Charles University in Prague
3rd Faculty of Medicine

Summary report of the doctoral dissertation

Circulating tumor cells in personalized cancer therapy

Cirkulující nádorové buňky v personalizované onkologické terapii

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Prague 2021

Doctoral Study Programmes in Biomedicine

Charles University in Prague and The Czech Academy of Sciences

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Summary report was sent on (date): ……...…….…….

The dissertation defense will take place on (date)**:** ……..……..……

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Content

1. Souhrn 4
2. Summary 5
3. Introduction 6
4. Thesis aims 8
5. Material and Methods 9
   5.1. CTC enrichment and cultivation of CTC 9
   5.2. Cytomorphological analysis 9
   5.3. Gene expression analysis 9
6. Results 11
   6.1. Breast cancer 11
   6.2. Ovarian cancer 15
   6.3. Other cancer diagnosis 17
7. Discussion 19
8. Conclusion 24
7. References 26
8. List of publications 30

1. Souhrn

Cirkulující nádorové buňky (CTC) prokázaly velký potenciál stát se prognostickým a prediktivním biomarkerem u různých typů onkologických onemocnění. CTC by mohly pomoci detekovat pacienty s vyšším rizikem kratšího celkového přežití, přežití bez progrese nebo relapsu. Mohou být také užitečné při výběru terapie, protože v současné klinické praxi je léčba volena na základě charakteristik primárního nádoru. Pravidelné sledování počtu a charakteru CTC by mohlo být indikátorem terapeutické odpovědi v reálném čase a bylo by možné jej použít k cílené léčbě. Tyto informace by bylo možné implementovat do personalizované medicíny a každého pacienta s rakovinou léčit na základě individuálního profilu.

Ačkoli vzorek pacientů s CTC je snadno dostupný, jejich detekce CTC zůstává výzvou zejména kvůli jejich nízkému počtu v krevním oběhu a jejich heterogenní povaze. CTC mohou cirkulovat v krvi ve formě samostatných buněk nebo ve shlucích, které se obvykle nevyskytují tak často jako jednotlivé CTC, ale jejich metastatický potenciál je významně větší než u samostatných CTC. Kromě počtu CTC, taky molekulární charakter vykazoval dynamický vývoj a heterogenní povahu nejen mezi pacienty, ale také v rámci nádorové tkáně pacienta. Charakter primárního nádoru, CTC a metastáz není vždy konzistentní a během procesu léčby se mění, což může významně ovlivnit reakci na léčbu.

V této práci jsme použili metodu izolace CTC založenou na jejich velikosti u různých nádorových diagnóz a sledovali jsme počet a molekulární charakter CTC v pravidelných intervalech v průběhu léčby. Analýza cytomorfologie a genové exprese odhalila dynamický vývoj onemocnění v průběhu celého procesu léčby. Pomocí fluorescenční mikroskopie jsme pozorovali změny počtu nejen jednotlivých CTC, ale také CTC shluků. Vlastnosti CTC se také měnily mezi jednotlivými odběry krve. Zatímco některé CTC dokázaly během kultivace přežít jen několik dní, u jiných izolovaných CTC byl pozorován agresivní růst a založili jsme z nich dlouhodobé kultury. Analýzy genové exprese genů spojených s nádorem, epiteliálně-mezenchymálním přechodem, vlastnostmi podobnými kmenovým buňkám a chemorezistencí u CTC odhalily jejich přítomnost a dynamické změny v expresi markerů. Zjistili jsme, že charakter CTC nekoreluje s charakteristikami odpovídajícího primárního nádoru. Výsledky výzkumu CTC jsou shrnuty v publikacích, které jsou součástí této disertační práce.

2. Summary

Circulating tumor cells (CTC) have showed great potential to become both prognostic and predictive biomarker in various types of oncological diseases. CTC can help detect patients in higher risk of shorter overall survival, progression-free survival or relapse. They can be also helpful in therapy selection as in current clinical practice treatment is chosen based on primary tumor characteristics. Regular CTC counts and features monitoring can be real-time indication of therapy response and can be used to guide-targeted treatment. This information can be implemented to personalized medicine and each cancer patient can be treated based on individual profile.

However patients‘sample with CTC is easily accessible, their detection has remained challenge due to low CTC number in the circulation and heterogeneous nature. CTC can circulate in blood in the form of single cells or in clusters that usually represent minority in comparison with single CTC but their metastatic potential is significantly greater than of single CTC. Apart from CTC count, the molecular character showed dynamic development and heterogeneous nature not only between patients but also within the individual patient’s tumor tissue itself. Character of primary tumor, CTC and metastasis is not always consistent and has been changing during treatment process which can significantly impact response to therapy.

In this work, we used size-based CTC enrichment in various cancer diagnoses and monitored both CTC count and molecular character in regular intervals during treatment process. Cytomorphological and genes-expression analyses revealed dynamic disease development through whole treatment process. Fluorescent microscopy found count changes not only single CTC but also in CTC clusters presence. CTC behaviour also varied during withdrawals. While some of the CTC were able to survive only days during cultivation, there were enriched CTC fractions with aggressive growth and long-term cultures were established from them. Gene-expression CTC analyses of genes associated with tumor, epithelial-mesenchymal transition, stem cells-like features and chemoresistence revealed their presence and dynamic change in expression levels. We found CTC character does not correlated to corresponding primary tumor features. The outcomes of the CTC research are summarized in publications that are part of this doctoral thesis.

3. Introduction

Cancer is known as one of the most frequent diseases that are responsible for morbidity and mortality worldwide. In general, cancer disease is characterized by uncontrolled growth of cells. Cancer cells proliferate in an uncontrolled manner and may invade the other parts of the body where they can form tumors known as metastases. Currently, metastases are the main cause of cancer deaths. (Dillekås et al., 2019; Hsu et al., 2019) Though the primary cause of why some tumors metastasize and others do not, remains undetected. There is a group of cells, which directly bridges the primary tumor and metastatic tumor sites in the body. These cells are named circulating tumor cells (CTC). CTC are an infinite source of new information on tumor development in time. We believe more straightforward characterization of CTC on the molecular level could change the way how we think about the cancer in general.

The universal approach "one drug fits all" has been found to become ineffective in the term of toxicity risks and treatment failures for various diseases. Instead, individual patient approach in disease management has become more preferred. (Matsuzaki et al., 2020; Groisberg et al., 2018) The point of view, how to plan patients‘ cure, has been developed to be more individual, known as personalized or precision medicine which has been referred as "the process of tailoring medical treatment to the individual characteristics of each patient; to classify individuals into subpopulations, that differ in their susceptibility to a particular disease of their response to a specific treatment, so that preventive or therapeutic interventions can then be concentrated on those, who will benefit, sparing expense and side effects for those who will not." along with "The molecular methods that make personalized medicine possible, include testing for variation in genes, gene expression, proteins, and metabolites as well as new treatments that target molecular mechanisms. Test results are correlated with clinical factors – such as disease stage, prediction of future disease status, drug response, and treatment prognosis – to help physicians individualize treatment for each patient." (Martínez-Jiménez et al., 2020; Maier, 2019)

Application of personalized medicine includes testing of newly developed molecules targets from whole molecular pathways through one mutated protein and aims to segment heterogeneous subset of patients whose response to therapeutic intervention within each subset is homogenous. (Crisci et al., 2019; Wang and Deisboeck, 2019) Molecular-genetic methods and innovative technologies are useful tools to apply these personalized principles in clinical oncology, nowadays.

Tumor classification based on molecular characterization helps guide treatment of patients. The philosophy of personalized medicine has found to stop searching for one drug to treat all patients with a specific indication. It is necessary to focus on differencies in patients with the same disease who need to be treated differently, and to identify the best treatment for these patients and appropriate method to monitor the disease in a long term perspective. (Martínez-Jiménez et al., 2020; Chen et al., 2019)

Recently, the most frequently used tests include genetic alterations testing (e.g. TP53 gene) (Fortuno et al., 2020; Azzollini et al., 2018), mismatch repair genes testing (e.g. MLH1, MSH2, MSH6) (Antonarakis et al., 2019, Xavier et al., 2019) overexpression and/or amplification of the genes (e.g. ESR, PGR, HER2) (Schaffar et al., 2019; Gupta et al., 2018) or mutations predicting drug effectivity (e.g. KRAS, BRAF) (Midthun et al., 2019). The most updated information regarding new genes modifications may be found in The Cancer Genome Atlas (TCGA) of the National Cancer Institute (The Cancer Genome Atlas Program, 2019)

Regular testing of these genes status could offer real-time monitoring of individual tumor development. The way how to reach this target could be connection of patients' liquid biopsy (LB) with molecular-genetic analysis. CTC and cell-free tumor deoxyribonucleic acid (ctDNA) are tumor markers obtained from blood of cancer patients. Both of them are bringing a new hope into the cancer field how to use "liquid" information in the process of therapy choice and therapy effect monitoring. Current research has signified combined analysis of CTC and ctDNA increases sensitivity and provides real-time information about individual patients' disease status. (Gorges et al., 2019; McNamara et al., 2019)

Thus, CTC represent rare cells which were previously a part of tumor tissue, were shedded from it, survived in circulation and are still able to form metastases. Since CTC are present in patients' blood, they are a suitable tool for regular patients monitoring. After their enrichment several scenarios are available regarding their processing. We are able to observe their consecutive behaviour (growth, division, differentiation or ability to be part of/ to create clusters) or compare them with known tumor features from biopsy. This information can significantly contribute to overall picture of the patients' disease in real-time. (Amantini et al., 2019; Heeke et al., 2019)

Although wide spectrum of tumors has been studied for CTC, this thesis is focused preliminary on breast cancer (BC) patients. Population of CTC in blood system is very low. Their enrichment from blood is a crucial step which makes their characterisation even more challenging. In addition, we hypothesized that viable CTC can offer more information about cancer patients' disease status in comparison with the fixed ones. We were able to enrich viable CTC, cultivate them *in vitro* and monitor their behaviour and stage by cytomorphologic methodologies. Additional CTC- features could be obtained by gene expression analysis of tumor- and chemoresistance-associated genes. These analyses are performed regularly during treatment process. Results from these tests are then individually evaluated, correlated with current primary tumor characteristics and compared to patients' clinical outcomes. Long-term CTC monitoring enables to study both prognostic and predictive potential of CTC.

Breast carcinoma belongs to the most extensively studied areas within the wide spectrum of cancer diseases. Large international meta-analyses have been focused on CTC role in this cancer type. Recently, prognostic relevance of CTC in both adjuvant treatment and metastatic settings in breast carcinoma diagnosis has been determined. Outcome of these intensive researches confirms CTC position in the field of biomarkers to be widely used in clinical practice. (Banys-Paluchowski et al., 2019)

4. Thesis aims

Doctoral thesis is focused on liquid biopsy – CTC examination and its translational research in oncological therapy personalization.

The aims of this thesis were CTC enrichment and molecular characterization and can be summarized into following points:

- To set- up size- based enriched CTC *in vitro* cultures of cancer patients CTC in various treatment process stages; before therapy, undergoing chemotherapy, radiotherapy and biological treatment (e.g. anti – HER2), which is the prerequisite of successful molecular characterization of CTC in different solid tumor cancer types

- To evaluate cytomorphology of CTC with focus on cytomorphological changes of CTC during ongoing therapy (e.g. chemotherapy, radiotherapy, anti- HER2 treatment) in different cancer types

- To analyse gene expression profile of tumor-associated, stem cell-like and chemoresistance-associated genes in CTC in early and metastatic BC patients undergoing NACT with respect of CTC population heterogeneity under the chemotherapy pressure

- To assess prognostic and predictive potential of CTC based on molecular data correlated to the observed clinicopathological criteria in eBC patients

- To compare characteristics of primary tumor and CTC in BC patients undergoing NACT with respect of evolution of CTC in the mean of and HER2 status

5. Material and Methods

Patients' blood withdrawal as a part of liquid biopsy examination was followed by blood processing within 24 hours. After CTC enrichment, cytomorphology and gene expression analysis were applied for detailed CTC characterization.

5.1. CTC enrichment and cultivation of CTC

Patients' blood withdrawal as a part of liquid biopsy examination was followed by blood processing within 24 hours. After CTC enrichment, cytomorphology and gene expression analysis were applied for detailed CTC characterization.

Enrichment of CTC was done by size-based separation protocol at room temperature. Until the blood was processed, it was kept at 4-8ºC (temperature in the fridge). Approximately 6-8ml (volume was dependent on each study blood) of peripheral blood was filtered using Metacell® device (Metacell s.r.o., Czech Republic). Cells captured on the filter were either cultured *in vitro* or stored for later gene expression studies.

After whole peripheral blood filtration, membrane with captured cells was washed by RPMI media (SIGMA-ALDRICH, USA) and placed into cultivation plate. 4 ml of RPMI media supplemented by fetal bovine serum (FBS) - 10% (SIGMA-ALDRICH, USA), Amphotericin B (SIGMA-ALDRICH, USA) and penicillin-streptomycin (SIGMA-ALDRICH, USA) antibiotics to avoid contamination were added on the membrane. The cells were cultured *in vitro* under standard conditions (37ºC, 5% CO_2) for 3-5 days. Long-term cultivation was performed in the cases of massively growing cancer cells.

In the case of enriched cells storage, the cells with the membrane were directly after the filtration put into the 600 µl of Buffer RLT (QIAGEN, Germany) + β-mercaptoethanol (VWR, USA) and stored at -20ºC.

5.2. Cytomorphological analysis

After 3-5 days of *in vitro* cultivation, cytomorphological analysis of cells by fluorescent microscope was performed. Nucleus and cytoplasm of viable cells were stained by vital fluorescent dyes NucBlue® Live ReadyProbes® Reagent (Thermo Fisher Scientific, USA) and Celltracker™ Green CMFDA Dye (Thermo Fisher Scientific, USA), respectively. Stained cells were captured (magnification x40) by C software, Olympus IX51 fluorescent microscope with built in camera (Olympus U-RFL-T power supply unit) and identified using standardized cytopathologic criteria for CTC, which include: nuclei larger than 10µm, proliferation, presence of tridimensional cell-sheets, high nuclear/cytoplasmic ratio, prominent nucleoli, irregular nuclear membrane, visible cytoplasm, cells size above 15 µm. After cytomorphological analysis, cells captured on the membrane (membrane fraction) and meanwhile growing under the membrane (bottom fraction) were both disrupted by 600 µl of Buffer RLT + β-mercaptoethanol and stored at -20 ºC, respectively.

5.3. Gene expression analysis

White blood cells enrichment from whole peripheral blood

In total, 800 µl of Buffer EL (Qiagen, Germany) and 200 µl of peripheral blood were mixed together and incubated for 10-15 min on ice. After centrifugation of this suspension at 400 x g

for 10 min at 4ºC supernatant was removed and discarded. Pellet of the cells was resuspended with 400 µl of Buffer EL by pipetting and this mixture was centrifuged at 400 x g for 10 min at 4ºC. Supernatant was completely removed and discarded. This procedure was completed by adding 600 µl of Buffer RLT supplemented by β-mercaptoethanol to the cells pellet. This suspension was mixed by pipetting and stored at -20ºC until RNA/DNA was isolated.

RNA isolation and reverse transcription reaction

All of the samples for RNA isolation were stored in Buffer RLT + β-mercaptoethanol solution. After defrosting (if there were stored in freezer, at -20ºC), RNeasy Mini Kit (Qiagen, Germany) was used for RNA isolation. Particular fractions of cells were analysed (1. white blood cells (WBC), 2. enriched cells (virgin CTC), 3. cultured cells on the membrane (membrane fraction CTC) and 4. cultured cells invading the membrane and setting up a colony under the membrane (bottom fractions).

Sample types differed in studies. Concentration of RNA was measured by NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, USA).

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

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

Analysis of gene expression

After the reverse transcription reaction, differences between particular fractions were detected by qPCR analysis of tumor- and/or stem-cells like- and/or resistance-associated genes. For gene expression monitoring in the samples TaqMan™ Gene Expression Assays (Thermo Fisher Scientific, USA) were used. Conditions in the cycler were set according to manufacturing instructions.

Results of genes expression were processed, evaluated and charted by GenEx software (MultiD Analyses AB). Several groups had been set in measured data which were based on patients, fraction type (WBC, virgin CTC, membrane fraction CTC and bottom CTC fraction), HR and HER2+ primary tumor status, results of cytomorphology and genes expression analysis and monitored genes. Beta-actin (ActB) gene was selected as reference gene due to its stability and enough amount presence in all of the tested samples. Normalization to this reference genes was performed which corresponds to ΔCq calculation. Relative quantities process was the next step of pre-processing. Quantity our samples were given to relation with WBC fraction of corresponding withdrawal. This step corresponds to ΔΔCq calculation. Relative RNA amounts are showed in $\log_2$ values. Data in particular groups were compared visually in bar graphs by Mann-Whitney test (2-tailed). Hierarchical clustering of samples and genes was combined displaying the measured intensities in a heat map. (GenEx User Guide; Bredemeier et al., 2017)

6. Results

The research accompanying this PhD thesis was aimed to CTC in various cancer diagnoses with main focus on breast cancer. Peripheral blood samples were withdrawn from cancer patients and CTC were enriched by size-based separation. Further processing of CTC included their microscopic and molecular analyses that confirm their presence and provide detailed characterisation.

6.1. Breast cancer

Following questions were asked regarding CTC studies in BC patients
Does CTC character differ from primary tumor features?
Does the cancer cells morphology and molecular profile develop in the course of disease?
Are the molecular markers of the disease changing during treatment process?
Is the evolution of BC primary tumor molecular heterogeneity detectable by CTC molecular characterization?
Do CTC reflect real-time disease status?
Could CTC contribute to assess disease prognosis?
Does expression of chemoresistance associated genes on CTC indicate worse therapy response during NACT?

Results of BC research are described below for each scientific paper
Publication I
CTC positivity was also evaluated in relation to particular tumor types. We had found the highest CTC positivity in the peripheral blood of patients with ESR+ and HER2+ tumors corresponding to 90% and 91%, respectively. The lowest, 76% was detected in patients with TNBC primary tumors. (Table 1)
Table 1: CTC + samples proportions regarding primary tumor types

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

plus (+) represents positive

Consistence of ESR+, HER2+ and TNBC expression was also studied. Our results showed relatively low concordance between primary and CTC, 1/13 and 1/27 in the ESR+ tumor type throughout the whole monitoring type. Higher accordance between primary tumor and CTC was observed in HER2+ and TNBC tumors not only during NACT but also within whole monitoring period. (Table 1 and Table 2)

Table 2: Status of primary tumor and CTC in relation to tumor types and neoadjuvant chemotherapy

Primary tumor	CTC			
	ESR+	ESR-	HER2+	HER2-
	NACT - any time	NACT - any time	NACT - any time	NACT - any time
ESR+ 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 %)
ESR- HER2-	0/18 - 0/28	18/18 (100 %) - 28/28 (100 %)	6/18 (33 %) - 9/28 (32 %)	12/18 (67 %) - 19/28 (68 %)
ESR+ HER2+	0/10 - 5/26 (19 %)	10/10 (100 %) - 21/26 (81 %)	9/10 (90 %) - 13/26 (50 %)	1/10 (10 %) - 13/26 (50 %)
ESR- 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 (+) represents positive; minus (-) represents negative

Monitoring of CTC was performed in a regular manner from diagnosis through NACT to post-surgery follow-up period. As part of this process, real-time CTC characteristics were examined by gene expression analysis. It had been showed CTC features were changing not only in the case of tumor-specific characteristics but also in chemoresistance-associated features. Our results indicated that several epithelial markers and chemoresistance-associated genes expression during NACT had seemed to be linked with positive clinical effect of treatment. Surprisingly, CTC negativity was detected only in the cases of relevant clinical response (response rate as set by ultrasound) (RR=2 or 3), after AC (patient no. 2, 8 and 14) or TAX (patient no. 2, 5, 7, 13 and 17) therapy. On the contrary, CTC of patients with poor clinical response (RR=0 or 1) had usually higher expression of two or more chemoresistance-associated genes detected and number of these genes was increased throughout treatment.

Except chemoresistance-associated markers, other characteristics of CTC had been monitored. Changes of ESR and HER2 expression status during NACT were dynamic. Several different scenarios occurred. Higher HER2 gene expression level was detected only before NACT (patient no. 16), during therapy (patient no.12), in several peripheral blood withdrawals (patient no. 3, 4, 5, 6, 8, 9, 10) or in none (patient no. 13, 14, 15, 17, 18, 19, 20) of them. On the other hand, ESR was detected in 4 CTC samples of 3 eBC patients (patient no. 7, 8, 12) in our research.

Changes in epithelial and stem-cells like features were detected. Most of the patients exhibited exclusively CTC with higher levels of epithelial markers before NACT (KRT19, EpCAM, mucin 1 (MUC1), etc.). Increased stem cells-like (CD44, CD24) characteristics expression was observed in CTC during or after NACT therapy. There were patients with elevated stem cells-like markers expression also before NACT.

After NACT, the surgery had taken place and status of both disease and CTC was monitored in several patients. CTC were observed in 100% at different time points. Regarding CTC characteristics, in patients with ESR+ tumor type CTC with epithelial features were detected

more frequently in comparison with stem cells-like. Although certain trends in disease behaviour were observed, development of both CTC and tumor tissue(s) was individual in each patient monitored.

Publication II

Overall result of CTC positivity examination in this study was 76% from the patients with BC. (Figure 1) Patients were the divided into groups based on their stage, histopatological characteristics, menopausal status, tumor size, nodal involvement, grade, hormonal receptors and HER2 status of primary tumor and therapy state.

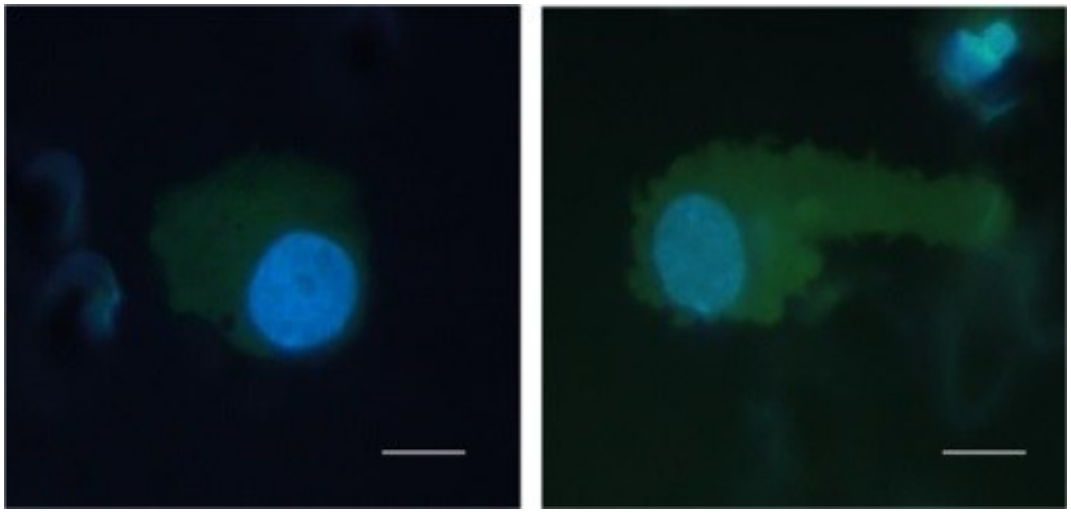

Figure 1: CTC enriched from peripheral blood of breast cancer patients after cultivation on the membrane after vital fluorescent staining with NucBlue™ and CellTracker™. Bar represents 10 µm (adapted from Jakabova and Bielcikova et al., 2017)

Regarding histopathological characteristics we had identified the lowest CTC positivity, 44.4%, in *ductal carcinoma in situ* (DCIS**)** patients group. The highest percentage, 82%, was showed within grade 3 (G3) patients' group. The menopausal and premenopausal group showed 55.5% and 78.4% of CTC - positive samples, respectively. Impact of therapy on CTC presence had been also studied. We found high CTC occurrence in the groups before therapy and surgery which were 82.3% and 86.6%, respectively. Group of patients who were undergoing hormonal therapy showed 52.9% CTC positivity. CTC had been examined also in the relationship with primary tumor and HR and HER2 status. The highest CTC positivity was detected in HR- HER2+ and the lowest in patients with HR+ HER2- primary tumors corresponding 100% and 70.3%, respectively. In patients'groups with primary tumors characteristics as ESR+ PGR- and ESR+ PGR+, CTC were identified in 60% (9/15) and 73% (68/93) of tested samples, respectively. In ESR+ tumors CTC occurred in 96.7% (30/31) of samples.

The second part of this study was a regular long-term monitoring of 20 BC patients during the course of the disease. CTC samples were analysed by both cytomorphological and molecular examination. Gene expression tests have been focused on ESR and HER2 status of CTC. This analysis had revealed difference between primary tumor and CTC in ESR and HER2 expression. Although 12 of these patients had been diagnosed with TNBC, HER2 status changed from HER2- to HER2+ in at least four cases (33.3%). Similarly, 50% (3/6) of

patients who had been initially HER2+ changed to HER2-. In the case of ESR status; it had been changed only from ESR+ to ESR- (3/3) in our patients' sample.

Publication III

The results of the research in reported paper is processed in the form of case study reports. Patients' no. 1, 2, 3 and 4have been diagnosed with BC and their monitoring outcomes are dscribed below.

Patient No. 1 (TNBC; 34 years old, woman) underwent 3 blood withdrawals presented in this study. There were 2 CTC examinations during neoadjuvant treatment and 1 CTC analysis after primary tumor surgery. At the time of diagnosis gene expression of KRT18, KRT19, MUC1 and MRP1 increased in CTC. After AC (doxorubicin + cyclophosphamide) treatment KRT18, KRT19, VIM, ALDH and MRP1, MRP7 and ERCC1 were elevated. Primary tumor grew from 28 mm to 32 mm (35 mm according to final histology) during neoadjuvant therapy. The last withdrawal was performed 1 month after surgery, increased level of CD44 and MRP1 was reported. None of the CTC examination had confirmed increased expression level of HER2 or HR.

Patient No. 2 (TNBC; 44 years old, woman) underwent long-term CTC monitoring. Before start of NACT following genes were increased in enriched CTC-fraction: KRT18, KRT19, MUC1 and MRP1. Surprisingly, elevated level of HER2 in CTC was also confirmed. Although after surgery there were still CTC detected, after radiotherapy treatment CTC were no longer present in peripheral blood of this patient. During adjuvant chemotherapy (capecitabine) treatment, patient was evaluated as CTC-positive again. This withdrawal had revealed CTC clustering and increased expression of mesenchymal markers, such as VIM and ALDH. Expression of HER2 was confirmed in CTC only before the therapy.

Patient No. 3 (TNBC; 29 years old, woman) was tested for CTC presence exclusively in the postoperative period (adjuvant setting (AS)). During AS the doxorubicin + cyclophosphamide – docetaxel (AC-D) treatment, CTC have expressed epithelial characteristics; in the follow-up period mesenchymal features were in prevalence. First withdrawal was provided at the start of AS (AC-D treatment) and EpCAM, KRT19, ESR and MRP1 gene expression was increased in the CTC fraction. During therapy, CTC expressed MUC1, KRT19, HER2 (not ESR anymore), MGB, MRP1 and MRP7, at the end of treatment epithelial characteristics and MRP1 with ERCC1 were increased. None of the HR or HER2 showed elevated expression. In the follow-up period, expression of mesenchymal genes started to be increased even though number of CTC had a decreasing tendency.

Patient No. 4 (BC - ESR+, PGR+, HER2+; 39 years old, woman) underwent 6 blood withdrawals for the purpose of CTC examination and all of them were evaluated as CTC positive. Although at the time of diagnosis two genes, HER2 and KRT19 were increased, after NACT (AC therapy) epithelial (EpCAM, KRT19, MUC1), mesenchymal (CD44), HER2 and chemoresistance-associated (MRP1, MRP5, ERCC1) genes were increased. After another therapy with docetaxel + herceptin (DH), genes CD24, CD44, KRT19, ESR and MRP1 expressed elevated expression levels. Although CTC features were changing, size of the primary tumor diminished from 31x25 mm to 12x15 mm. During another treatment, firstly with tamoxifen + herceptin and later with tamoxifen alone, the patient generalized into central nervous system (CNS) and CTC expressed KRT18, KRT19, CD24, CD44, MRP1, Adenosine

Triphosphate - Binding Cassette Subfamily C Member 2 (MRP2) and MRP5. Shortly after CNS metastasis diagnosis the patient died.

6.2. Ovarian cancer

Following questions were asked regarding CTC studies in CaOV patients
Is enrichment of CTC by MetaCell® effective enough for obtaining of viable CTC and representative heterogeneous CTC sample?
Could CTC examination significantly enhance in the CaOV staging process in patients with recurrent disease?
Is there a difference in molecular character of CTC developing under different environment conditions (e.g. chemotherapy, radiotherapy, *in vitro* culture)?

Results of CaOV research are described below for each scientific paper
In our patients' sample in **Publication IV**, 32 (58%) of them were assessed as CTC-positive and 24 (42%) were included in CTC-negative group based on cytomorphological examination. (Figure 2)

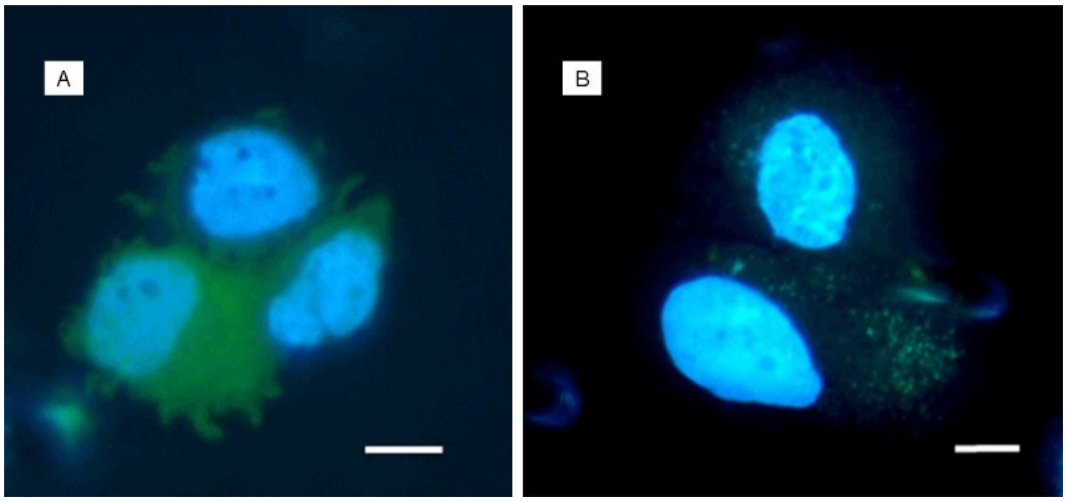

Figure 2: Cultivated CTC on the membrane from ovarian cancer patients after enrichment and short-term cultivation in publication IV. Cells were stained by NucBlue™ and CellTracker™ and captured by fluorescent microscope. The bar represents 10µm. (adapted from Kolostova et al., 2016)

Our results in **Publication V** showed that 77 out of 118 (65.2%) CaOV patients, enrolled into our study, were evaluated as CTC-positive based on cytomorphological evaluation. Although no significant correlation between CTC and lymph nodes involvement was found in this study, in the case of more severe disease status (higher grade, worse FIGO stage) CTC were detected in more than 2/3 of the cases.
Out interest in **Publication V** had been also focused on correlation of CTC presence with other patients'clinicopathological characteristics. Patients with ascites, peritoneal carcinomatosis and residual disease had been evaluated as CTC positive in 73.6% (39 out of 53), 70.5% (43 out of 61) and 75.0% (42 out of 56) in our study.

Another important CaOV marker CA125 had been studied in the context of CTC presence in **Publication V**. We concluded elevated level of CA125 could be a reliable marker of peritoneal spread and CTC presence could be a significant marker of haematogenous CaOV spread.

After cytomorphological analysis, genes expression analysis was performed in samples of 40 CaOV patients in **Publication IV**. Four fractions for every patient have been tested: peripheral blood leukocytes (fraction 1 sample type 1), CTC fraction stored immediately after enrichment procedure (fraction 2 sample type 2), cultured CTC fraction grown on the membrane (fraction 3 sample type 3) and CTC fraction which pass through the membrane and grew at the bottom of cultivation plate (fraction 4 sample type 4). Relative gene expression for monitored markers regarding different sample types is shown on the figures below. (Figure 3 and Figure 4)

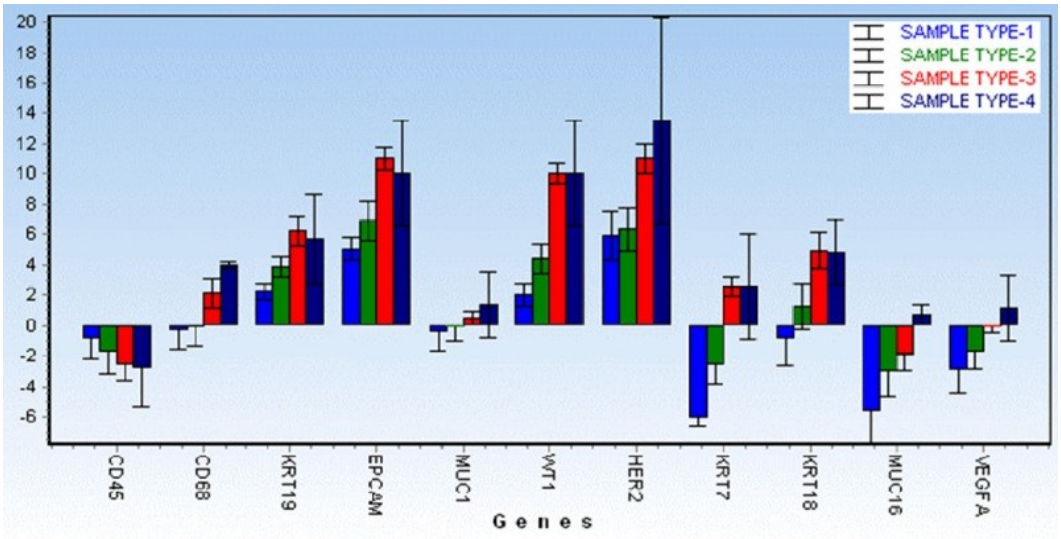

Figure 3: Relative gene expression of monitored markers regarding each sample types in publication IV. Sample type-1, whole peripheral blood; sample type-2, CTC fraction stored immediately after enrichment procedure; sample type-3, cultured CTC fraction grown on the membrane; sample type-4, CTC fraction which pass through the membrane and grew at the bottom of cultivation plate. (adapted from Kolostova et al., 2016)

The results of gene expression analyses showed noticeable increase of tumor-associated genes in both CTC - enriched and also CTC - cultivated fractions. Single CTC enrichment by size-based MetaCell® device had resulted in higher level of cytokeratin-7 (KRT7), KRT18, KRT19, EpCAM, wilms tumor 1 (WT1) and mucin 16 (MUC16) in CTC fraction if compared to peripheral blood samples (sample type 1). (Figure 3) Results had become more powerful in the case of enriched CTC fraction after short-term *in vitro* cultivation. Gene expression analyses revealed elevated level of KRT7, KRT18, KRT19, EpCAM, MUC1 and MUC16 in fraction 3 in comparison with peripheral blood with statistically significant differences ($p < 0.02$). (Figure 3 and Figure 4) These results had strongly indicated not only presence of cancer cells in our samples but also revealed their detailed characteristis.

In the next step, gene expression analysis of chemoresistance-associated genes was performed. The analysis included following genes: MRP1, MRP2, MRP4, MRP5, MRP7, ATP binding cassette subfamily B member 1 (MDR1) and ERCC1. According to the findings, all of these genes were expressed in excess if compared to peripheral blood. (Figure 4)

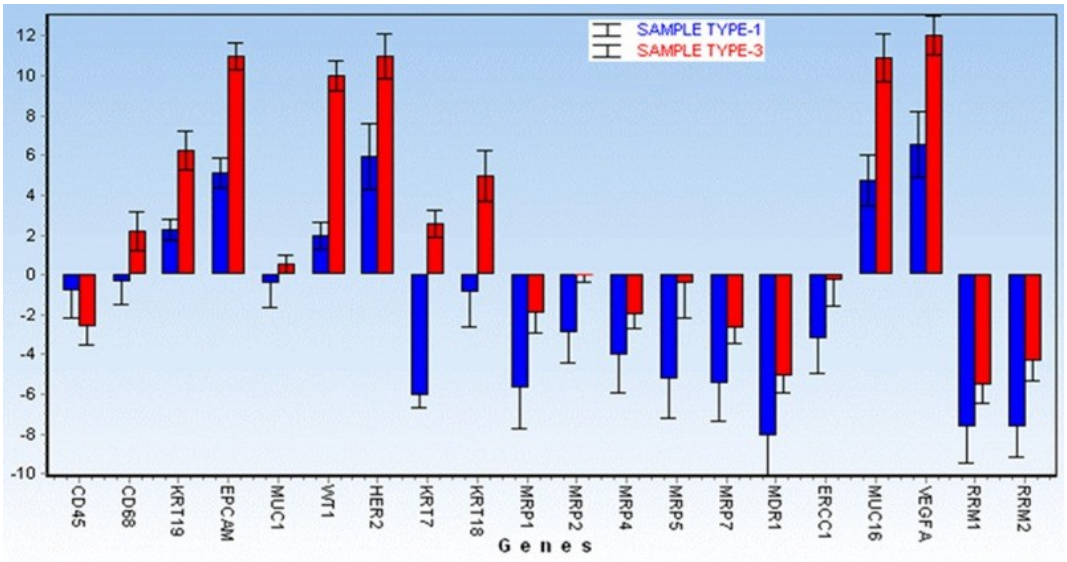

Figure 4: Relative gene expression of all of the monitored markers considering two sample types in publication - whole peripheral blood and cultured CTC fraction grown on the membrane. Sample type-1, whole peripheral blood; sample type-3, cultured CTC fraction grown on the membrane. (adapted from Kolostova et al., 2016)

6.3. Other cancer diagnosis

Following questions were asked regarding CTC study in various cancer diagnoses
The study should answer the question, how to use CTC in clinical practice based on the data with obtained in time following the dynamics of the cancer disease.
Do CTC reflect whole cancer disease heterogeneity?
Are CTC ready to be used in clinical practice at all?
Could be a personalized therapy implemented at all?

Results of research are described below
Publication III
Patient No. 5 (CRC; 35 years old, man) underwent CTC examination after primary surgery and two relapses. This withdrawal was evaluated as CTC positive where KRT18, KRT19, MUC1 and MRP4 genes showed increased expression. After FOLFIRI (leucovorin + fluorouracil + irinotecan) chemotherapy regimen and after the third relapse, another CTC analysis revealed higher expression of KRT18, KRT19, MUC1, VIM, Ribonucleotide Reductase Catalytic Subunit M1 (RRM1) and MRP1 genes. RAS status was examined with negative results for liver metastasis and CTC.
Patient No. 6 (Rectal adenocarcinoma, 50 years old, man) started with CTC examination after surgery and lungs generalization. All of the CTC analyses were evaluated as CTC positive. The cells expressed epithelial characteristics, chemoresistance profile showed MRP1 and ERCC1 in the first blood withdrawal and two additional genes (RRM1, Ribonucleotide Reductase Regulatory Subunit M2 (RRM2)) were elevated in the second withdrawal. The third CTC analysis was provided by means of cytomorphology only, the last CTC test revealed increased expression of KRT18, thyroid transcription factor 1 (TTF1), MRP1 and

MRP4 genes in CTC fraction. The disease progressed and new lesions in bones were detected. This patient died because of new lesions in CNS.

Patient No. 7 (Lung cancer, 47 years old, man) who's first CTC analysis was provided during carboplatin and pemetrexed therapy resulted in detection of elevated gene expression for: KRT18, KRT19, EpCAM, MUC1, MRP1 and ERCC1. Later on, CTC examination revealed not only mesenchymal characteristics, VIM expression, but also more chemoresistance-associated markers, such as MRP1, MRP2, MRP4, MRP7 and ERCC1.

Patient No. 8 (Duplicate tumor CRC and PC; 57 years old, man) monitoring CTC analysis revealed there are cells expression epithelial (KRT18, KRT19), stem cells-like (VIM, ALDH), prostate (AMACR) and vascular endothelial growth factor (VEGF) markers in the patient peripheral blood.

7. Discussion

Cancer is a systemic disease and its initial prognosis and predictions are not always consistent with the current disease development. Patients can experience increase in tumor volume or metastasis forming despite complete surgical removal of the primary tumor, receiving targeted medicinal products or general improvements in medical care.

For sure, a regular monitoring of the disease status is a necessary part of the treatment schedule and tumor biopsies and imaging methods are considerably involved in. These procedures have often an invasive character, especially in cases where tumor is not easily accessible due to its location. (Schaffner et al., 2020; Liikanen et al., 2018; Dasgupta et al., 2017)

Cancer cells released from primary or metastatic tumor mass reaching patients'blood seem to be a useful marker for real-time tumor disease monitoring. However, their occurrence is very rare and their fate in circulation rather unclear. Although it has been suggested that CTC half-life in bloodstream is about 1-2 hours, these cells can be detected 8-22 years after mastectomy in BC patients. (Cote and Datar, 2016)

In fact, CTC examination relies on peripheral blood withdrawal which is a minimally invasive procedure. Peripheral blood, seen as a source of CTC for LB analysis, is the only prerequisite of the regular CTC-monitoring during whole treatment process. (Yamada et al., 2019; Zapatero et al., 2017) There are usually several treatment options considered for a patient before the final decision of the therapeutic schedule is agreed. Biomarkers are significant help within this decision processes. (Coates et al., 2015; Burstein et al., 2014) A biomarker has been defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or biological responses to a therapeutic intervention. (Kessler et al., 2015)

Enrichment of viable CTC is believed to play a significant role in experimental functional assays. CTC present a great challenge due to their natural characteristics such as incidence, heterogeneity or adaptability. Occurrence of CTC in patients' blood has been estimated to be 1 - 10 CTC in 10^6-10^8 WBC or 1-100 CTC may be found in 1 ml of whole blood. (Ecke and Thomas, 2019; Garg et al., 2018) Also, CTC are highly heterogeneous population considering both biological and physical properties. Enrichment strategies relying on a single CTC-feature has not obtained reproducibility and clinical relevance so far. A significant ratio of CTC is able to survive in unfavourable environment in circulation by several mechanisms such as phenotype alteration which avoids them from immune system attack. (Luo et al, 2018; Yang et al., 2017)

Numerous methods have been developed for CTC enrichment and detection in the last decade. A size-based enrichment protocol (Metacell®) has been implemented in clinical studies of various cancer types. This technology allows obtaining of viable and unmodified CTC without implementation of pre-processing procedures or any physical force during enrichment. The artificial overpressure or vacuum which are used in the most of the size-based enrichment methods to regulate the filtration flow, can result in cells damage, too. (Bhagwat et al., 2018; Kang et al., 2017) The size-based captured cells are heterogeneous, vivacious and contain both single cells and clusters that enable further downstream cellular

and molecular analysis including set up of both short- and long-term *in vitro* cultures as has been discussed in presented **publications I-V**.

Research of CaOV CTC (**Publication V**) evaluated 118 patients with CaOV who were candidates for surgery or surgical diagnostics. 77 out of 118 (65.2%) patients were evaluated as CTC positive and short-term *in vitro* CTC cultures were established. Gene expression analysis confirmed heterogeneous expression profile of captured cells. The results confirmed that CA125 level and CTC may behave as two independent biomarkers for lymphogenic and haematogenous dissemination in CaOV patients, respectively.

In the study of Rao et al., CTC positivity was detected in 87% of patients with CaOV if CTC were captured by microfluidic system with immunomagnetic beads. (Rao et al., 2017) Other results have been reported by Obermayr et al. who observed CTC positivity at the time of diagnosis and 6 months after completion of adjuvant therapy in 26.5% and 7.7% of CaOV patients, respectively. CTC enrichment was provided by density gradient centrifugation and followed by multi-marker immunostaining. (Obermayr et al., 2017) Gebauer et al. reported CTC positivity in 32.3% of CaOV patients using CellSearch system and confirmed presence of CTC was not correlated with FIGO stage, nodal status or grading factors in CaOV patients. (Gebauer et al., 2017)

In the molecular study of CTC in patients with CaOV (**Publication IV**) cytomorphological and molecular analysis of enriched cells was performed. 56 patients with CaOV were enrolled into this study and 32 out of 56 (58%) were evaluated as CTC positive. Gene-expression analysis confirmed heterogeneity of the population of CTC enriched and cultured by size-based Metacell® protocol examinating tumor-associated genes expression. We found increased expression of EpCAM, KRT7, KRT18, MUC16 and WT1 genes in CTC-enriched fraction and elevation in EpCAM, KRT7, KRT18, KRT19, MUC1, MUC16 and WT1 genes expression after 3 days of CTC - *in vitro* culture. This study has also supported the idea of multi-marker instead of single-marker analysis. In the recent paper of Balakrishnan et al. enriched CTC were used to set up a short-term cluster culture. This study had confirmed that captured cells were cytokeratines positive and CD45-negative after 14 days of *in vitro* culture. (Balakrishnan et al., 2019) Outcomes of these studies support the hypothesis of immune cells depletion during *in vitro* culture.

Similar analysis was also performed in the study of 167 patients with various BC stages (**Publication II**). CTC examination revealed CTC positivity in 127 patients. Further cytomorphological and tumor-associated gene expression analysis revealed the highest CTC occurrence was identified in the group undergoing surgery and before the start of neoadjuvant treatment. In this study HER2 and ESR receptors, routinely examined in primary tumor, were evaluated in CTC. These markers are being used to stratify BC patients with early disease and are used as predictive markers for targeted therapies, too. (Schaffner et al., 2020) It has been widely accepted that expression status of these key receptors differs between primary tumor and metastasis. (Aaltonen et al., 2017) **Our testing also found that status of these markers can differ between primary tumor and CTC.** Research in this area is still not delivering consistent results. Although some of the studies demonstrated significant discordances in receptors status on CTC and in primary tumor tissue, other studies reported similarities between them. (Braun et al., 2019; Wang et al., 2017; Beije et al., 2016One of the possible explanations could be coexistence of cells that display different phenotypic characteristics.

(Castro-Giner and Aceto, 2020; Jordan et al., 2016) In any case, this discordance can significantly affect treatment response of BC patients.

Long-term CTC monitoring was performed in the study presented as collection of case reports (**Publication III**) where patients with BC, CRC, prostate and lung cancer were involved. Individual cases were analysed for CTC and clinical perspective of the CTC test results. In this publication, several aspects of CTC monitoring are described. Cytomorhological evaluation was concentrated on showing CTC numbers in responders and non-responders. The CTC numbers differed in the patients with the same disease type undergoing the same treatment. Size-based label-free enrichment was used and heterogeneous CTC population was obtained. In total, 24 out of 34 (70.6%) samples were evaluated as CTC-positive. Molecular analysis revealed that EpCAM, KRT18/19 and MUC1 gene expression was increased in 2, 23 and 7 out of 34 samples, respectively. This study supports the idea of the long-term cancer patients monitoring. Continuous CTC-monitoring after primary treatment is a tool how to follow the CTC-persistence in the blood. Persistence of CTC after primary treatment significantly increases relapse risk. Recent study of Trapp et al. published that CTC presence 2 years after chemotherapy significantly increased a risk of relapse in eBC patients. (Trapp et al., 2019) Similar outcomes have been reported by Sparano et al. who studied association of CTC with late recurrence of ESR+ BC patients. They reported that CTC positivity 5 years after diagnosis had provided independent prognostic information for clinical recurrence. (Sparano et al., 2018)

The last presented study of eBC patients (**Publication I**) included a long-term monitoring for both cytopathological and molecular CTC character of 20 patients diagnosed with eBC. These patients underwent CTC examination in the time of diagnosis, during NACT (after AC therapy, after TAX treatment), after surgery and in the next follow-up period. We monitored CTC during the course of treatment and found that CTC-positivity is not associated with tumor volume. The study confirmed that receptors status is not always the same in primary tumor and CTC. According to our results, during NACT, the highest concordancy rate in receptor expression was observed in patients with TNBC and HER2+ primary tumors. On the other hand, CTC of patients with ESR+ primary tumors showed the lowest concordancy rate regardless of their HER2 status. Long-term monitoring revealed each of the patients monitored experienced an individual disease development. Our publications emphasize importance of personalized treatment.

Although chemotherapy regimens are often an essential part of the cancer treatment, its success rate can be low due to intrinsic or acquired chemoresistance mechanisms. (Briz et al., 2019) Monitoring of genes associated with chemoresistance has become an important part of our CTC studies. We monitored gene-expression of chemoresistance-associated genes in different cancer case studies and in the study of early BC patients (**Publications II and III**). In the **Publication III** a prolonged CTC-monitoring revealed an expression of different chemoresistance-associated genes for various diagnoses. The development of chemoresistance has been confirmed also in eBC patients (**Publication I).** Despite importance of individual features of the disease progress, some of the findings were significant and common for several patients. In non-responders group, two or more chemoresistance-associated genes often showed increased expression and these CTC characteristics changed dynamically during treatment process. Worse therapy outcome was observed in patients with CTC that expressed

chemoresistance markers. Frequent overexpression of MRP1, MRP2 or MRP7 was observed in our patients although another 9 patients with TAX chemoresistance showed elevated expression of MRP2, MRP7 or MDR1 before or during TAX therapy. Based on our observations, resistance patients expressed elevated levels of MRP1 during AC and MRP1 and MRP7 during TAX treatment. ERCC1 overexpression was detected in 4, 3 and 2 patients before, during and after NACT, respectively and MRP1 was co-expressed in 8 of the 9 cases. We have also observed epithelial signs of CTC and minimal chemoresistance-associated genes expression during NACT correlated with positive clinical effect of AC or TAX.
Recent research of Shlyakhtunou et al. confirmed CTC heterogeneity in primary non-mBC patients. This study has also revealed elevated expression of MRP7, MRP1, MDR1 and MRP5 in 37/69 (54%), 37/69 (54%), 24/69 (35%) and 14/69 (21%) of BC patients included in this study, respectively. (Shlyakhtunou, 2018) Gradilone et al. classified patients into "drug sensitive" and "drug resistant" groups based on MRPs expression and found that the resistant group has significantly shorter PFS than the sensitive patients. Detailed analysis also confirmed that patients whose CTC expressed two or more MPRs were having shorter PFS than those who expressed zero or one of the MRPs. Furthermore, this research had clearly connected MRP1 and MRP7 with resistance to AC and TAX, respectively, which is in concordance with our results. (Gradilone et al., 2010) In another study Kasimir-Bauer et al analysed ERCC1 in CTC. 72% of residual CTC obtained from peripheral blood of BC patients after NACT expressed ERCC1 which indicated them as therapy resistant population. Awareness of this information may help clinicians with decision of further therapy. (Kasimir-Bauer et al., 2016) Approaches which would be able to pair treatment targeting the predominant, drug-sensitive population in addition to various subsets of drug-resistant and drug-tolerant cells may lead to the most-durable responses achievement. (Dagogo-Jack and Shaw, 2017)

EMT and stem cell-like characteristics of cancer cells have been also considered as major importance in metastatic progression. (Schaffner et al., 2020) Previous studies confirmed presence of tumor cells which survive in the blood, pass through EMT and can exist in intermediate stages expressing both epithelial and mesenchymal markers. This cell phenotype has been found to contribute to resistance of anti-cancer therapies including AC and TAX. Part of this heterogeneous CTC population may manifest stem cells character. The stem cell character promotes therapy resistance, survival facilitation or metastases. (McInnes et al., 2015; Yu et al.,2013) Although CTC with elevated expression level of EMT or intermediate phenotype occured more frequently in patients with metastatic in comparison with early-stage disease, their presence is not rare so their significance should not be underestimated. (Lowes and Allan, 2018; Pal et al., 2015)
Markers that characterize these features were included into our testing. We have identified these types of cells by CD24, CD44 in study of eBC patients (**Publication I)** and together with ALDH1 and VIM genes-expression analysis in study of case reports (**Publication III).** We observed differences in stem cells markers expression in ESR+ and other BC subtypes in study of eBC patients (**Publication I)**. Although in the cases of ESR+ primary tumor CTC with CD24/CD44 characteristics were detected more often after surgery in comparison with epithelial features (patient no. 1, 2, 7, 14 or 15), patients with more aggressive tumor types

(HER2+ primary tumor diagnosis) showed CTC with these markers overexpressed from the beginning of disease (patient no 9, or 12). However in study of eBC patients (**Publication I)** most of the TNBC patients overexpressed CD24 and/or CD44 markers in their CTC from the time of diagnosis (patient no. 10, 11, 13, 16, 17, 19, 20), study of case reports (**Publication III)** showed 3 TNBC case reports each with different EMT and stem cell-like features during CTC development. In the patient no.1 we observed changes in CTC regarding EMT and stem cells-like characteristics. None of these markers was overexpressed in the CTC from the first blood withdrawal, VIM, ALDH1 and CD44 gene expression was elevated in the second, third and fourth blood collection, respectively. The expression level of CD24 and CD44 elevated from the diagnosis time point and during the most of her blood withdrawals in the CTC of patient no. 2. As next, VIM and ALDH1 markers were overexpressed later during the monitoring, after 15 months. Recent study of Akkiprik et al. reported CTC with stem-cells properties were found in most HR+, HER2- cases and their high incidence was also observed in the cases of early metastasis. (Akkiprik et al., 2020)

Our studies declare patients individual CTC development in time considering the different markers monitored. Most of the BC patients included in our studies overexpressed EMT and stem cells-like markers in the corresponding CTC. Presented studies have also contributed to the hypothesis that circulating cancer cells may become a helpful tool for individual patients‘disease characterization and provide benefit for early detection and therapy personalization as transition status between epithelial and mesenchymal cells subpopulation changed dynamically. (Mansoori et al., 2017; Grosse-Wilde et al., 2015)

Despite all efforts, metastasis may take place after a long period after surgical tumor removal without any symptoms appearance. Metastasis has been major cause of cancer-related death. Understanding and proper monitoring of this process may bring disease treatment process improvement. (Chitty et al., 2018; Gomis and Gawrzak, 2017)

CTC showed to reflect cancer disease more accurately than biopsies, they are highly heterogeneous and more affortable in comparison with classical biopsies. Additional studies are needed to explore their use and potential in cancer disease monitoring, treatment and patients‘life quality improvement. (Brown et al., 2019; Ye et al., 2019; Jie et al., 2017; Aaltonen et al., 2017; Gkountela et al., 2016)

8. Conclusion

The presented PhD thesis was focused on CTC, their isolation, *in vitro* culture, cytomorphological description and molecular characterization. Based on our results, following could be concluded:

1. Size-based enrichment (MetaCell®) of CTC in patients diagnosed with solid tumor diagnoses was performed and successfully implemented into the diagnostic processes, reflecting purposes of special timing for CTC-tests in different cancer treatment periods (neoadjuvancy, adjuvancy, palliative). Patients with BC, CaOV, lung, CRC and prostate cancer were included into the testing. The enriched CTC (bigger than 8μm) were captured in a viable stage and were in the good fitness cultured for both, short and long time period *in vitro.*
2. Enriched and cultivated cells were observed using vital fluorescent microscopy. Following cytomorphological criteria were applied and evaluated to identify tumor cells in CTC samples: nuclei larger than 24μm, cell size at least 1.5 times larger than white blood cells, irregular nuclei and nuclear membrane, anisonucleosis, high nuclear/cytoplasmic ratio, presence of 3-dimensional cell sheets, presence of prominent nucleolus or several nucleoli. Significant differencies in CTC morphology of BC patients during treatment included also prevalent changes in CTC number and increased frequency of CTC clusters formation.
3. Gene expression profiling of both tumor- and chemoresistance-associated genes was provided in CTC collected during NACT, surgery and in follow-up period in BC patients. CTC expression profile has been analyzed reflecting disease and treatment dynamics. In patients who did not respond to NACT, two or more chemoresistance-associated genes showed elevated expression. The molecular characteristics of CTC were changing dynamically during treatment process.
4. It was confirmed that molecular characteristics of tumor and CTC in BC patients may not be always concordant either in the time of diagnosis or during treatment period. This has been confirmed in every of the presented publications (I-V). In the **Publication III**, CTC of TNBC patients have been HR and/ or HER2 negative in 78.5% of tested cases. In the **Publication V**, the change from HER2 negative to HER2 positive status and *vice versa* was shown for 33.3% and 50% of BC patients, respectively. Status of HR (ESR) changed from ESR+ to ESR- in all of the patients

monitored (3/3). Based on the results from **Publication IV** a high concordance rate of CTC and primary tumor was confirmed for TNBC and HER2+ BC patients. The highest discordance was revealed in tumors with ESR+ status independent of HER2 status.

We can conclude that majority of CTC showed ESR- and HER2-negativity in HER2-negative primary tumors. On the other hand, in HER2-positive primary tumors, discordance of HER2 was detected in 50% of tested CTC samples.

5. Gene expression analysis of CTC identified several CTC subpopulations in BC patients' samples. We were able to identify CTC with epithelial, mesenchymal, stem cell-like and chemoresistance features during long-term monitoring of CTC (up to 36 months in total).
6. Long-term monitoring in combination with real-time clinical assessment (ultrasonography) had allowed us to conclude that CTC of NACT responders overexpressed epithelial genes. In the case of the NACT non-responders, increased expression of chemoresistance-associated genes in CTC of these patients was detected.

7. References

AALTONEN, Kristina E., Vendula NOVOSADOVÁ, Pär-Ola BENDAHL, Cecilia GRAFFMAN, Anna-Maria LARSSON and Lisa RYDÉN. Molecular characterization of circulating tumor cells from patients with metastatic breast cancer reflects evolutionary changes in gene expression under the pressure of systemic therapy. *Oncotarget*. 2017, **8**(28), 45544–45565. DOI: 10.18632/oncotarget.17271. ISSN 1949-2553.

AKKIPRIK, Mustafa, Sinan KOCA, M. Ümit UĞURLU, et al. Response Assessment With Molecular Characterization of Circulating Tumor Cells and Plasma MicroRNA Profiling in Patients With Locally Advanced Breast Cancer During Neoadjuvant Chemotherapy. *Clinical Breast Cancer*. 2020, **20**(4), 332-343.e3. DOI:10.1016/j.clbc.2020.02.006. ISSN 15268209.

AMANTINI, Consuelo, Maria Beatrice MORELLI, Massimo NABISSI, et al. Expression Profiling of Circulating Tumor Cells in Pancreatic Ductal Adenocarcinoma Patients: Biomarkers Predicting Overall Survival. *Frontiers in Oncology*. 2019, **9**(874), abstract. DOI: 10.3389/fonc.2019.00874. ISSN 2234-943X.

ANTONARAKIS, Emmanuel S., Farah SHAUKAT, Pedro ISAACSSON VELHO, Harsimar KAUR, Eugene SHENDEROV, Drew M. PARDOLL and Tamara L. LOTAN. Clinical Features and Therapeutic Outcomes in Men with Advanced Prostate Cancer and DNA Mismatch Repair Gene Mutations. *European Urology*. 2019, **75**(3), abstract. DOI: 10.1016/j.eururo.2018.10.009. ISSN 03022838.

AZZOLLINI, Jacopo, Milena MARIANI, Bernard PEISSEL and Siranoush MANOUKIAN. Increased access to TP53 analysis through breast cancer multi-gene panels: clinical considerations. *Familial Cancer*. 2018, **17**(3), 317-319. DOI: 10.1007/s10689-017-0020-z. ISSN 1389-9600.

BALAKRISHNAN, Ajay, Deepak KOPPAKA, Abhishek ANAND, et al. Circulating Tumor Cell cluster phenotype allows monitoring response to treatment and predicts survival. *Scientific Reports*. 2019, **9**(1), 1-8. DOI: 10.1038/s41598-019-44404-y. ISSN 2045-2322.

BANYS-PALUCHOWSKI, Malgorzata, Andreas HARTKOPF, Franziska MEIER-STIEGEN, Wolfgang JANNI, Erich-Franz SOLOMAYER and Tanja FEHM. Circulating and Disseminated Tumour Cells in Breast Carcinoma. *Geburtshilfe und Frauenheilkunde*. 2019, **79**(02), 177-183. DOI: 10.1055/a-0753-7331. ISSN 0016-5751.

BEIJE, Nick, Wendy ONSTENK, Jaco KRAAN, et al. Prognostic Impact of HER2 and ER Status of Circulating Tumor Cells in Metastatic Breast Cancer Patients with a HER2-Negative Primary Tumor. *Neoplasia*. 2016, **18**(11), 647-653. DOI: 10.1016/j.neo.2016.08.007. ISSN 14765586.

BHAGWAT, Neha, Keely DULMAGE, Charles H. PLETCHER, et al. An integrated flow cytometry-based platform for isolation and molecular characterization of circulating tumor single cells and clusters. *Scientific Reports*. 2018, **8**(1), 1-14. DOI: 10.1038/s41598-018-23217-5. ISSN 2045-2322.

BROWN, Hannah K., Marta TELLEZ-GABRIEL, Pierre-François CARTRON, François M. VALLETTE, Marie-Francoise HEYMANN and Dominique HEYMANN. Characterization of circulating tumor cells as a reflection of the tumor heterogeneity: myth or reality?. *Drug Discovery Today*. 2019, **24**(3), abstract. DOI: 10.1016/j.drudis.2018.11.017. ISSN 13596446.

BURSTEIN, Harold J., Sarah TEMIN, Holly ANDERSON, et al. Adjuvant Endocrine Therapy for Women With Hormone Receptor–Positive Breast Cancer: American Society of Clinical Oncology Clinical Practice Guideline Focused Update. *Journal of Clinical Oncology*. 2014, **32**(21), 2255-2269. DOI: 10.1200/JCO.2013.54.2258. ISSN 0732-183X.

CASTRO-GINER, Francesc and Nicola ACETO. Tracking cancer progression: from circulating tumor cells to metastasis. *Genome Medicine*. 2020, **12**(1), 1-12. ISSN 1756-994X. DOI: 10.1186/s13073-020-00728-3

COATES, A. S., E. P. WINER, A. GOLDHIRSCH, R. D. GELBER, M. GNANT, M. PICCART-GEBHART, B. THÜRLIMANN and H.-J. SENN. Tailoring therapies—improving the management of early breast cancer: St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2015. *Annals of Oncology*. 2015, **26**(8), 1533-1546. DOI: 10.1093/annonc/mdv221. ISSN 0923-7534.

COTE, Richard J. and Ram DATAR. *Circulating tumor cells: advances in basic science and clinical applications*. 1. New York: Springer, [2016]. Current cancer research. ISBN 978-1-4939-3361-7.

CRISCI, Stefania, Filomena AMITRANO, Mariangela SAGGESE, et al. Overview of Current Targeted Anti-Cancer Drugs for Therapy in Onco-Hematology. *Medicina*. 2019, **55**(8), 1-3. DOI: 10.3390/medicina55080414. ISSN 1010-660X.

DAGOGO-JACK, Ibiayi and Alice T. SHAW. Tumour heterogeneity and resistance to cancer therapies. *Nature Reviews Clinical Oncology*. 2017, **15**(2), abstract. DOI: 10.1038/nrclinonc.2017.166. ISSN 1759-4774.

DASGUPTA, Arko, Andrea R. LIM and Cyrus M. GHAJAR. Circulating and disseminated tumor cells: harbingers or initiators of metastasis?. *Molecular Oncology*. 2017, **11**(1), 40-61. DOI: 10.1002/1878-0261.12022. ISSN 15747891.

DILLEKÅS, Hanna, Michael S. ROGERS and Oddbjørn STRAUME. Are 90% of deaths from cancer caused by metastases? *Cancer Medicine*. 2019, **8**(12), 5574-5576. DOI: 10.1002/cam4.2474. ISSN 2045-7634.

ECKE, Thorsten and Thomas OTTO. Diagnostic, Prognostic and Predictive Biological Markers in Bladder Cancer. ECKE, Thorsten a Thomas OTTO. *Diagnostic, Prognostic and Predictive Biological Markers in Bladder Cancer: Illumination of a Vision* [online]. 2019. Switzerland: MDPI, 2019, s. 161 [cit. 2019-12-01]. ISBN 978-3-03897-635-6. (https://www.mdpi.com/books/pdfview/book/1145)

FORTUNO, Cristina, Tina PESARAN, Jill DOLINSKY, Amal YUSSUF, Kelly MCGOLDRICK, David GOLDGAR, Paul A. JAMES and Amanda B. SPURDLE. Differences in patient ascertainment affect the use of gene-specified ACMG/AMP phenotype-related variant classification criteria: evidence for TP53. *Human Mutation*. 2020. DOI: 10.1002/humu.23972. ISSN 1059-7794.

GARG, Neha, Trisha M. WESTERHOF, Vick LIU, Robin LIU, Edward L. NELSON and Abraham P. LEE. Whole-blood sorting, enrichment and in situ immunolabeling of cellular subsets using acoustic microstreaming. *Microsystems & Nanoengineering*. 2018, **4**(1), 1-9. DOI: 10.1038/micronano.2017.85. ISSN 2055-7434.

GEBAUER, Gerhard, Malgorzata Joanna BANYS-PALUCHOWSKI, Hans NEUBAUER, et al. Clinical relevance of circulating tumor cells in ovarian, fallopian tube and peritoneal cancer. *Journal of Clinical Oncology*. 2017, **35**(15_suppl), e17080-e17080. DOI: 10.1200/JCO.2017.35.15_suppl.e17080. ISSN 0732-183X.

GKOUNTELA, Sofia, Barbara SZCZERBA, Cinzia DONATO and Nicola ACETO. Recent advances in the biology of human circulating tumour cells and metastasis. *ESMO Open*. 2016, **1**(4), 1-9. DOI: 10.1136/esmoopen-2016-000078. ISSN 2059-7029.

GOMIS, Roger R. and Sylwia GAWRZAK. Tumor cell dormancy. *Molecular Oncology*. 2017, **11**(1), 62-78. DOI: 10.1016/j.molonc.2016.09.009. ISSN 15747891.

GORGES, Katharina, Lisa WILTFANG, Tobias GORGES, et al. Intra-Patient Heterogeneity of Circulating Tumor Cells and Circulating Tumor DNA in Blood of Melanoma Patients. *Cancers*. 2019, **11**(11), abstract. DOI: 10.3390/cancers11111685. ISSN 2072-6694.

GRADILONE, A., G. NASO, C. RAIMONDI, et al. Circulating tumor cells (CTC) in metastatic breast cancer (MBC): prognosis, drug resistance and phenotypic characterization. *Annals of Oncology*. 2010, **22**(1), 86-92. DOI: 10.1093/annonc/mdq323. ISSN 0923-7534.

GROISBERG, Roman, Hossein MAYMANI and Vivek SUBBIAH. Immunotherapy and next-generation sequencing guided therapy for precision oncology: what have we learnt and what does the future hold? *Expert Review of Precision Medicine and Drug Development*. 2018, **3**(3), abstract. DOI: 10.1080/23808993.2018.1480898. ISSN 2380-8993.

GROSSE-WILDE, Anne, Aymeric FOUQUIER D'HÉROUËL, Ellie MCINTOSH, et al. Stemness of the hybrid Epithelial/Mesenchymal State in Breast Cancer and Its Association with Poor Survival. *PLOS ONE*. 2015, **10**(5), abstract. DOI: 10.1371/journal.pone.0126522. ISSN 1932-6203.

GUPTA, Swati, Navin R. MANI, Daniel E. CARVAJAL-HAUSDORF, et al. Macrodissection prior to closed system RT-qPCR is not necessary for estrogen receptor and HER2 concordance with IHC/FISH in breast cancer. *Laboratory Investigation*. 2018, **98**(8), abstract. DOI: 10.1038/s41374-018-0064-1. ISSN 0023-6837.

HEEKE, Simon, Baharia MOGRABI, Catherine ALIX-PANABIÈRES and Paul HOFMAN. Never Travel Alone: The Crosstalk of Circulating Tumor Cells and the Blood Microenvironment. *Cells*. 2019, **8**(7), 1-12. DOI: 10.3390/cells8070714. ISSN 2073-4409.

HSU, Kuo-Feng, Chun-Yu CHEN, Tzi-Shiang CHU, et al. Scalp melanoma with rectus abdominis metastasis. *Medicine*. 2019, **98**(28), abstract. DOI: 10.1097/MD.0000000000016395. ISSN 0025-7974.

CHEN, Fengju, Darshan S. CHANDRASHEKAR, Sooryanarayana VARAMBALLY and Chad J. CREIGHTON. Pan-cancer molecular subtypes revealed by mass-spectrometry-based proteomic characterization of more than 500 human cancers. *Nature Communications*. 2019, **10**(1), abstract. DOI: 10.1038/s41467-019-13528-0. ISSN 2041-1723.

CHITTY, Jessica L., Elysse C. FILIPE, Morghan C. LUCAS, David HERRMANN, Thomas R. COX and Paul TIMPSON. Recent advances in understanding the complexities of metastasis. *F1000Research*. 2018, **7**(2018), 1-18. DOI: 10.12688/f1000research.15064.2. ISSN 2046-1402.

JIE, Xiao-Xiang, Xiao-Yan ZHANG and Cong-Jian XU. Epithelial-to-mesenchymal transition, circulating tumor cells and cancer metastasis: Mechanisms and clinical applications. *Oncotarget*. 2017, **8**(46), 81558-81571. DOI: 10.18632/oncotarget.18277. ISSN 1949-2553.

JORDAN, Nicole Vincent, Aditya BARDIA, Ben S. WITTNER, et al. HER2 expression identifies dynamic functional states within circulating breast cancer cells. *Nature*. 2016, **537**(7618), 102-106. DOI: 10.1038/nature19328. ISSN 0028-0836.

KANG, Yoon-Tae, Il DOH, Jiyoung BYUN, Hee Jin CHANG and Young-Ho CHO. Label-free Rapid Viable Enrichment of Circulating Tumor Cell by Photosensitive Polymer-based Microfilter Device. *Theranostics*. 2017, **7**(13), 3179-3191. DOI: 10.7150/thno.19686. ISSN 1838-7640.

KANKEU FONKOUA, Lionel and Nelson YEE. Molecular Characterization of Gastric Carcinoma: Therapeutic Implications for Biomarkers and Targets. *Biomedicines*. 2018, **6**(1), abstract. DOI: 10.3390/biomedicines6010032. ISSN 2227-9059.

KASIMIR-BAUER, Sabine, Ann-Kathrin BITTNER, Lisa KÖNIG, Katharina REITER, Thomas KELLER, Rainer KIMMIG and Oliver HOFFMANN. Does primary neoadjuvant systemic therapy eradicate minimal residual disease? Analysis of disseminated and circulating tumor cells before and after therapy. *Breast Cancer Research*. 2016, **18**(1), 1-15. DOI: 10.1186/s13058-016-0679-3. ISSN 1465-542X.

KESSLER, Larry G, Huiman X BARNHART, Andrew J BUCKLER, et al. The emerging science of quantitative imaging biomarkers terminology and definitions for scientific studies and regulatory submissions. *Statistical Methods in Medical Research*. 2015, **24**(1), 9-26. DOI: 10.1177/0962280214537333. ISSN 0962-2802.

LIIKANEN, Jenni S., Marjut H. LEIDENIUS, Heikki JOENSUU, Jaana H. VIRONEN and Tuomo J. MERETOJA. Prognostic value of isolated tumour cells in sentinel lymph nodes in early-stage breast cancer: a prospective study. *British Journal of Cancer*. 2018, **118**(11), 1529-1535. DOI: 10.1038/s41416-018-0052-7. ISSN 0007-0920.

LOWES, Lori E. and Alison L. ALLAN. Circulating Tumor Cells and Implications of the Epithelial-to-Mesenchymal Transition. *Adv Clin Chem*. Elsevier, 2018, 2018, **2018**(83), 121-181. Advances in Clinical Chemistry. DOI: 10.1016/bs.acc.2017.10.004. ISBN 9780128152072.

LUO, Y. T., J. CHENG, X. FENG, S. J. HE, Y. W. WANG and Q. HUANG. The viable circulating tumor cells with cancer stem cells feature, where is the way out?. *J Exp Clin Cancer Res*. 2018, **37**(1), 1-13. DOI: 10.1186/s13046-018-0685-7. ISSN 1756-9966.

MAIER, Manfred. Personalized medicine—a tradition in general practice! *European Journal of General Practice*. 2019, **25**(2), 63-64. DOI: 10.1080/13814788.2019.1589806. ISSN 1381-4788.

MANSOORI, Maryam, Zahra MADJD, Leila JANANI and Arezoo RASTI. Circulating cancer stem cell markers in breast carcinomas: a systematic review protocol. *Systematic Reviews*. 2017, **6**(1), 1-6. DOI: 10.1186/s13643-017-0660-y. ISSN 2046-4053.

MARTÍNEZ-JIMÉNEZ, Francisco, Ferran MUIÑOS, Inés SENTÍS, et al. A compendium of mutational cancer driver genes. *Nature Reviews Cancer*. 2020, **2020**, 1-18. DOI: 10.1038/s41568-020-0290-x. ISSN 1474-175X.

MATSUZAKI, Juntaro, Hitoshi TSUGAWA and Hidekazu SUZUKI. Precision Medicine Approaches to Prevent Gastric Cancer. *Gut and Liver*. 2020, **2020**(January), abstract. DOI: 10.5009/gnl19257. ISSN 1976-2283.

MCINNES, Linda M., Natalie JACOBSON, Andrew REDFERN, Anthony DOWLING, Erik W. THOMPSON and Christobel M. SAUNDERS. Clinical Implications of Circulating Tumor Cells of Breast Cancer Patients: Role of Epithelialâ€"Mesenchymal Plasticity. *Frontiers in Oncology*. 2015, **5**(42), 1-7. DOI: 10.3389/fonc.2015.00042. ISSN 2234-943X.

MCNAMARA, M.G., T. JACOBS, M. FRIZZIERO, R. PIHLAK, A. LAMARCA, R. HUBNER, J.W. VALLE and E. AMIR. Prognostic and predictive impact of high tumor mutation burden (TMB) in solid tumors: A

systematic review and meta-analysis. *Annals of Oncology*. 2019, **30**(Supplement 5), abstract. DOI: 10.1093/annonc/mdz239. ISSN 09237534.

MIDTHUN, Lauren, Shagufta SHAHEEN, Jeremy DEISCH, Maheswari SENTHIL, James TSAI and Chung-Tsen HSUEH. Concomitant KRAS and BRAF mutations in colorectal cancer. *Journal of Gastrointestinal Oncology*. 2019, **10**(3), abstract. DOI: 10.21037/jgo.2019.01.10. ISSN 20786891.

OBERMAYR, Eva, Natalia BEDNARZ-KNOLL, Beatrice ORSETTI, et al. Circulating tumor cells: potential markers of minimal residual disease in ovarian cancer? a study of the OVCAD consortium. *Oncotarget*. 2017, **8**(63), 106415–106428. DOI: 10.18632/oncotarget.22468. ISSN 1949-2553.

PAL, Sumanta K., Miaoling HE, Timothy WILSON, et al. Detection and Phenotyping of Circulating Tumor Cells in High-Risk Localized Prostate Cancer. *Clinical Genitourinary Cancer*. 2015, **13**(2), 130-136. DOI: 10.1016/j.clgc.2014.08.014. ISSN 15587673.

SHLYAKHTUNOU, Y. Expression of the genes of multiple drug resistans ABC-transporters in circulating tumor cells in primmary nonmetastatic breast cancer. *European Journal of Cancer*. 2018, **92**(Supplement 3), S147. DOI: 10.1016/S0959-8049(18)30666-X. ISSN 09598049.

SCHAFFAR, Robin, Christine BOUCHARDY, Pierre Olivier CHAPPUIS, Alexandre BODMER, Simone BENHAMOU, Elisabetta RAPITI and Sushanta K BANERJEE. A population-based cohort of young women diagnosed with breast cancer in Geneva, Switzerland. *PLOS ONE*. 2019, **14**(9), abstract. DOI: 10.1371/journal.pone.0222136. ISSN 1932-6203.

SCHAFFNER, Florence, Jean-Louis MERLIN and Nikolas VON BUBNOFF, ed. *Tumor Liquid Biopsies: Recent Results in Cancer Research*. 215. Switzerland: Springer Nature Switzerland AG 2020, 2020. ISBN 978-3-030-26438-3.

SPARANO, JA, A O'NEILL, K ALPAUGH, AC WOLFF, DW NORTHFELT, C DANG, GW SLEDGE and KD MILLER. Abstract GS6-03: Circulating tumor cells (CTC) five years after diagnosis are prognostic for late recurrence in operable stage II-III breast cancer. *Cancer Research*. 2018, **78**(4 Supplement), GS6-03-GS6-03.

TRAPP, Elisabeth, Wolfgang JANNI, Christian SCHINDLBECK, et al. Presence of Circulating Tumor Cells in High-Risk Early Breast Cancer During Follow-Up and Prognosis. *JNCI: Journal of the National Cancer Institute*. 2019, **111**(4), 380-387. DOI: 10.1093/jnci/djy152. ISSN 0027-8874.

WANG, Zhihui and Thomas S. DEISBOECK. Dynamic Targeting in Cancer Treatment. *Frontiers in Physiology*. 2019, **10**(2019), 1-2. DOI: 10.3389/fphys.2019.00096. ISSN 1664-042X.

WANG, Hongxia, Nikolas H. STOECKLEIN, Peter P. LIN and Olivier GIRES. Circulating and disseminated tumor cells: diagnostic tools and therapeutic targets in motion. *Oncotarget*. 2017, **8**(1), 1884–1912. DOI: 10.18632/oncotarget.12242. ISSN 1949-2553.

XAVIER, Alexandre, Maren Fridtjofsen OLSEN, Liss A. LAVIK, Jostein JOHANSEN, Ashish Kumar SINGH, Wenche SJURSEN, Rodney J. SCOTT and Bente A. TALSETH-PALMER. Comprehensive mismatch repair gene panel identifies variants in patients with Lynch-like syndrome. *Mol Genet Genomic Med*. 2019, **7**(8), abstract. DOI: 10.1002/mgg3.850. ISSN 2324-9269.

YAMADA, Takeshi, Akihisa MATSUDA, Michihiro KOIZUMI, et al. Liquid Biopsy for the Management of Patients with Colorectal Cancer. *Digestion*. 2019, **99**(1), 39-45. DOI: 10.1159/000494411. ISSN 0012-2823.

YANG, Fang, Jing XU, Lin TANG an Xiaoxiang GUAN. Breast cancer stem cell: the roles and therapeutic implications. *Cellular and Molecular Life Sciences*. 2017, **74**(6), 951-966. DOI: 10.1007/s00018-016-2334-7. ISSN 1420-682X.

YE, Qianwei, Sunbin LING, Shusen ZHENG and Xiao XU. Liquid biopsy in hepatocellular carcinoma: circulating tumor cells and circulating tumor DNA. *Molecular Cancer*. 2019, **18**(1), 1-2. DOI: 10.1186/s12943-019-1043-x. ISSN 1476-4598.

YU, M., A. BARDIA, B. S. WITTNER, et al. Circulating Breast Tumor Cells Exhibit Dynamic Changes in Epithelial and Mesenchymal Composition. *Science*. 2013, **339**(6119), 580-584. DOI: 10.1126/science.1228522.

ZAPATERO, A., A. Gomez CAAMANO, M. D. L. A. Cabeza RODRIGUEZ, et al. Monitoring of Circulating Tumor Cells in Patients With High-Risk Nonmetastatic Prostate Cancer Treated With Radiation Therapy and Hormones: Preliminary Data of an Observational Prospective Phase 2 Study. *International Journal of Radiation Oncology*Biology*Physics*. 2017, **99**(2), abstract. DOI: 10.1016/j.ijrobp.2017.06.1272. ISSN 03603016.

8. List of publications

I. **JAKABOVA Anna,** Zuzana BIELCIKOVA, Eliska POSPISILOVA, Lubos PETRUZELKA, Piotr BLASAK, Vladimir BOBEK and Katarina KOLOSTOVA. Characterization of circulating tumor cells in early breast cancer patients receiving neoadjuvant chemotherapy. *Therapeutic Advances in Medical Oncology* [Accepted on 09 June 2021, Impact Factor in 2021: 7.03]

II. **JAKABOVA, Anna**, Zuzana BIELCIKOVA, Eliska POSPISILOVA, Rafal MATKOWSKI, Bartlomiej SZYNGLAREWICZ, Urszula STASZEK-SZEWCZYK, Milada ZEMANOVA, Lubos PETRUZELKA, Petra ELIASOVA, Katarina KOLOSTOVA and Vladimir BOBEK. Molecular characterization and heterogeneity of circulating tumor cells in breast cancer. *Breast Cancer Research and Treatment*. 2017, **166**(3), 695-700. ISSN 0167-6806. doi:10.1007/s10549-017-4452-9 [Impact Factor in 2017: 3.855]

III. BIELCIKOVA, Zuzana, **Anna JAKABOVA**, Michael PINKAS, Milada ZEMANOVA, Katarina KOLOSTOVA and Vladimir BOBEK. Circulating tumor cells:: what we know, what do we want to know about them and are they ready to be used in clinics? *American Journal of Translational Research*. 2017, **9**(6), 2807-2823. [Impact Factor in 2017: 3.255]

IV. KOLOSTOVA, Katarina, Michael PINKAS, **Anna JAKABOVA**, Eliska POSPISILOVA, Pavla SVOBODOVA, Jan SPICKA, Martin CEGAN, Rafal MATKOWSKI and Vladimir BOBEK. Molecular characterization of circulating tumor cells in ovarian cancer. *American Journal of Cancer Research*. 2016, **6**(5), 973-980. [Impact Factor in 2016: 3.265]

V. KOLOSTOVA, Katarina, Rafal MATKOWSKI, Marcin JEDRYKA, Katarzyna SOTER, Martin CEGAN, Michael PINKAS, **Anna JAKABOVA**, Jiri PAVLASEK, Jan SPICKA and Vladimir BOBEK. The added value of circulating tumor cells examination in ovarian cancer staging. *American Journal of Cancer Research*. 2015, **5**(11), 3363-3375. [Impact Factor in 2015: 2.125]