 9). The validation of both prognostic significance of pCR and prognostic significance of CTCs after NACT is needed. We still don't know if the CTCs persistence or the information about pCR is more important for disease relapse prediction.

In the follow-up monitoring of patients we detected CTCs in 100 % of them. Moreover, CTCs were more often CD24/CD44-positive so probably more aggressive and resistant to therapy. We also saw CTCs-positive cases treated with adjuvant anti-HER2 therapy or hormonal therapy but with ER and/or HER2-CTCs.

The presence of CTCs after tumor resection or after completion of adjuvant therapy could lead to disease relapse and has prognostic impact (Rack B. et al., 2014). However, prognostic significance of CTCs presence in patients treated with NACT is bound to pre-treatment CTCs-positivity in some studies (Bidard FC. et al., 2013) but to both pre- and post-treatment CTCs presence in another (Pierga JY. et al., 2008). We observed the presence of CTCs long time after the tumor resection. We also detected expression of MRP1 gene as the most frequently seen elevated CA-gene after AC-based therapy. Expression of MRP7 (TAX-associated resistance) was not so frequent.

Detection of multi-resistant CTCs (with expression of MDR1 or two or more MRP genes including ERCC1) supports a possible scenario of SCs-like CTCs selection after cytostatic therapy. Although we didn’t assess the SCs markers beside CD24/ CD44 in cases of ER+ disease we detected CTCs with epithelial signs more often before/during NACT and CTCs with CD24/CD44 properties after surgery. On the other hand, it seems that aggressive subtypes of BC are more frequently CD24/CD44 positive from the beginning of the disease.

Clinical use of CTCs testing is still the problem. Promising design of new studies (Bidard FC. et al., 2013) focusing on predictive power of CTCs is the main prerequisite for definition of coherent conclusions. On the other hand, targeting a single mutation in the metastatic disease releasing a wide range of tumor cells

subclones would not lead to the clinical effect.

In the MOSCATO trial new mutations in metastatic tumor lesions were identified in 49 % of patients but only 19 % of patients were practically treated and the targeted therapy have helped to improve prognosis of 33 % of them (Massard C., 2015). The use of MRD in palliative indication is the closest to the clinical practice. cfDNA- navigated therapy based on the detection of certain types of mutations in NSCLC patients is already part of clinical care. We expect the same usage of CTCs in another diagnoses (e.g. the determination of RAS status in CRC or ARV7 in CRPC patients) and in cases of primary disease, which are not approachable for biopsy verification. Finally, typing of tumors of unknown primary site or typing of MRD in patients with duplicate tumor disease is further step how to improve the therapeutic outcome of cancer treatment.

Clinical use of CTCs testing is still the problem. Promising design of new studies (Bidard FC. et al., 2013) focusing on predictive power of CTCs is the main prerequisite for definition of coherent conclusions.

6. CONCLUSIONS

We can conclude that:

1. **By comparing of two CTC-isolation and detection approaches (AdnaTest® and MetaCell® -qPCR completed), higher CTCs detection rates have been observed by using combined detection method including MetaCell® size- based filtration and qPCR-based detection of TA- genes expressions in the CTC- enriched fraction (see chapter 4.1).**

	MetaCell	**Adnatest**
	CTC positivity (%)	CTC positivity (%)
Before NACT	85	35
Before surgery	72	5
After surgery at any time of follow-up period	**100**	26

2. **The presence and properties of CTCs do not correlate with conventional clinicopathological parameters of the primary tumor. The presence of CTCs in an independent prognostic parameter.**

   - The correlation of CTCs presence with the tumor size, hormonal receptor status, and lymph node involvement was not statistically significant in the adjuvant setting.

3. **High discordancy rate in status of ER and HER2 among primary tumors and CTCs was observed.**

   - During NACT CTCs with a quite high rate of concordancy in TNBC a HER2+ BC patients were detected. The highest concordancy rate among BC subtypes was seen in TNBC (67 - 100 %). The worst concordancy was seen in PBC tumors with the status of ER+ independently on HER2 status. The discordancy rate increased after systemic therapy.

4. **CTCs count is independent to the local response in patients undergoing NACT, but CTCs characterinstics predicted tumor response.**

   - The effect of the whole NACT was accompanied by a non-significant decrease in CTCs positivity (85 % of CTCs-positive patients before NACT and 72 % of CTCs-positive patients before operation).

   - In responders, CTCs were more frequently not present (in CTC-negative patients) or had epithelial characteristics. In non-responders, expressions of 2 or more CA-genes were detected repeatedly. Patients showing chemoresistancy have had elevated MRP1 during AC therapy mostly and MRP1 and MRP7 in combination during TAX therapy.

5. **It could be assumed that CTCs character is more important than single CTC-number.**

   - Tumor response in patients undergoing NACT was small if chemoresistant CTCs populations were present.

   - Few case reports were described to shown potential of CTCs – testing as a complementary method in clinical practice. Beside the prediction of NACT response, CTCs could be used in the same indication adjuvantly and in palliative therapy in different malignant diseases.

7. LIST OF ABBREVIATIONS

ABCB1/ABCG2 members	ATP-binding cassette (ABC) transporter family
AC	Antracyclins
ACT	Adjuvant chemotherapy
ActB	Actin B
AKT	Serine-threonine protein kinase
ALDH1	Aldehyde dehydrogenase 1 (marker for CSCs)
AMACR identified as	α-methylacyl coenzyme A racemase, a gene being overexpressed in prostate cancer
ARV7	Androgen receptor splice variant 7
AT	Adjuvant therapy
BC	Breast cancer
Bcl-2 the Bcl-2	B-cell lymphoma 2 is the founding member of family of regulator proteins that regulate cell death
BL-1	Basal-like type 1
BL-2	Basal-like type 2
Bp	Base pairs
CA-genes	Genes associated with chemoresistance
c-Src	Proto-oncogene tyrosine-protein kinase
CD24	Cell adhesion molecule
$CD44^+$	Cell-surface glycoprotein involved in cell–cell interactions, cell adhesion and migration
$CD44^+/CD24^-$ reported	A subpopulation of breast cancer cells has been to have stem/progenitor cell properties
CD45	Marker of white blood cells
CD68	Marker of monocytes/macrophages
$CD8^+$ T-cell	Cytotoxic T lymphocyte
CEE™	Cell Enrichment and Extraction™
cf-DNA	Cell free-deoxyrinonucleotid acid

CGH	Comparative genomic hybridization
CK	Cytokeratin
CK19	Cytokeratin 19
CNS	Central nervous system
CRC	Colorectal cancer
CRCP	Castrate resistant prostate cancer
CSCs	Cancer stem cells
ct-DNA	Circulating tumor-deoxyrinonucleotid acid
CTCs	Circulating tumor cells
CTM	Circulating tumor microemboli
CXCL1	Gene, which encodes a member of the CX subfamily of chemokines. The encoded protein is a secreted growth factor that signals through the G-protein coupled receptor, CXC receptor 2.
CXCR1	Cytokine receptor to interleukin-8
C80	Tumors of unknown primary site
DCCs	Early disseminated cancer cells
δEF-1	Transcription factor ZEB1, repressor of E-cadherin
DEP-FFF	Dielectrophoresis field flow fractions
DFS	Disease free survival
DNA	Deoxyribonucleotid acid
DTCs	Disseminated tumor cells
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EGP2	Epithelial glycoprotein 2
EMT	Epithelial-mesenchymal transition
EpCAM	Epithelial cell adhesion molecule
ER	Oestrogen receptor
ER-	Oestrogen receptor negative
ER+	Oestrogen receptor positive
ERCC1	DNA excision repair protein (predictor of cisplatin

	resistance)
ESR	Oestrogen receptor gene
FAST	Fiber-optic array scanning technology
FCM	Flow cytometry
FDA	US Food and Drug Administration
FISH	Fluorescent in situ hybridization
FOLFOX	Chemotherapeutical regimen basef on 5-fluorouraxil, oxaliplatin and calciumfolinat used for CRC patients treatment
FOLFIRI	Chemotherapeutical regimen basef on 5-fluorouraxil, irinotecan and calciumfolinat used for CRC patients treatment
FOXC2	Forkhead box protein C2, FOX transcription factors are expressed during development and are associated with a number of cellular and developmental differentiation processes
FoxO-3	Transcription factor, a member of family FoxO
FSMW	Functionalized structured medical wire
GA733.2	Epithelial cell adhesion molecule (EpCAM)
GLI-1	Glioma-associated oncogene, effectors of Hedgehog signaling
G	Grade of tumor cells differentiation
G1	Low degree of cell differentiation
G2	Middle grade of cell differentiation
G3	Low grade of cell differentiation
GS	Gleason score
HB-Chip	Herringbone-chip
HD-CTC	High definition CTCs images
Hedge-hog	Signaling pathway that transmits information to embryonic cells required for proper cell differentiation

HER2	Human epidermal growth factor receptor type 2
HER2-	Human epidermal growth factor receptor type 2 negative
HER2+	Human epidermal growth factor receptor type 2 positive
IHC	Immunohistochemical
ICC	Immunocytochemistry
IDC	Invasive ductal carcinoma
IL-6	Interleukin-6
IL-8	Interleukin-8
IM	Immunomodulatory type
ISET™	Isolation by size of epithelial tumor cells
JAK-STAT	Janus kinase/signal transducers and activators of transcription pathway is the principal signaling mechanism for a wide array of cytokines and growth factors
Ki-67	Nuclear protein associated with the cellular proliferation
KRT19	Keratin 19
LAR	Luminal/androgen-receptor positive type
LABC	Localy advanced breast cancer
M	Mesenchymal Type
M0	No distant metastases
M1	Metastatic disease
MAPK	Mitogen-activated protein kinase
MBC	Metastatic breast cancer
MEMS	Micro electromechanical system
MET	Menechymal-to-epitelial transition
MGB	Mammaglobin
mir-93	Micro-RNA functions as tumor suppressor, located in MCM7 gene
ML	Mesenchymal/stem-like type

MMP	Matrix metalloproteinases
MP	Auxin response factor 5 mediates cell-to-cell movement
MRD	Minimal residual disease
MRP	Multidrug resistance-associated proteins encoded by the adenosine triphosphate (ATP) binding cassette (ABC) transporter genes
MSS	Microsatelite stability
mRNA	Mediator ribonucleotid acid
MUC-1	Mucin 1
NACT	Neoadjuvant chemotherapy
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells is a protein complex that controls transcription of DNA, cytokine production and cell survival
NGS	Next-generation sequencing
N0	Negative lymph nodes
N1	Positive lzmph nodes
Notch	Signaling pathway involved in the generation of cell diversity and SCs maintenance in different systems
NSCLC	Non-small cell lung cancer
NST	Invasive carcinoma of not specified type
OS	Overall survival
p1B	Acidic ribosomal protein P1B
PBC	Primary breast cancer
PC	Prostate cancer
pCR	Pathologic complete remission
PDGF	Platelet derived growth factor
PFS	Progression free survival
PGR	Progesterone receptor gene
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase

PR	Progesterone receptors
PR-	Progesteron receptor negative
PR+	Progesteron receptor positive
PS2	Oestrogen-responsive pS2 gene
PTEN	Phosphatase and tensin homolog
RAS	Family of KRAS, HRAS and NRAS oncogenes
RAS_{wt}	Wild type form of RAS genes
qRT-PCR	Quantitative real time polymerase chain reaction
RB1	Tumor suppressor gene
RFA	Radiofrequent ablation
ROS1	Tyrosine kinase receptor encoded by the gene ROS1
RRM1/2	Ribonucleotide reductases M1/M2 are associated with gemcitabine /vinorelbin and probably also platinum chemosensitivity in cancer cell
RT-PCR	Reverse-transcription polymerase chain reaction
SCs	Stem cells
SIP1	Smad interacting protein 1, intracellular mediator transforming growth factor-beta
Smads	Intracellular proteins that transduce extracellular signals from transforming growth factor beta ligands to the nucleus
Snail	Family of transcription factors that promote the repression of the adhesion molecule E-cadherin to regulate epithelial to mesenchymal transition
TA	Transit amplifying cells
TA-genes	Tumor associated genes
TAX	Taxanes
TDC	Terminally differentiated cells
TGF-β	Transforming growth factor beta
TNBC	Triple negative breast cancer
TNF-α	Tumor necrosis factor alfa

TRAIL	TNF-related apoptosis-inducing ligand is a protein functioning as a ligand that induces the process of cell death called apoptosis
TRR	Tumor response rate
TTP	Time to progression
Twist	Twist-related protein 1 plays an essential role in cancer metastasis
uPA	Urokinase plasminogen activator
VEGF	Vascular endothelial growth factor
VIM	Vimentin
WBC	White blood cells
Wnt	Signaling by the Wnt family of secreted glycolipoproteins via the transcription co-activator β-catenin controls embryonic development and adult homeostasis.
β- katenin	Dual function protein, involved in regulation and coordination of cell–cell adhesion and gene transcription

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9. LIST OF PUBLICATIONS

9.1 Publications in extenso on the topics of the doctoral thesis

I. Ušiaková Z, Mikulová V, Pintérová D, Brychta M, Valchář J, Kubecová M, Tesařová P, Bobek V, Kološtová K. Circulating tumor cells in patients with breast cancer: monitoring chemotherapy success. In Vivo 2014;28(4):605-14. [IF 0.974]

II. Bielcikova Z., Jakabova A., Pospisilova E., Pinkas M., Zemanova M., Petruzelka L., Kolostova K., Bobek V. Character of circulating tumor cells in early breast cancer patients receiving neoadjuvant chemotherapy [in review process Nature Methods]

III. Bielčiková Z, Jakabová A, Pinkas M, Zemanová M., Kološtová K., Bobek V. Circulating tumor cells: what we know, what do we want to know about them and are they ready to be used in clinics? AJTR 2017 [IF 3.22] [accepted May 2017]

IV. Jakabova A., **Bielcikova Z**, Pospisilova E, Zemanova M, Petruzelka L, Kološtová K, Bobek V. Heterogeneity of circulating tumor cells in breast cancer patients [in review process to American Journal of Cancer Research]

9.2 Other original publications unrelated to the topics of the doctoral thesis

with an IF:

Buchler T, Pavlik T, Melichar B, Bortlicek Y, **Usiakova Z,** Dusek L, Kiss I, Kohoutek M, Benesova V, Vyzula R, Abrahamova , Obermannova R. Bevacizumab with 5-fluorouracil, leucovorin, andoxaliplatin versus bevacizumab with capecitabine and oxaliplatin for metastatic colorectal carcinoma: results of a large registry-based cohort analysis. BMC Cancer 2014, 14:323. [IF 4.46]

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9.4 Conference abstracts / posters unrelated to the topics of the doctoral thesis

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9.5 Monography

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ANNEXES

Full- text publications

Publication I: **Ušiaková Z**, Mikulová V, Pintérová D, et al. Circulating tumor cells in patients with breast cancer: monitoring chemotherapy success. In Vivo 2014;28(4):605-14.

Publication II: **Bielcikova Z.,** Jakabova A., Pospisilova E., et al. Character of circulating tumor cells in early breast cancer patients receiving neoadjuvant chemotherapy [in review process Nature Methods]

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