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Summary report of the doctoral dissertation

Circulating tumor cells in breast cancer patients

Cirkulující nádorové buňky u pacientek s karcinomem prsu

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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 (disseminated) tumor cells in women with breast cancer 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 the principle CTC-character does not correlate to the clinicopathological characteristic of the primary disease and change dynamically in time.

Finally, we tried to implicate the CTCs testing in the clinical practice in our department of oncology. CTCs-examination is indicated only as a complementary test. The summary of potential clinical applications of the CTCs-testing was published in other article.

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 klinickopatologických 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.

Konečně, CTCs vyšetření se nám podařilo částečně implementovat do běžné klinické praxe. Vyšetření CTCs je indikováno jako doplňková metoda. Možné využití CTCs v klinické praxi je shrnuto v další publikaci.

1. INTRODUCTION

Breast cancer (BC) is the group of malignant tumors with the highest incidence among 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 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 primary BC patients. The new molecular classification based on the recognition of intrinsic biological subtypes within the BC spectrum (Goldhirsch A. et al., 2011) was adopted in 2011. The state of the art in BC treatment does not incorporate tumor biology information into the therapy decision yet.

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 tissue of probably different organs. It can have the appearance of CTCs, circulating DNA fragments (cfDNA/ctDNA) or other fragments of tumor cells (miRNA/circulating exosomes) or disseminated tumor cells (DTCs) detected mainly in bone marrow. In all below discussed text CTCs as the one type of MRD will be discussed. DTCs are also discussed in some chapters.

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.

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 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 aggressiveness of the primary disease. CTCs were detected in 2 - 55 % of PBC patients and 40 - 80 % of MBC patients.

The standard pathological examination of the primary tumor is inadequate for the risk of disease relapse prediction. The removal of primary tumor mass may not be sufficient to ensure that the patient is cured. 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.

The presence of CTCs has the significant impact on PBC/MBC patient prognosis regardless the BC subtype and detection technique (Cristofanilli M. et al., 2004, Dawood S. et al., 2008, Zhang L. et al., 2012, Pierga J. et al., 2012, Wallwiener M. et al., 2013, Zhang L. et al. 2012, Gradilone A. et al., 2011, Weigelt B. et al., 2003, Tewes M. et al., 2009, Reinholz MM. et al., 2011, Janni WJ. et al., 2016, Pierga JY. et al., 2008, Bidard FC. et al., 2010, Xenidis N. et al., 2006, Rack B. et al., 2014, Xenidis N. et al., 2013, Franken B. et al., 2012, Pachmann K. et al., 2008, Xenidis N. et al., 2007). Detection of DTCs usually not correlates with the CTCs presence. DTCs in the bone marrow of BC patients are an independent significant predictor for poor prognosis (Braun S. et al., 2005).

CTCs/DTCs may also provide useful predictive information to guide treatment decision. We know that the presence (number) of CTCs differ in patients responding and non-responding to the therapy, but the SWOG s0500 trial did not support the assumption that the early chemotherapy change has the clinical benefit in patients with MBC and persistent CTCs after the first cycle of therapy (Smerage JB. et al., 2014). We have still little data about the real

predictive power of CTCs but it seems that CTCs with stem-cells (SCs) and or EMT characteristics are related to non-respoding patients (Aktas B, et al., 2009, Yu M. et al., 2013, Kasimir-Bauer S. et al., 2016). Some studies indicate the potential clinical significance of monitoring biomarkers in CTCs during treatment or at disease progression (Liu Y. et al., 2013). The use of CTCs-based biomarkers to guide therapy, however, has not been incorporated into practice due to the need for further technical and clinical validation.

Concordance/discordance between primary tumor/CTCs and/or metastasis in molecular characterisation is the key point to predict therapy effectiveness and potentially better patients outcome. The predictive ability of CTCs biomarker analysis has predominantly been assessed in relation to HER2, with variable and inconclusive results. Limited data exist for other biomarkers, such as the oestrogen receptor (ER). In addition, it is necessary to define and validate the most accurate and reproducible method for CTCs molecular analysis.

No optimal method for CTCs detection 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). Until now the only FDA-approved system for detection of CTCs in breast, prostate and colorectal cancer is CellSearch (Riethdorf S. et al., 2007). The cut-off $\geq$ 5 cells per 7.5 ml of blood was prospectively identified in a set of MBC patients and cut-off $\geq$ 1 cell per 7.5 ml of blood in PBC patients. 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. 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.

In 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).

2. AIMS OF THE THESIS

The main aims of my thesis:

1. To examine and attest CTCs isolation and detection methods in clinical setting for different tumor types.

2. To monitor MRD by means of CTC- analysis in patients with BC in different therapeutical subgroups (neoadjuvant, adjuvant, palliative).

3. To define prognostic, predictive and diagnostic utility of CTCs in clinical practice.

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

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.

- *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.

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

- *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 NACT.

- *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.

3. METHODS

3.1 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.

mRNA isolation from lysed, enriched cell is performed, next done reverse transcription results in cDNA. The analysis of three tumor-associated transcripts HER2, MUC1 and EpCAM is performed in a multiplex PCR using prepared cDNA from enriched CTCs. 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.

3.2 CTCs testing by using MetaCell® – Size-based CTC enrichment

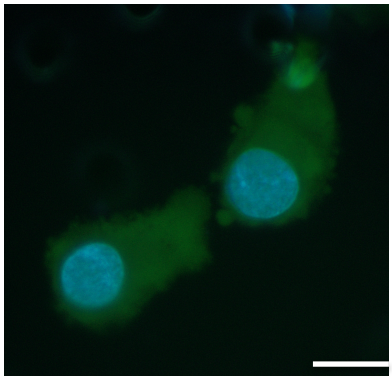
First, cytomorphological evaluation of captured viable cells was evaluated. Second, qPCR analysis of mRNA isolated from captured CTCs –like cells were provided. A set of tumor-associated (TA)-genes and genes associated with the chemoresistance (CA-genes) was analysed. Gene expression of the CTC-enriched 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^{o}C. This fraction can be assigned as CTC- enriched fraction without *in vitro* culture. Captured cells were cultured *in vitro* under standard conditions (37^{o}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. 1). 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^{o}C for subsequent gene expression analysis.

Figure 1: CTCs identified according the cytomorphological analysis by vital fluorescence microscopy in BC- patient undergoing adjuvant treatment. Bar represents 10μm.



Patients' blood collections were classified as CTCs positive by combined cytomorhological 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.

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 1 bellow).

Table No. 1: 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

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 (significance level $p < 0,05$ if not set automatically by GenEx).

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.**
- Results as described in study I (Ušiaková Z., Mikulová V. et al., 2014) and in study II (Bielcikova Z., Jakabova A. et al., 2017 under review process) ⇒ The CTCs detection rate by using MetaCell® is higher than the one if AdnaTest® was used (see Table 2 bellow). This does not neccesarily means that the sensitivity of the MetaCell test is higher (the right complementary test of both methods was not done).

Table 2: Comparison of CTC positivity using MetaCell® and AdnaTest®

	MetaCell	**Adnatest**
	CTC positivity (%)	CTC positivity (%)
Before NACT	85 (17/20)	35
During NACT	88 (15/17)	
Before surgery	72 (13/18)	5
After surgery at any time of F-U period	100 (19/19)	26

NACT:neoadjuvant chemotherapy, F-U: follow-up

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 disease in BC patients.**

- Results as described in study I (Ušiaková Z., Mikulová V. et al., 2014) ⇒ 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+ PBC, (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 (see Table 3).

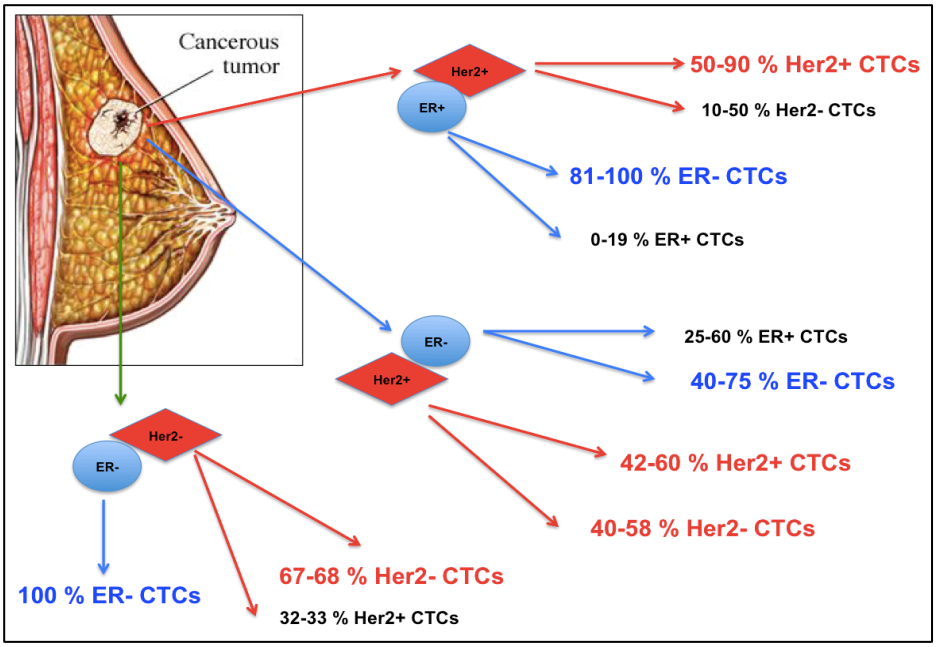
Table 3: Concordance of HER2 status in CTCs and primary tumor.

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)

HER2+...HER2 positive, HER2- ...HER2 negative

- Results as described in study II (Bielcikova Z., Jakabova A. et al., 2017 under review process)⇒ We monitored CTCs with a quite high rate of concordancy in TNBC a HER2+ BC patients during NACT. The worst concordancy was seen in ER+ PBC independently on HER2 status. The monitoring of CTCs status during NACT and after surgery shows that the majority of CTCs are ER- and HER2- in HER2- PBC, in HER2+ PBC, CTCs are predominantly ER- and HER2+ during NACT but they are loosing HER2+ status in time after surgery (see Figure 2).

Figure 2: CTCs retain aggressive properties predominantly (more often after the therapy than during therapy)



HER2+...HER2 positive, HER2- ...HER2 negative, ER+ ....ER positive, ER- ...ER negative

- Results as described in study III (Jakabova A., Bielcikova Z. et al., 2017 under review process) ⇒ The CTCs positivity in all groups was 76 %. There was no significant difference between tested groups if taking different therapy stages into account. 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 the primary disease response and CTCs response to NACT

- C. *Hypothesis: The systemic response to the treatment is independent from the local response in BC patients undergoing NACT.*
- **C. Objective: The comparison of the primary disease response and CTC – response in BC patients treated with NACT.**
- Results as described in study II (Bielcikova Z., Jakabova A. et al., 2017 under reveiw process) ⇒ We declare that the tumor volume reduction is not in connection to the CTCs positivity rate. The effect of the whole NACT was accompanied by a non-significant decrease in CTCs positivity

(see Table 4).

Table 4: Tumor volume reduction in comparison to CTC-positivity (CTC+) in blood

	Reduction of tumour volume							
	R=0	CTC+	R=1	CTC+	R=2	CTC+	R=3	CTC+
Before AC								
Before TAX	1/20 (5)	100 %	7/20 (35)	85 %	4 /20 (20)	75%-100%	8/20 (40)	87%-75%
Before surgery	3/17 (18)		9/17 (53)		1/17 (6)		4/17 (23)	

AC: antracyclins, TAX: taxanes, R0-R1: tumor volume reduction < 50%, R2: tumor volume reduction = 50%, R3: tumor volume reduction> 50%

4.4 Predictive value of CTCs

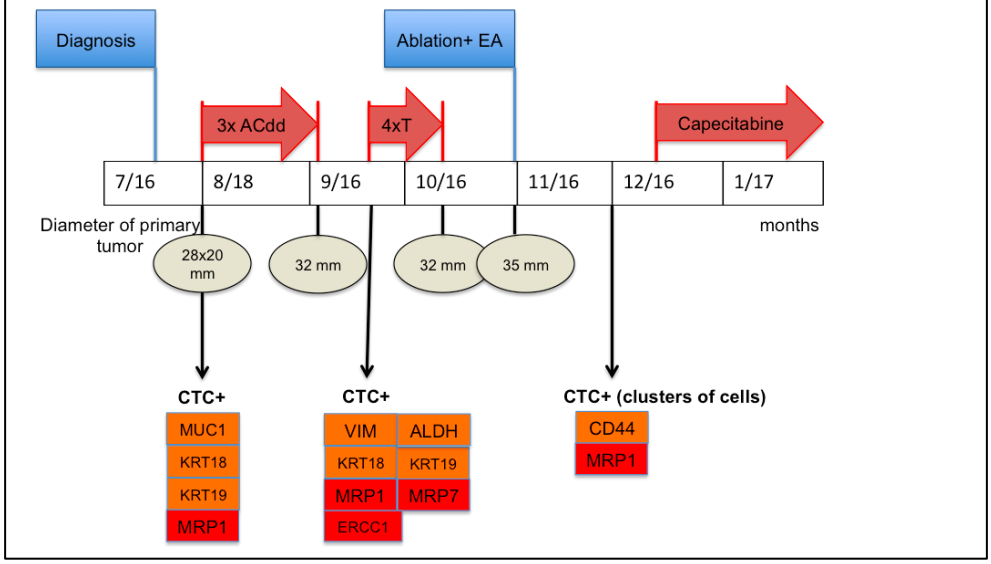
- *D. Hypothesis: CTCs have predictive value in BC disease management.*
- **D. Objective: The comparison of CTCs characteristics among responding and non-responding BC patients undergoing NACT.**
- Results as described in study II (Bielcikova Z., Jakabova A. et al., 2017 under reveiw process) ⇒ The bigger 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 antracyclins (AC) therapy and MRP1 and MRP7 genes during taxanes (TAX) therapy. In responders, CTCs were more frequently negative or had epithelial characteristics.

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 and the presentation of our original data and current experience.**
- Summary of relevant results as described in study IV (Bielcikova Z., Jakabova A., et al 2017) ⇒ The example of the potential clinical application of CTCs-testing during NACT in BC patient is documented bellow (Figure 3).

Figure 3: 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 (see table 1)

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.

By using AdnaTest technology **(original article Ušiaková Z., Mikulová V. et al., 2014)** CTCs were detected 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 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 **(original data presented in Ušiaková Z., Mikulová V. et al., 2014)** we reported results in the range of mentioned data: 35 % of CTCs- positive patients before and 5 % after NACT.

If using MetaCell CTCs positivity in 85 % of patients before starting NACT and 72 % after NACT is reported **(see original article Bielcikova Z., Jakabova A. et al., 2017 under review process).** 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. 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. On the other hand, we keep in mind that we miss data comparing both methods in one sample set.
As next, 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 **(original article Ušiaková Z., Mikulová V. et al., 2014)**. As the AdnaTest doesn'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 **(original data Bielcikova Z., Jakabova A et al., 2017)**. 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.

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.

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 **(original article Bielcikova Z., Jakabova A. et al., 2017).** 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.

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

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.

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.

Molecular analysis of a sufficient number of individual CTCs could lead to the finding of predictive markers, which are a prerequisite for the personalization of treatment in both palliative and neo/adjuvant indication. Comparison of CTCs genotype with the genotype of primary and secondary tumors opens the possibility of monitoring the evolution of the disease in a particular patient during the treatment period or during disease progression. Moreover, it allows the research of potential therapeutic targets in real time. We suggest (**original article Bielcikova Z., Jakabova A. et al., 2017)** possible indications for future clinical use of CTCs monitoring in patients with different diagnoses. In the form of case reports original data of our current experience with CTCs testing were presented.

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).**

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 predict the 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 two or more CA-genes were detected repeatedly. Patients showing chemoresistance have had elevated MRP1 during antracyclin therapy mostly and MRP1 and MRP7 in combination during taxanes 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 the 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.

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8. LIST OF PUBLICATIONS

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