

## Original Article

# Isolation, primary culture, morphological and molecular characterization of circulating tumor cells in gynecological cancers

Katarina Kolostova<sup>1</sup>, Jan Spicka<sup>2</sup>, Rafal Matkowski<sup>3,4</sup>, Vladimir Bobek<sup>1,5,6</sup>

<sup>1</sup>Department of Laboratory Genetics, Institute of Laboratory Diagnostics, University Hospital Kralovske Vinohrady, Srobarova 50, Prague, Czech Republic; <sup>2</sup>Department of Biochemistry, Institute of Laboratory Diagnostics, University Hospital Kralovske Vinohrady, Srobarova 50, Prague, Czech Republic; <sup>3</sup>Division of Surgical Oncology and Department of Oncology, Wroclaw Medical University, Plac Hirszfelda 12, 53-413 Wroclaw, Poland; <sup>4</sup>Lower Silesian Oncology Centre, pl. Hirszfelda 12, 53-413 Wroclaw, Poland; <sup>5</sup>3rd Department of Surgery, First Faculty of Medicine Charles University in Prague and University Hospital Motol, Prague, Czech Republic; <sup>6</sup>Department of Histology and Embryology, Wroclaw Medical University, Chalubinskiego 6a, 50-368 Wroclaw, Poland

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**Abstract:** The focus of the study was to implement a new workflow for circulating tumor cells (CTCs) characterization that would allow the analysis of CTCs on a cytomorphological and molecular level in patients with diagnosed gynecological cancer. Our findings may be useful in future cancer patient management. The study introduces a size-based enrichment (MetaCell<sup>®</sup>) method for the separation of viable CTCs, followed by CTCs culturing *in vitro* and gene expression characterization. It is based on the observation of CTCs and DTCs (Disseminated Tumor Cells) in several case studies of ovarian, endometrial and cervical cancer by means of cytomorphology and gene expression profiling. The viability of the enriched CTCs was estimated using vital and lethal fluorescence nuclear staining. This type of staining may be predictive for the success rate of subsequent CTC growth *in vitro*. To identify CTCs in the enriched CTC fraction, cytomorphological evaluations based on vital fluorescence staining were followed by gene expression analysis of tumor-associated (TA) genes. Cytokeratin expression (KRT7, KRT19) was analyzed in combination with MUC1, MUC16, CD24, CD44 and ALDH1. Gene expression analysis has shown that short-term *in vitro* culture enhanced the differentiation process of the captured CTCs growing on a membrane. On the other hand, redundant white blood cells captured on the membrane were eliminated during a short-term culture. The most frequently elevated genes in ovarian cancer (serous type) are EPCAM, KRT19 and MUC1. It has been demonstrated that CTC presence revealed by cytomorphological evaluation may be usefully complemented by TA-gene expression analysis, to increase the sensitivity of the analysis.

**Keywords:** CTCs, gynecological cancers, ovarian cancer, cervical cancer, endometrial cancer, cultivation, *in vitro*

## Introduction

Circulating tumor cells (CTCs) are viable solid-tumor cells that have disseminated from the primary tumor and are carried through the circulatory system [1]. CTCs are relatively rare, and can be found at frequencies of approximately one tumor cell per 10<sup>7</sup> blood elements [2]. The presence of CTCs in cancer patients is associated with a poor prognosis [3-5]. There is, however, limited data on the prognostic potential and prevalence in patients with gynecological cancers.

Identification of CTCs by isolating and separating them from the peripheral blood of patients with solid tumors may help to personalize cancer treatment. In the near future CTCs will be a tool aiding the overall survival and prognosis of cancer patients by improving clinical management. Using currently available methods, the identification of CTCs in peripheral blood is quite challenging. The majority of methods are targeted at the isolation and enumeration of CTCs from previously stabilized peripheral blood [6]. The study of fixed CTCs provides little data about their functional capability, METASTA-

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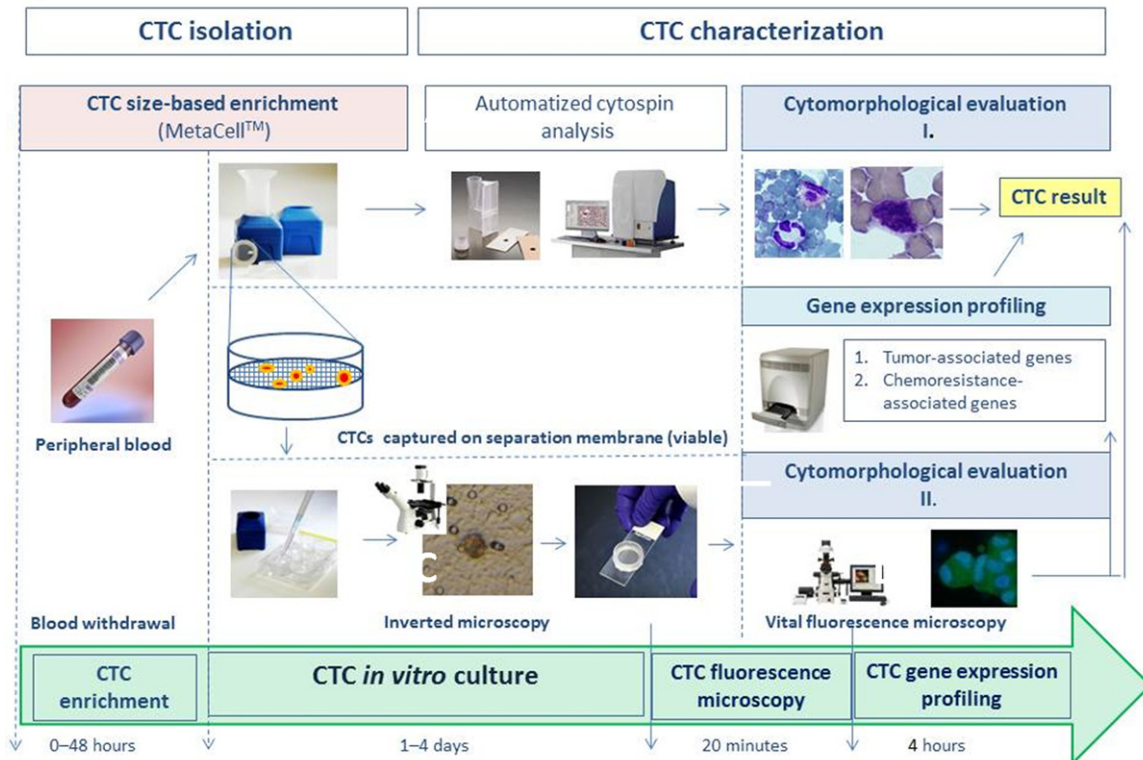


Figure 1. CTC isolation and characterization workflow.

Table 1. Gene expression analysis report

Genes tested by quantitative qPCR (PBW vs. CTC-enriched fraction)										
		T	↑		T	↑		T	↑	
1	ACTB	X		14	CHGA			27	SCGB	X
2	CD45	X		15	MLANA			28	CD24	X X
3	CD68	X		16	S100B			29	CD44	X
4	EPCAM	X	X	17	VIM			30	ALDH	X
5	MUC1	X		18	VEGF			31	MRP1	
6	TTF1			19	WT1			32	MRP2	
7	KRT5			20	CD56			33	MRP4	
8	KRT6			21	CD57			34	MRP5	X
9	KRT7	X	X	22	SYP			35	MRP7	X
10	KRT18			23	HER2	X		36	MDR1	
11	KRT19	X	X	24	ER	X		37	ERCC1	
12	KRT20			25	PR	X				
13	EGFR			26	MUC16	X	X			

T: genes tested by qPCR, ↑: genes with elevated amount of RNA (relative RNA amount).

TIC potential and treatment-induced changes. The *in vitro* culturing of CTCs can therefore allow their metastatic potential and the drug sensitivity of tumor cells to be investigated.

The focus of our study was to implement a new CTC-characterization workflow (Figure 1) that should allow the analysis of CTCs on a cytormorphological and molecular level. The study outcome has the potential for use in cancer patient management. Our study introduces a size-based enrichment method for the separation of viable CTCs followed by culturing *in vitro* and geneexpressioncharacterization. The approach is demonstrated on CTCs in patients with gynecological cancers, specifically ovarian, cervical and endometrial cancers.

### Materials and methods

#### Patients

Patients diagnosed with gynecological tumors (of the three types mentioned above) were involved in the study: one patient with ovarian cancer, one patient with endometrial cancer and one patient with cervical cancer. Approximately 8 ml of venous blood was drawn from the antecubital veins of each

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**Table 2.** The list of Taqman probes

Gene	Taqman probe specification
ACTB	Hs01060665_g1
CD45	Hs04189704_m1
CD68	Hs02836816_g1
KRT7	Hs00559840_m1
KRT19	Hs01051611_gH
MUC1	Hs00159357_m1
MUC16	Hs01065189_m1
EPCAM	Hs00158980_m1
HER2	Hs01001580_m1
ER	Hs00174860_m1
PR	Hs01556702_m1
SCGB (Mammaglobin)	Hs00935948_m1
CD24	Hs03044178_g1
CD44	Hs01075861_m1
ALDH1	Hs00946916_m1
ABCC1 (MRP1)	Hs01561502_m1
ABCC2 (MRP2)	Hs00166123_m1
ABCC4 (MRP4)	Hs00988717_m1
ABCC5 (MRP5)	Hs00981087_m1
ABCC10 (MRP7)	Hs00375701_m1
MDR1	Hs00184500_m1
ERCC1	Hs01012158_m1

patient and placed into S-Monovette tubes (Sarstedt AG & Co., Numbrecht, Germany) containing 1.6 mg EDTA/ml blood as an anticoagulant. The samples were processed at room temperature using an isolation procedure completed within 24 hours of the blood draw.

The ethics committees of all participating universities and hospitals approved the study as compliant with the Declaration of Helsinki. All patients also provided written consent.

### *CTC enrichment and culture*

A simplified size-based separation method for viable CTC enrichment from unclotted peripheral blood (PB) has recently been introduced (MetaCell®, MetaCell s.r.o., Ostrava, Czech Republic) [7, 8]. This process is based on the filtration of PB through a porous polycarbonate membrane (with pores of 8 µm diameter). The minimum and maximum volume of the filtered PB may be adjusted up to 50 ml. An 8 ml PB sample from patients suffering from gynecological cancer was transferred into the filtration tube. Gradual transfer of the blood in several steps is preferred to prevent clogging of the

membrane filter. The PB flow is driven naturally by blood viscosity and supported by capillary action of the absorbent material touching the membrane filter. Afterwards the membrane filter, which is kept in a plastic ring, is transferred into a 6-well cultivation plate; 4 ml RPMI media is added to the filter top and CTCs are cultured on the membrane *in vitro*, under standard cancer-cell culture conditions (37°C, 5% atmospheric CO<sub>2</sub>) and observed by inverted microscope. The CTCs grow in an FBS-enriched RPMI medium (10%) for a minimum of 3 days on the separating membrane. The grown cells can be analyzed by means of histochemistry (May-Grünwald staining - MGG) and immunohistochemistry using specific antibodies to determine the cell origin (pancytokeratin 1-FITC conjugated antibody (Sigma), cytokeratin7 antibody (Dako)) and unspecific, DAPI staining). A novelty that we introduced was a cytomorphological analysis method based on vital fluorescent staining, which enables us to use stained cells for further downstream analysis (e.g. gene expression profiling). The cells captured on the membrane are stained with unspecific nuclear stain (NucBlue™, Life Technologies) and unspecific green cytoplasmic stain (CellTracker™, Life Technologies).

Alternatively, the CTC-enriched fraction can be transferred from the membrane directly onto a microscopic slide with a cytospin centrifuge. The slide is then left to dry for 24 hours and automated cytomorphological analysis can then be performed (Cellavision DM96, Sysmex). The workflow is shown schematically in **Figure 1**.

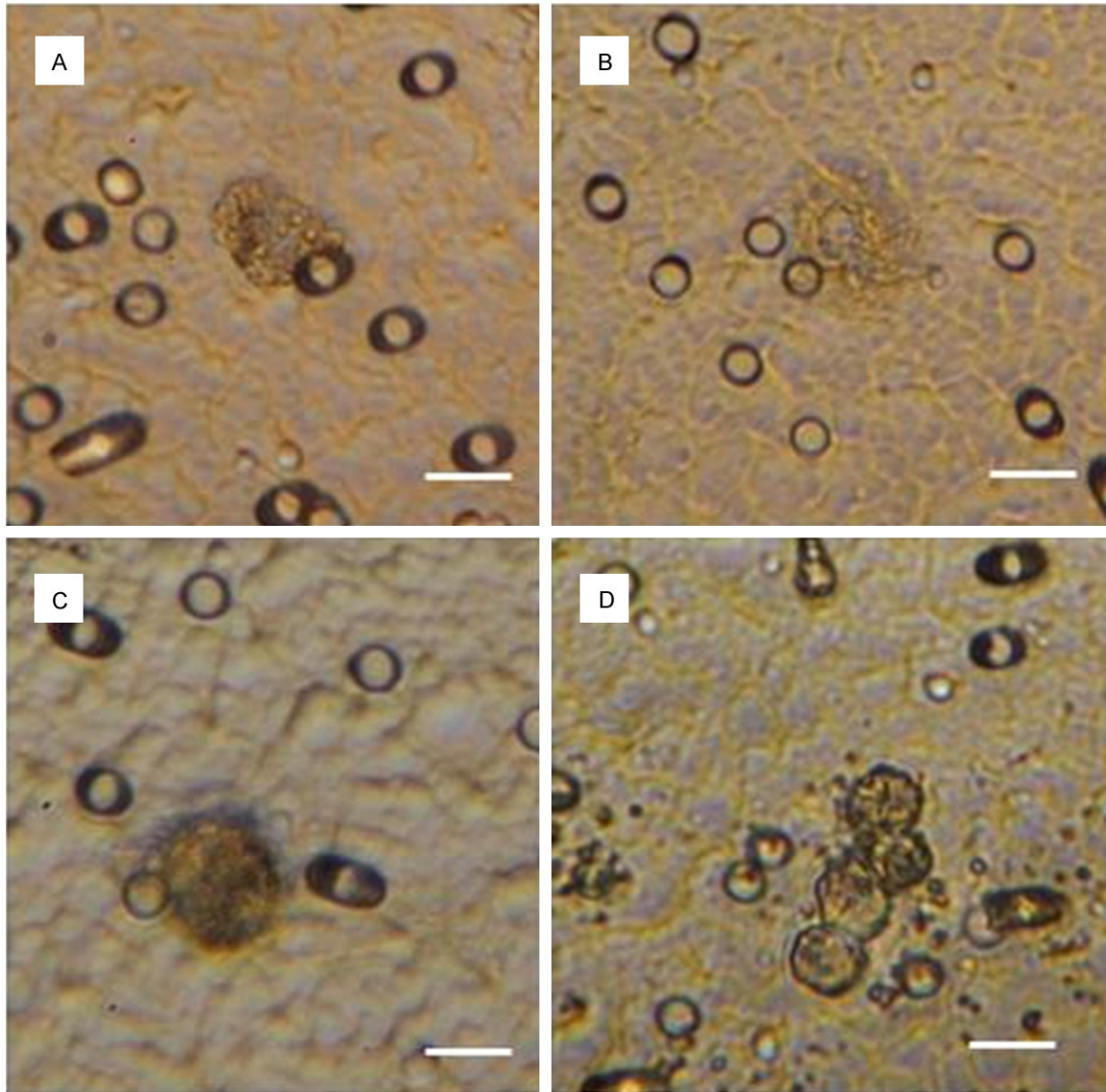
### *Cytomorphological analysis*

The cells, either fixed or viable, and stained on the membranes, were examined using microscopy in three steps: 1. screening at x20 magnification to locate cells; 2. observation at x40 magnification for detailed cytomorphological analysis; 3. observation at x100 magnification for a subcellular-level analysis.

Isolated cells and/or clusters of cells of interest were selected, photographed digitally, and the images were then examined by an experienced researcher and/or pathologist. CTCs were defined as cells with the following characteristics: (A) with a nuclear size ≥10 µm; (B) irregular nuclear contour; (C) presence of visible cytoplasm; (D) prominent nucleoli; (E) high nuclear-



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**Figure 2.** CTCs isolated from patients with: (A) ovarian cancer; (B) endometrial cancer; (C, D) cervical cancer. Scale bar 10  $\mu$ m.

cytoplasmatic ratio (NC-ratio, which changes anyway with the *in vitro* culture time) (F) high deformability/plasticity (growth through the membrane to the bottom of the well and setting up of new colonies); and (G) proliferation.

### Gene expression analysis (GEA)

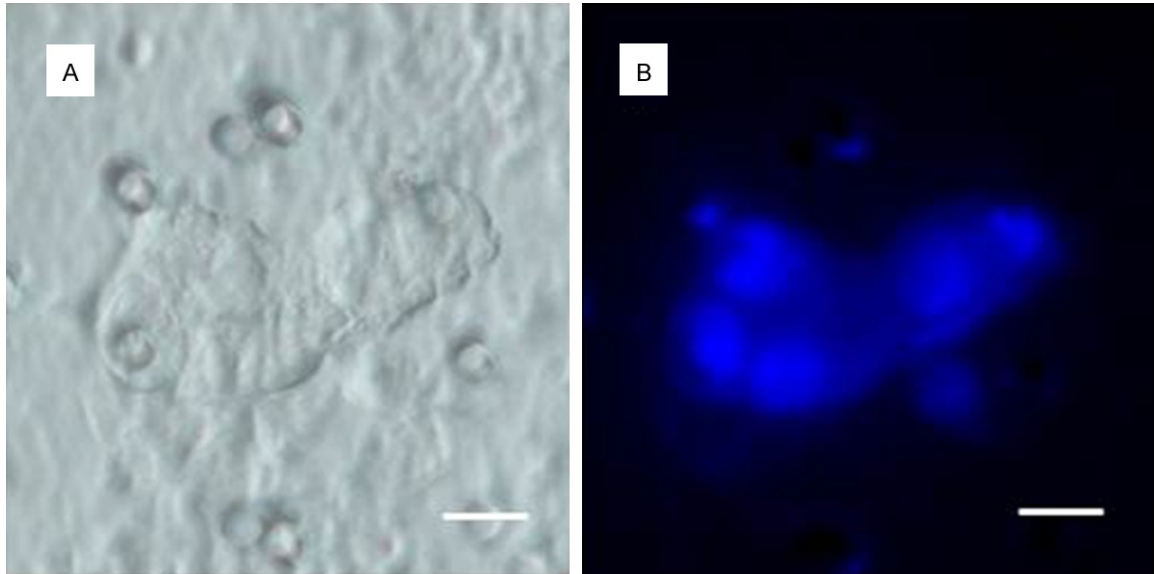
To confirm the origin of the cells on the separation membrane, CTC-gene expression analysis can be performed. Gene expression analysis (GEA) allows up to 20 tumor-associated (TA) markers in RNA from different cell fractions to be tested within a single quantitative polymerase chain reaction (qPCR) run. Differential

diagnostic markers (TA genes) for qPCR testing are chosen depending on the patient's clinical status and primary tumor histology. The following genes were included in the TA/qPCR tests: (ACTB, CD45, CD68, EPCAM, MUC1, MUC16, mammaglobin, HER2, ER, PR, CD24, CD44, ALDH1).

The purpose of the GEA analysis is to compare gene expression of the TA markers in the CTC-enriched fractions to that in the blood as a whole.

In detail, RNA is isolated from the explanation for PBW (white blood cell fraction) and the CTC-

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**Figure 3.** Comparison of: (A) inverted microscope and (B) fluorescence microscope images of DTCs. DTCs were enriched out of the ascitic fluid of an ovarian cancer patient. For visualization of the nucleus a vital nuclear stain (NucBlue™) is used. Scale bar 10  $\mu$ m.

enriched fraction on the membrane. The RNA can be isolated from the CTC fraction immediately after the separation process (these are so-called “virgin” CTCs) or/and from the CTC fraction grown for a minimum of three days on the separation membrane *in vitro* (the so-called “membrane fraction”). Some of the cells grown on the membrane *in vitro* may overgrow the membrane and set up new cell colonies on the culture-well bottom. These cells are analyzed as the “bottom fraction”.

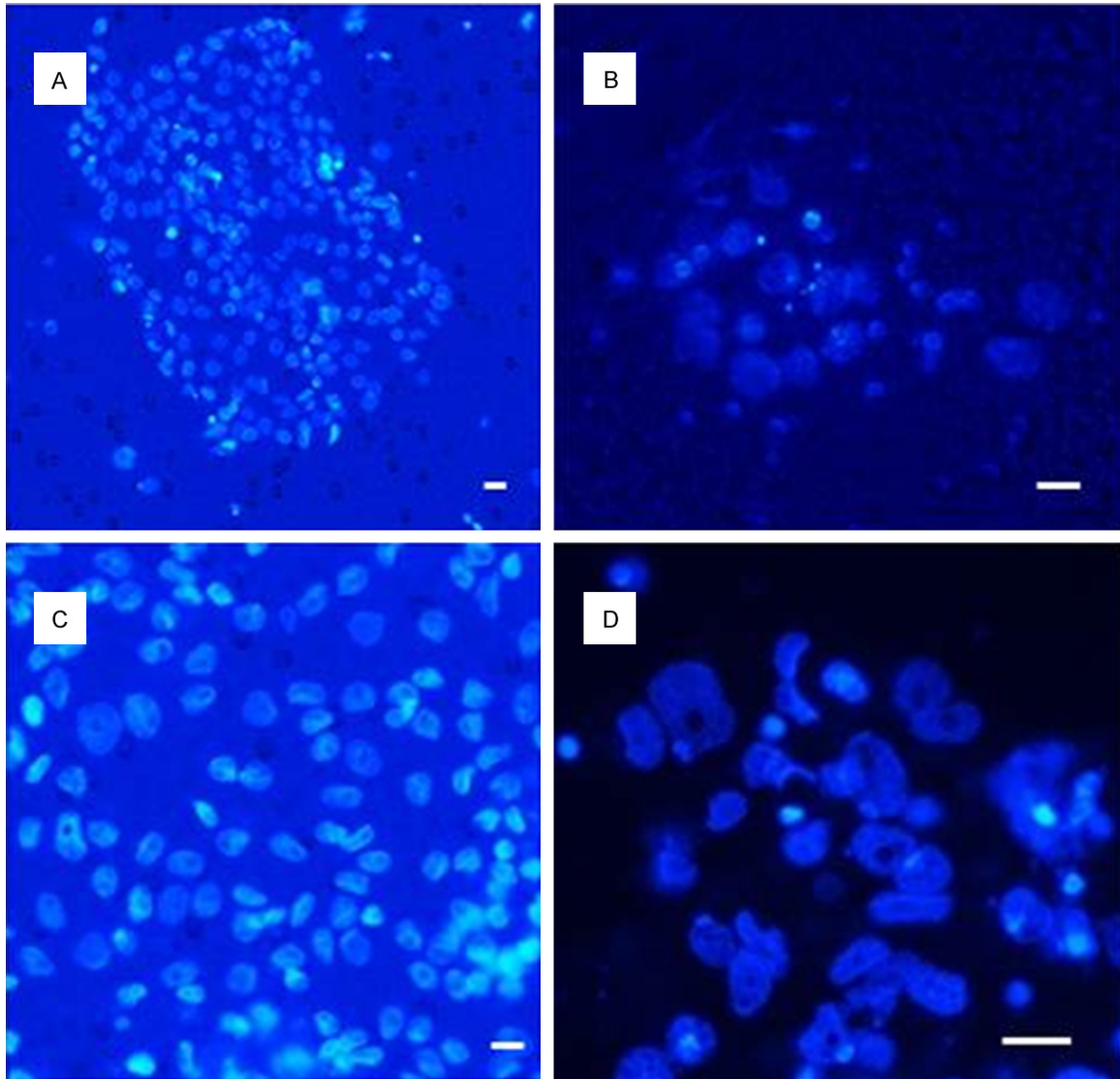
Finally, the CTC-gene expression analysis allows identification of the relative amount of TA markers in the whole blood and in CTC-enriched fractions. One possible approach to report the collected gene expression data (Cycle of quantification [Cq] values) is to compare the relative differences ( $\Delta Cq = Cq_{TA-gene} - Cq_{Control\ gene}$ ) in CTC fraction and white blood cell fraction (PBW) ( $\Delta = \Delta Cq_{CTC\ fraction} - \Delta Cq_{PBW\ fraction}$ ). In detail, TA-gene expression ( $Cq_{TA-gene}$ ) is normalized to control gene ( $Cq_{Control\ gene}$ ) in CTC fraction ( $\Delta Cq_{CTC\ fraction} = Cq_{TA-gene} - Cq_{Control\ gene}$ ). Similarly, difference in PBW fraction ( $\Delta Cq_{PBW\ fraction} = Cq_{TA-gene} - Cq_{Control\ gene}$ ) is calculated.

If the relative amount of tumor-associated gene is increased in CTC fraction if compared to the PBW, the results table is marked with an X (see **Table 1**). If TA genes are highly expressed in the CTC fraction, a subsequent analysis of chemo-

resistance-associated (CHA) genes is performed. The following genes are included in the qPCR-testing: (ERCC1, MDR1, MRP1, MRP2, MRP4, MRP5, MRP10). Molecular analysis allows identification of which type of chemotherapy could be used as an effective treatment. In future, the GEA analysis should make it possible to develop and implement a personalized cancer therapy.

The cells on the membrane are lysed in RLT-buffer with beta-mercapto-ethanol (Qiagen). RNA is then isolated using the RNeasy Mini Kit (Qiagen). The RNA from the whole blood is isolated with a modified procedure, and the quality/concentration of RNA is measured with NanoDrop (ThermoScientific). As there are only a few hundred cells on the membrane, the median concentration of RNA is quite low (5-10 ng/ $\mu$ l). For cDNA production we used the High Capacity cDNA Reverse Transcription Kit (Life Technologies). For gene expression analysis we employed Taqman chemistry including Taqman MGB probes for all the above-mentioned genes (Life Technologies). (A list of Taqman probes employed is given in **Table 2**).

Statistical analysis of the gene expression data was performed using multivariate techniques: hierarchical clustering and principal component analysis (PCA) using the GenEx Pro v.6 Software (MultiD, Sweden). The cut-off value



**Figure 4.** (A, C, D) DTCs and (B) CTCs from an ovarian cancer patient after 10 days of *in vitro* culture (NucBlue™ - nuclear staining). Scale bar 10  $\mu$ m.

was set as 40 Cq. Missing data were obtained by interpolation. Off-scale data (above the cut-off value of 30 Cq) were replaced by maximal Cq value of the tested gene plus 0.5 (when maximal Cq was below the cut-off value). The relative RNA-quantity data between the PBW and CTC fractions in patient cohorts were compared by non-parametric Man-Whitney test (not applicable if reporting on individuals).

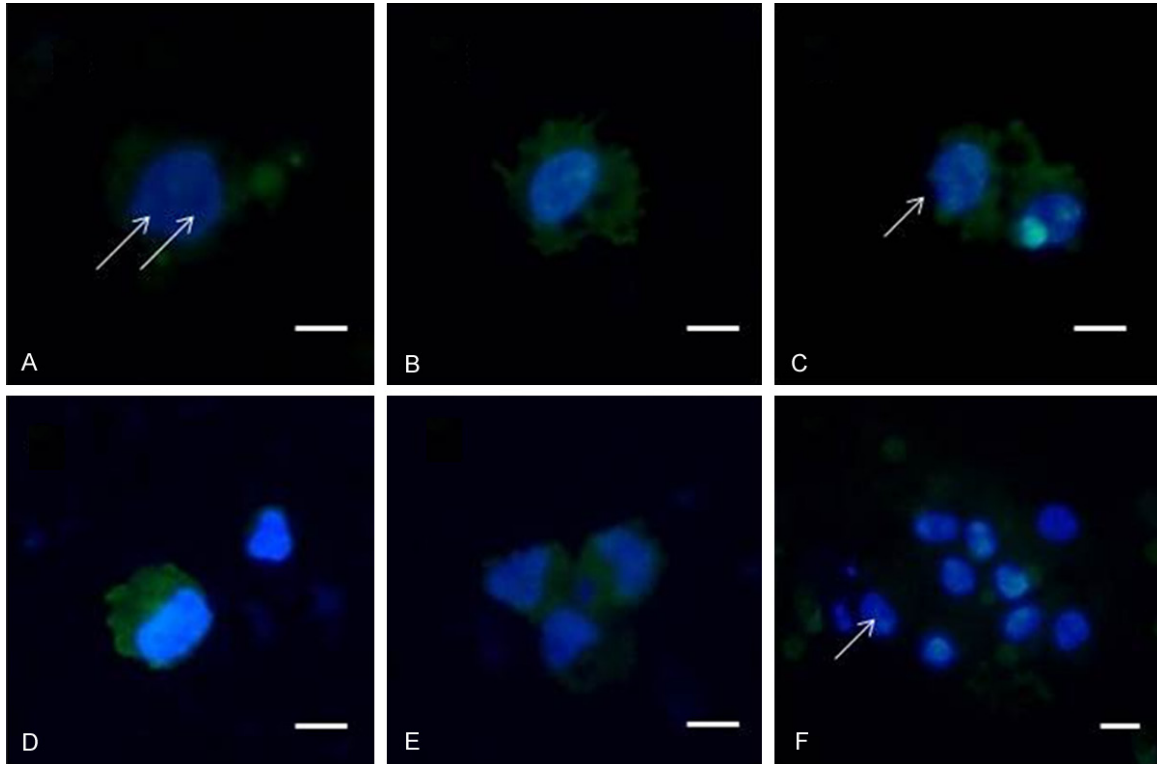
### Results

We report successful implementation and optimization of the workflow for CTC characterization. The study is based on the description of CTCs and DTCs in several case studies of ovar-

ian, endometrial and cervical cancer by means of cytomorphology and gene expression profiling data.

After blood filtration, the membrane kept in a plastic ring was transferred to a 6-well plate and the CTCs were observed by inverted microscope (**Figure 2**). The CTCs were cultured *in vitro* for a minimum of 3 days. The cells on the membrane were co-incubated with unspecific fluorescent vital stains and the morphology was evaluated after 3 days of culturing and digital images were captured (**Figures 3-5**). Thanks to their vital staining the cells can be used immediately after microscopy for mRNA isola-





**Figure 5.** CTCs from an ovarian cancer patient, stained for subcellular cytomorphology evaluation with vital nuclear stain (NucBlue™) and unspecific green cytoplasmic stain (Celltracker™). Prominent nucleoli are arrowed. Scale bar 10  $\mu$ m.

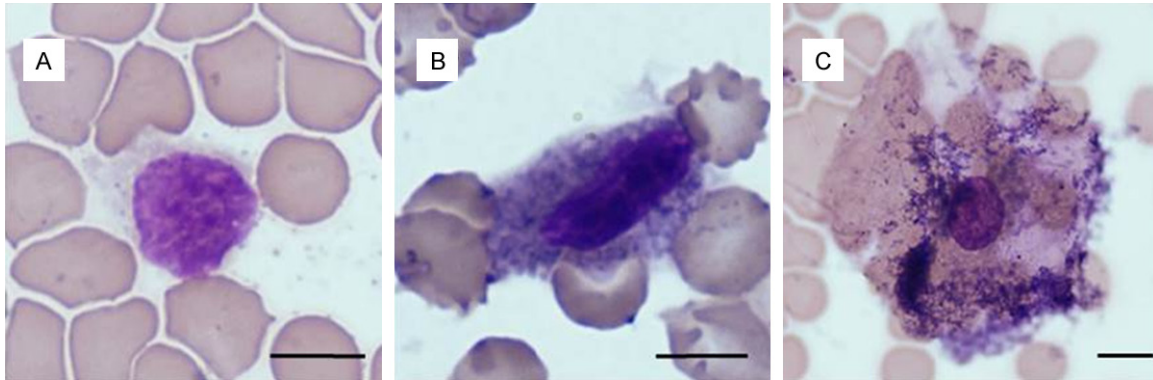
tion and gene expression profiling. As shown in **Figure 6** it is possible to follow the procedure of filtration by cytospin-slide preparation. All the captured cells were washed from the separation membrane, transferred using a cytospin to a slide, and analyzed automatically with a Cellvision DM 96 analyzer (Sysmex). In practical terms, this means that the machine takes high quality photographs and the analytical specialist's only task is to evaluate the cell morphology on the digitized images (**Figure 6**).

Similarly, DTCs from the peritoneum can be analyzed. We report a successful *in vitro* culture of DTCs, but the success of *in vitro* growth is dependent on the viability of the cells. The viability of the captured cells can be estimated using vital (NucBlue™) and lethal (NucGreen™ Dead) nuclear staining (**Figure 7**). The green nuclei can be found in dead cells, the cells with blue nuclei may grow further. This type of staining may be predictive of the success of subsequent *in vitro* growth. The DTC cultures may then be used for additional chemotherapy testing (**Figure 8**).

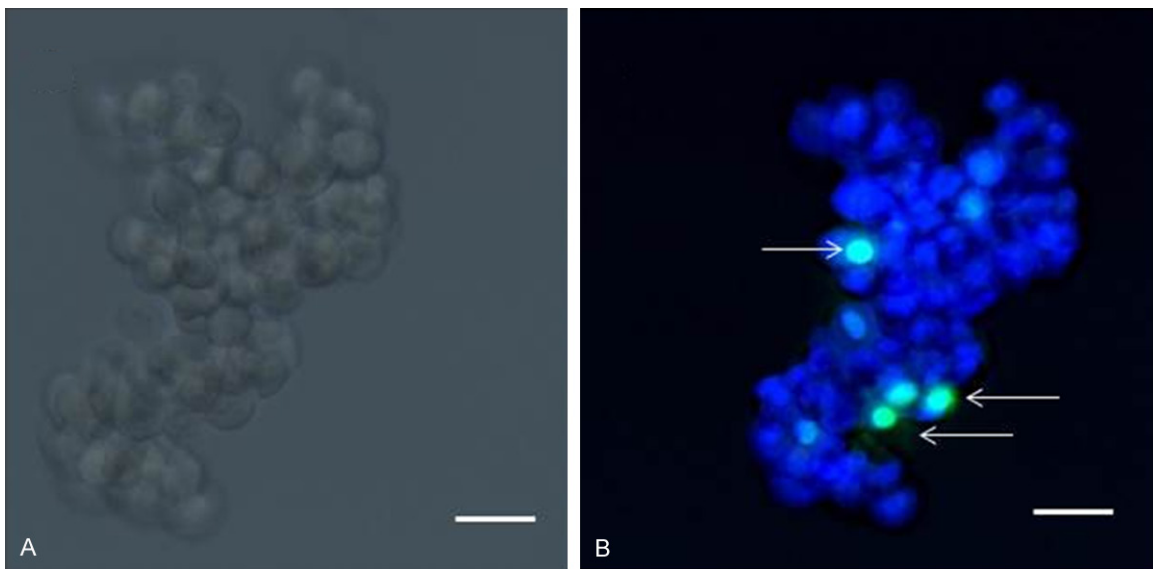
To identify CTCs in the enriched CTC fraction, an RNA relative quantity of TA genes was evaluated. Following the generally adopted procedure, we looked for cells expressing EpCAM. Since CTCs undergo epithelial mesenchymal transition (EMT), cytokeratin expression (KRT7, KRT19) was analyzed in combination with MUC1 [9, 10]. Additionally, MUC16 was found a promising marker of ovarian tissue [11]. CD24, CD44 and ALDH1 were tested for the stemness of the captured cells. This could not be achieved without reporting expression of CD45, CD68 and a control gene (ACTB). It is quite challenging to find a relevant control gene for gene expression analysis of CTCs as they change their phenotype relatively quickly. Similarly, it is quite challenging to find a general pattern characterizing gene expression in CTCs for all the included patients. It has been shown that the approach to analyze gene expression based on comparison within one individual could report more relevant information on CTC character.

We expected that the ratios of control gene and TA genes would change in enriched fractions. Ultimately, it was shown that *in vitro* culture

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**Figure 6.** CTCs from an ovarian cancer patient analyzed automatically by Cellviation DM 96 analyzer after May-Grünwald staining. Note the different cytomorphology of the captured cancer cells. Scale bar 5  $\mu$ m.



**Figure 7.** DTCs from an ovarian cancer patient (ascites) - (A) Inverted microscope image; (B) Fluorescence microscope image. Viable cells are stained with NucBlue™, dead cells (arrowed) with NucGreen™. Scale bar 10  $\mu$ m.

enhanced the differentiation process of the captured CTCs and on the other hand white blood cells died during a short-term culture. The detection of CTCs by cytomorphological evaluation could be further enhanced with verification by complementary GEA of TA-gene expression in CTC-enriched fractions. The most frequently elevated genes in ovarian cancer (serous type) are EPCAM, KRT19, MUC1.

An example of GEA data shown by cluster analysis is shown in **Figure 9**.

### Discussion

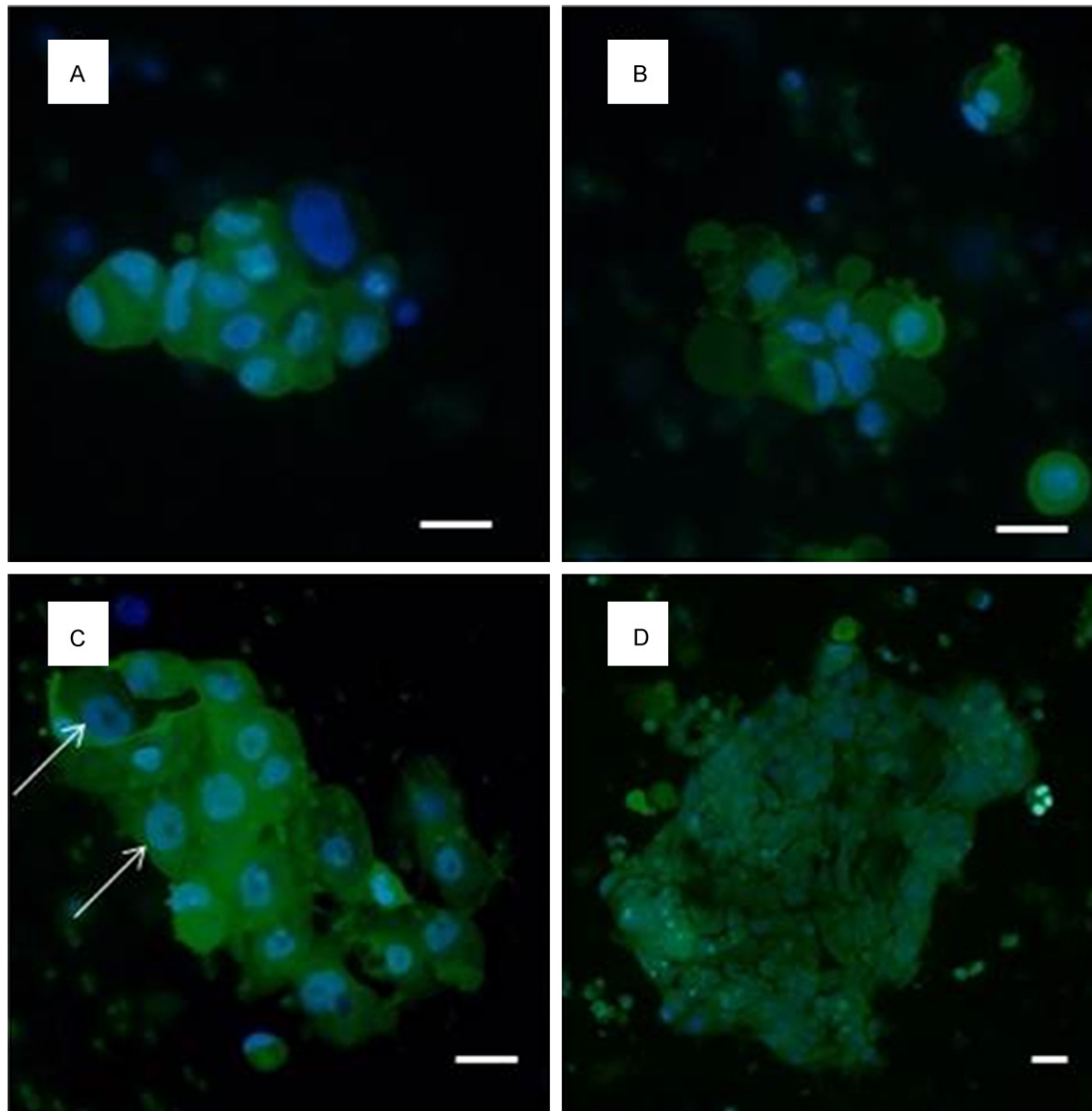
The early detection and characterization of CTCs is important as it is a general tool aimed

at monitoring and preventing the development of overt metastatic disease [12, 13].

Although the rate at which tumor cells are released into the bloodstream in cancer patients is unknown, experimental models indicate that millions are continuously dispersed through the body, though very few reach distant organs, and survive in a dormant state [14].

There are many strategies for detecting and isolating CTCs. Various approaches to the enrichment process are under development in laboratories all over the world [15, 16]. Most of capture technologies are dependent on the epithelial character of solid tumors, which is demonstrated by EpCAM expression [17]. This





**Figure 8.** In vitro cultured DTCs isolated from ascitic fluid from an ovarian cancer patient stained with vital nuclear stain (NuCBlue™) and unspecific green cytoplasmic stain (Celltracker™) to allow subcellular cytomorphology evaluation. Prominent nucleoli are arrowed. Scale bar 10  $\mu$ m.

technical issue leads to the underdetection of CTCs.

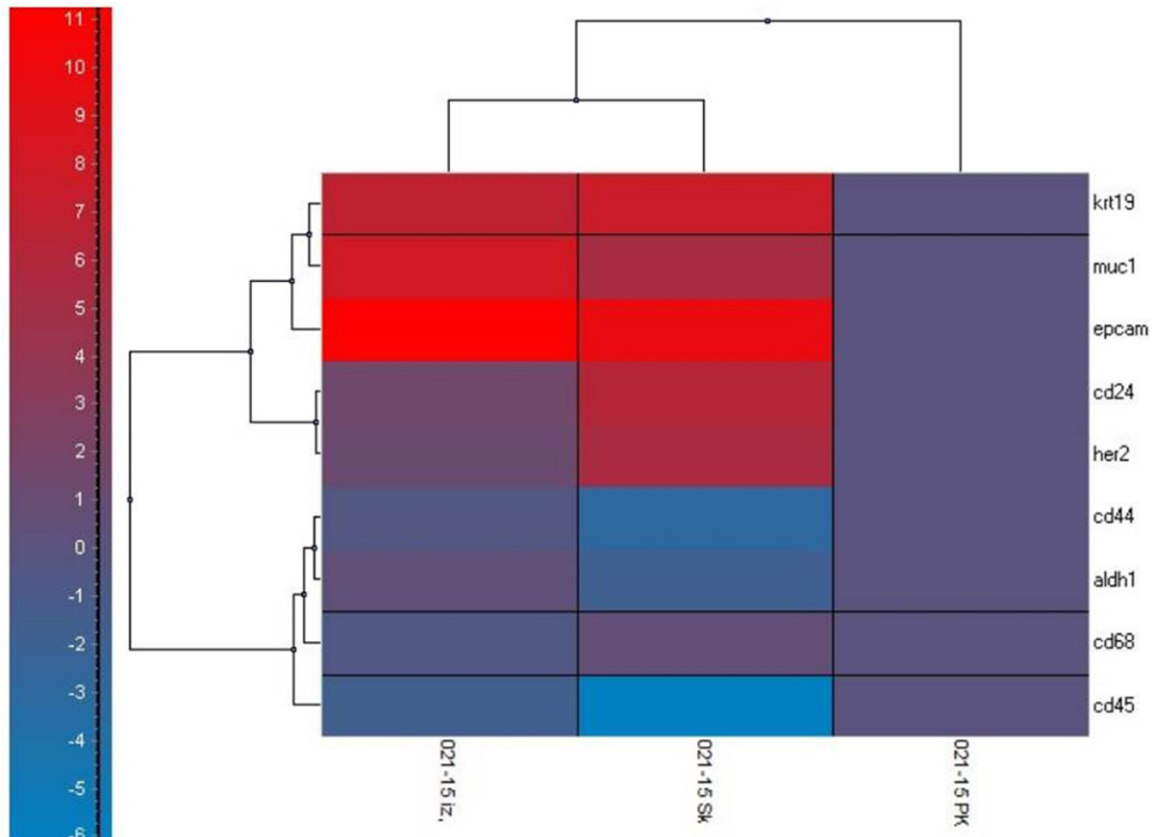
Another specific aspect is viability of the enriched CTCs. The captured CTCs lose their viability during the immunofluorescence detection process so that subsequent culturing of collected cells is disabled.

Quantification of CTCs could be of high value for clinicians as these CTCs generally represent the tumor (metastases) and facilitate real-time monitoring during systemic therapies by sequential peripheral blood sampling [18, 19].

In current clinical practice, the decision to employ targeted therapies is based solely on the analysis of the primary tumor although the therapy is directed against metastatic cells.

Furthermore, molecular characterization of CTCs might enable the identification of therapeutic targets and contribute to personalized anti-metastatic therapies. Molecular characterization of CTCs might be essential to identify therapeutic targets and contribute to more 'tailored' and personalized anti-metastatic therapies [20].

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**Figure 9.** Gene expression profiling data reported by hierarchical clustering shown in relative RNA-quantity values (scale at left). Gene expression level of TA genes in peripheral blood - the white blood cell fraction (PK) is compared to the CTC-enriched fraction (without in vitro culture) (PK izo) and the CTC-enriched fraction after in vitro culture (PK Sk). The following genes were tested: KRT19, MUC1, EpCAM, CD24, CD44, HER2, ALDH1, CD68, CD45, ACTB. Significant changes were seen in gene expression of EPCAM, MUC1 and KRT19 in the CTC-enriched fraction. After in vitro culturing of the CTC-enriched fraction, CD24 and HER2 expression increased, while CD44, ALDH1, CD68, CD45 expression fell. The fall of mRNA levels of these genes could be relevant to the dying of the white blood cells fraction.

CTC enrichment combined with an RT-PCR (qPCR) analysis could already be used for the identification of tumor-related markers (EpCAM, MUC1, and ERBB2), EMT-associated transcripts (PI3Ka (phosphatidylinositol 3-kinase alpha), Akt-2, or Twist1), or stem-cell markers such as ALDH1 (aldehyde dehydrogenase 1) [21].

qPCR is highly specific and sensitive and can detect the expression or mutation at a single cell level [22].

The testing of chemoresistance in cancer patients has its own place in treatment. The problem with implementing chemoresistance testing in the past was that it did not reflect the real-time situation but only the character of primary tumors. Testing metastatic tissue would of course be more beneficial but biopsy of metastatic sites is not usually feasible.

As reported in our study, a liquid biopsy (as CTCs are known) itself offers a way to overcome the above-mentioned obstacles. The captured CTCs can mirror the therapy effect - first by enabling numerical CTC analysis, second by qPCR testing of chemoresistance-associated genes. Of course, one could ask: why test, if in some cancer types the only therapy possible is based on just one drug? We believe that even information about inefficacy of drugs could be of value for patient management and could spare the patient many side effects. The main effect of chemoresistance testing in CTCs could provide a real-time message about the effectivity of administered chemotherapy until chemoresistance is developed.

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### Disclosure of conflict of interest

None to declare.

**Address correspondence to:** Dr. Vladimír Bobek, University Hospital, Kralovské Vinohrady, Department of Laboratory Genetics, Institute of Laboratory Diagnostics, Srobarova 50, 100 34 Prague, Czech Republic. Tel: +420 26716 3578; E-mail: vbobek@centrum.cz

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