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REVIEW ARTICLE

Circulating tumor cells in gynaecological malignancies

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Summary

New non-invasive approaches have developed for diagnosis and treatment of malignant diseases. Cells shed from the primary tumor circulating in the bloodstream with metastasis potential are called Circulating Tumor Cells (CTCs). These cells are easily acquired from the peripheral blood of patients, while several enrichment and isolation methods are available nowadays with different benefits and positive detection rates. A brief characterization of three major categories of detection is described (nucleic acid-based, physical properties-based, antibody-based). In this review we concentrate on gynecological malignancies and how CTCs could be used in the diagnosis of cancer, treatment management and its effective prognosis and early recurrence detection. Presence of CTCs in endometrial cancer patients show worse overall survival, while gene analysis could identify patients in need of systemic therapy after surgical treatment to prevent metastasis and recurrence. Based on the influence of human papillomavirus (HPV) in the etiology of cervical cancer, viral oncogene transcripts could be used as an ideal marker for cervical cancer cells detection. In ovarian cancer, CTCs could help in the differentiation from benign adnexal masses and show a high independence from other biomarkers such as CA125 and HE4. While detection of CTC after complete cytoreductive surgery could indicate invisible lesions, combination of tumor associated genes rises the specificity of CTC detection.

Key words: *biomarker, cervical cancer, circulating tumor cells, endometrial cancer, liquid biopsy, ovarian cancer*

Introduction

In the last two decades, big effort and hopes are put into the discovery of new non-invasive methods for diagnosis and understanding the pathophysiology of malignant diseases. Further development of these tools could help in diagnosis, prognosis, personalized therapy and evaluation of its effectiveness or even alert for recurrences in patients in the follow up period. Liquid biopsy which is easily acquired from patients allows to study the molecular architecture and behaviour of tumors in real time [1]. The tumor material is composed most often by circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), circulating tumor miRNA, proteins and exosomes and besides blood they could be present in several body fluids such as saliva, urine, cerebrospinal fluid, uterine aspirates, pleural effusions or even stool [2,3]. This review analyses the momentary state of circulating tumor cells in the malignancies of the female genital system. The studies used in this review are listed in Table 1.

CTCs are shed from the primary tumor into the bloodstream with potential ability of metastasis (Figure 1). Positive isolation and detection of CTCs have been validated as a prognostic factor in metastatic breast cancer and several other solid

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Table 1. List of studies used in this review

Type of cancer	*Authors*	*Year*	*Method*	*Number of patients*	*CTC isolated*	*CTC positivity rate*	*Notes*
Endometrial carcinoma	Kiss et al.	2018	Size based isolation (MetaCell)	92	69	75%	Independent from stage, grade, lymph node involvement
	Bogani et al.	2015	Immunomagnetic selection, immunofluorescence staining (CellSearch)	28	2	7%	Associated with myometrial invasion, lymph node positivity
	Ni et al.	2016	CellSearch	40	6	15%	Associated with cervical involvement
	Lemech et al.	2016	Detection of EpCAM (CellSearch)	30	18	60%	Stathmin expression as a biomarker for treatment response
	Kolbl et al.	2016	RNA isolation, cDNA - qPCR	6 cell lines 10 control	NA	NA	Suitable markers: Cytokeratin 19, claudin 4
	Obermayr et al.	2010	Microarray technology	25 EC, 25 CxCa, 23 CaOv	NA	44% CxCa, 64 EC, CaOv 19%)	CCNE2, DKFZp762E1312, EMP2, MAL2, PPIC, and SLC6A8
	Zhang et al.	2016	Flow cytometry	78	NA	NA	TTF-1 positive CTCs correlated with TNM stating, vascular infiltration, lymphatic metastasis
	Alonso-Alconada et al.	2014	EpCAM based immunoisolation, RTqPCR	34	NA	NA	EMT markers ETV5, NOTCH1, SNAI1, TGFB1, ZEB1 and ZEB2
Cervical carcinoma	Pfitzner et al.	2014	Digital-Direct-RT-PCR	10	3	30%	1 local and 2 systemic diseases
	Takakura et al.	2017	Conditionally replicative adenovirus targeting telomerase-positive cells	23	6	26%	CTCs negative for cytokeratins
	Wen et al.	2018	Magnetic beads separation, CEP8+/ DAPI+/CD45-	99	45	45.9%	Combination of CTC and SCC-Ag a significant predictive marker
Ovarian carcinoma	Suh et al.	2017	TSF - physical deformability	87	49	56.3% (44.2% benign, 100% early stage, 66.7% advanced stage)	Better than other modalities in detecting early stage
	Chebouti et al.	2017	AdnaTest ovarian cancer	91	NA	Before surgery (18% Epithelial, 30% EMT-like), after CHT (14% and 52%)	Emerge of PI3Ka and Twist
	Kuhlmann et al.	2014	AdnaTest ovarian cancer	143	20	14%	ERCC-1 Positive CTCs predict platinum resistance
	Obermayr et al.	2013	Microarray analysis, RT-qPCR	200	49	24.5%	PPIC gene correlates with poor DFS, OS and platinum resistance
	Kolostova et al.	2015	Metacell	118	77	65.2%	MUC1, EPCAM, KRT18, KRT 19 overexpressed
	Obermayr et al.	2017	Multimarker immunostaining, FISH	102	27	26.5%	CTC positivity associated with higher risk of death after optimal surgery
	Marth et al.	2001	Microbead coated with MOC-31 antibody	90	11	12%	PFS nit associated with CTCs in peripheral blood or bone marrow
	Kolostova et al.	2016	MetaCell	56	32	58%	KRT7, WT1, EPCAM, MUC16, MUC1, KRT18 and KRT19 overexpressed
	Judson et al.	2003	Anti-cytokeratin 8, 18, TFS-2, CK-7, CK-20, EGFR mad MiniMACS	64	12	18.7%	CTC positive patients had more grade 3 tumors, follow up had no correlation
	Lee et al.	2017	Electronically conductive chip	54	54	98.1%	CTC positive cluster associated with diminished OS in recurent disease and chemoresistance
	Lou et al	2017	EpCAM+, CK+, DAPI+, CD45 negat	49 (35 malignancy)	9	18.4% (malignant grup)	Only 17.5% positivity in OC, 80% in non-ovarian origin - metastatic tumours to ovary

tumors such as prostate, colorectal and lung cancer [4]. The main limitation of CTC is the low quantity of cells in the blood of cancer patients. The quantity of cells detected differs widely also by the method of isolation.

The broad heterogeneity of CTCs in cancer patients may play a dominant role in therapy resistance and recurrence of disease [5]. Disseminated and CTCs may undergo a broad range of biochemical changes and reversibly acquire fibroblastoid or mesenchymal traits described as epithelial-to-mesenchymal-transition (EMT) already published for breast cancer [6]. This mechanism is a key for malignant progression and is referred to as Oncogenic EMT. This allows tumor cells to gain invasive properties, develop metastatic growth characteristics and defend them during dissemination. Metastatic cells can, after reaching the distant organ, change back to their original epithelial phenotype, mesenchymal-epithelial-transition (MET), to support colonization [7].

CTC detection and isolation: methods and devices

To fully been able to benefit from CTCs, high purity isolation of viable CTCs and their detection is necessary. Isolation is the process when CTCs are separated from all other cells in the sample, while detection is the direct or indirect identification of tumor cells. The enrichment process may precede, when the majority of blood cells are removed from the sample to enhance relative CTC concentration. The most common methods are density gradient centrifugation, red blood cell lysis, positive or negative immunomagnetic separation and sized-based filtration [8]. Based on their working principles, isolation and detection could be classified in three major categories.

1. Nucleic acid-based methods for CTC detection

This method directly or indirectly detects the presence of CTCs by identifying specific DNA or mRNA molecules in the sample. Specific primers are enrolled on polymerase chain reaction (PCR) to target DNA or mRNA molecules known to be associated with cancer cells. CTC detection using mRNA is more effective due to short period of presence in the circulation (unstable molecule with rapid degradation) which means capture of living CTCs, while free DNA could deliver false positive result by capturing molecules released by necrotic or apoptotic cancer cells circulating longer period [9]. Nowadays, multiplex reverse transcription followed by primer specific PCR is widely used, in which expression of multiple transcripts

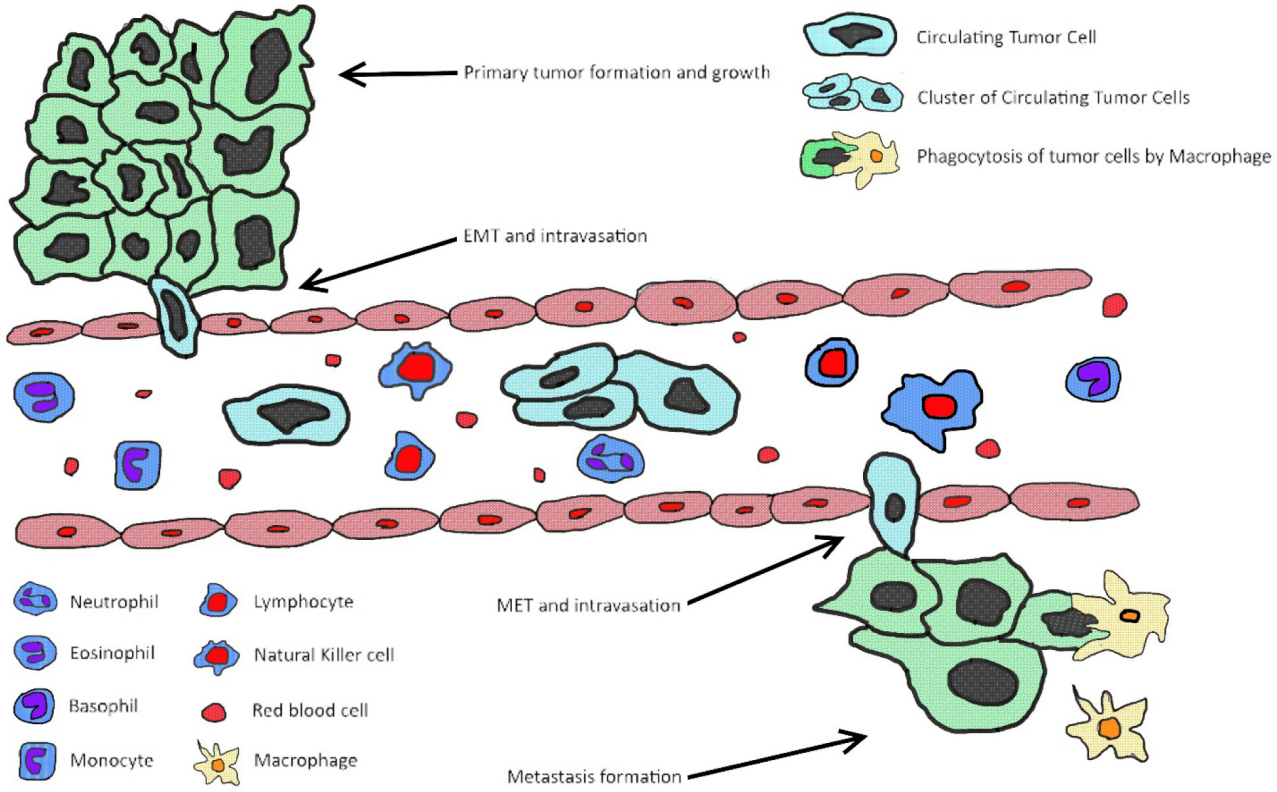

Figure 1. Presentation of potential metastasis: CTCs are shed from the primary tumor into the circulation via EMT process. After intravasation CTCs undergo MET and extravasation with metastasis formation.

could be measured providing improved sensitivity and specificity rated of heterogenic CTCs (commercially available AdnaTest kits – AdnaGen, Germany) [10]. Generally, thanks to the amplification principle of PCR, nucleic acid-based method could effectively pick out the signal from an extremely small amount of marker in a large sample (1 CTC in 5-10×10^6 nucleated blood cells or more than 5 mL of blood) thus offers the highest sensitivity for CTC detection [11]. The essential factor to reach this high sensitivity is the specificity of selected markers. Common markers of epithelial specific genes, such as cytokeratins or EpCAM, are widely used as they constitute malignancies and normally are absent in peripheral blood. Organ-specific markers, such as PSA, MUC1 or tumor specific markers, such as CAE, HER2, could help specify the correct cancer diagnosis [12]. The downside of this approach is possible false-positive result from tissue- and organ-specific markers originating from non-cancer cells that enter the bloodstream due to inflammation or invasive diagnostic biopsies [13]. Moreover, none of the recent markers used are entirely CTC-specific. The major drawback is the fact that CTCs must be lysed before the PCR process, making impossible for further analysis as observation or enumeration.

2. Physical properties-based methods for CTC isolation

These methods use the physical characteristics of cancer cells like density, size, mechanical plasticity and dielectric properties that could be used to isolate CTCs from samples.

a. Isolation of CTCs based on size and mechanical plasticity

This approach considers that cancer cells are larger than normal blood cell, thus it is selected throughout the filtration [14,15]. The simplest method is using track-edged filters or microfilters which are a porous membrane with 8 μm diameter holes that allow the blood cross but capture the bigger CTCs (ISET – Rarecells, France, ScreenCell systems – ScreenCell, France, MetaCell – MetaCell, Czech Republic). The advantage is that the captured cells remain intact allowing their subsequent morphological and molecular analysis [16-19]. This approach could be performed also in a microfluidic setting, where the separation results in a precisely defined topography of microstructures and the laminar flow in microchannels [20]. Advanced technology, such as CTChip by Clearbridge Biomedics, enables to isolate single CTCs with automatic vision-based enumeration and analysis. Methods using size-dependent hydrodynamic forces as formation of microscale vortices or Dean-coupled inertial migration has been also published [21,22], as well as active acoustophoresis technique that practices an external acoustic force to separate different cells in the microchannel [23]. The downside of this method is false-positive result in case of leucocytes capture, false negativity in case the cells become more plastic during EMT and altered functions of isolated CTCs due to mechanical stress during isolation [24-26].

b. Electrokinetic isolation of CTCs

Cells are electrically neutral, but in the electric field polarisable and electric dipoles moments are induced in them [27]. The magnitude and direction of these dipole moments depend on the polarity and conductivity of cell membrane and cytoplasm, cells phenotype, physiological state and morphology [28]. Factors affecting this method is the gradual change of dielectric characteristics due to ion leakage, thus the isolation should be completed as fast as possible. Unfortunately, the process is still relatively slow [29].

3. Antibody-based methods for CTC detection and isolation

The most common method for detection as well as isolation of CTCs. The principle is the antibody-antigen specific binding, mainly done by immunochemistry, but other techniques like Raman spectroscopy, photoacoustic flowmetry and nuclear magnetic resonance have been investigated [30-32]. CTCs are captured to the antibody-mediated matrix most often in a form of magnetic particles or microchannels. The performance of this method depends on the antigen it represents. For detection of CTCs most widely EpCAM and different subtypes of CK are used, while more organ- and tumour-specific markers, such as CEA, EGFR, PSA, HER2, MUC1 could be applied. Up to this date, no marker met the high specificity required for the ideal detection and isolation of CTCs.

a. Immunochemistry methods for CTC detection

Although still not achieving ideal performance in practice, it is considered the most reliable and specific method of CTC detection. CTCs are often referred as CK positive /DAPI positive/ CD45 negative cells [33]. While CD-45 negativity rules out white blood cells, DAPI excludes cell fragments and debris. Flow cytometry, including fluorescence-activated cell sorting (FACS) and the more popular image cytometry mainly referring immunofluorescence microscopy is used in this method. The lat-

ter could incorporate several markers and different molecular (FISH) or cytomorphological (N/C ratio) assays which improve the specificity of detection and integrate automated digital microscopy and computerized post-processing for better practical use [34]. CellSearch system (Menarini, Italy) is the only FDA approved assay for CTC detection. About 99% detection sensitivity was reached by the HD-CTC array, which without the enrichment process, could detect CTC aggregates with high clinical significance in micrometastasis development as well [35-36]. Living CTCs for prognostic significance for a variety of carcinomas could be detected by a novel approach called EPISPOT [37].

b. Immunomagnetic methods of CTC isolation

Magnetic field can be successfully used to isolate CTCs if their magnetic characteristics are selectively modified. Cancer cells can be tagged by antibody-conjugated magnetic microbeads or nanoparticles that bind to a specific surface antigen [38]. In a non-uniform magnetic field the tagged cells migrate towards areas of higher magnetic flux density where they are captured [39].

c. Adhesion-based methods for CTC isolation

This method focuses on an adhesion surface, whose biochemical and topographical properties have been modified to attract and capture cancer cells. This can be performed in static or in continuous-flow microfluidic modes [40]. In the first mentioned, the sample is left incubated on a collagen-coated surface. During incubation CTCs with invasive characteristics tend to invade the surface and are captured, while the rest non-target cells are washed off [41]. The second is achieved by flowing the sample through a straight microchannel coated with antibody against CTCs so the target cells can effectively interact with the capture surface [42].

Endometrial cancer

Cancer of the corpus uteri (EC) is the 7th most commonly diagnosed cancer in female population worldwide with 382,100 estimated new cases and 89,900 deaths in 2018 [43]. In the developed countries it represents the fourth leading cancer in women and the most common malignancy of the female genital tract. In the United States 63,230 new cases and 11,350 deaths were estimated in 2017 [44]. In Europe, the number of new cases was about 100,000 with an incidence of 13.6 per 100,000 in 2012 [45].

Despite the absence of a reliable screening tool, EC is most often diagnosed in early stage because of symptomatic postmenopausal uterine bleeding. Hematogeneous spread is in correlation with deep myometrial invasion [46]. Surgery is the primary treatment method, in addition with adjuvant radiotherapy and chemotherapy in advanced and high-risk cases.

The largest study was published by Kiss et al, in which blood from 92 patients with various grades and stages of EC was isolated for CTCs. Positivity reached 75% of patients and a method described a successful size-based separation method with high detection rate of viable CTCs with proliferation potential (Metacell®). In addition, there was no significant difference between CTC presence and differentiation level (grade), stage of disease and lymph node involvement [47].

Other studies involved rather a smaller number of high-risk EC patients with EpCAM-positive CTCs isolated by CellSearch. Bogani et al isolated CTCs in 2 EC patients from 28 (7% positivity). Both patients were in stage IIIC and CTCs presence was significantly correlated with myometrial invasion and lymph node positivity [48]. Association in CTCs and cervical involvement was published by Ni et al. From 40 EC patients 6 were positive for CTCs (15% positivity), whereas 3 patients had FIGO stage I and 3 patients had stage III with no significant difference in the quantity of cells. Also, no significant correlation was found between CTCs and serum CA125/human epididymis protein 4 (HE4) levels. One patient with type II stage I had repeated CTC examination after the first dose of adjuvant therapy [49]. Another study was provided by Lemech et al in which demonstrated 18 CTC positive EC patients from 30 (60% positivity). CTC correlated with higher stage disease, worse survival, non-endometroid histology over endometroid and tumour size bigger than 5 cm. In addition, CTCs and FFPE tissue blocks were placed for immunohistochemistry staining of EpCAM and stathmin primary antibodies and put in correlation with CTC status. Stathmin was overexpressed in all CTC-positive patients whose tissue was stained (7 patients). This could mean that stathmin has potential as a marker of PIK3K pathway activity which is one of the most studied pathways in EC with aberrations including oncogenic PIK3CA mutations and PTEN loss of function [50].

Further studies observed the presenting genes in CTCs in patients with high-risk EC. Due to the high expression in the investigated cell lines, Cytokeratin 19 and claudin 4 were identified as a suitable gene marker for CTCs in endometrial adenocarcinoma [51]. Obermayr et al conducted a multimarker analysis of six genes (CCNE2, DKFZp762E1312, EMP2, MAL2, PPIC and SLC6A8)

which were positively identified in 64% in a group of 25 EC patients [52]. The expression of thyroid transcription factor (TTF-1) in CTCs was strongly correlated with TNM staging, vascular infiltration and lymphatic spread. Progression-free survival rate and median survival time decreased in the TTF-1 positive cohort, while recurrence rate was significantly lower in the negative group [53]. Finally, Alonso-Alconada et al described the association of molecular CTC-phenotype with plasticity, stemness and epithelial-to-mesenchymal transition (EMT) features which promotes CTC dissemination. Markers of EMT show higher expression in ETV5, NOTCH1, SNAI1, TGFB1, ZEB1 and ZEB2. Expression of ALDH and CD44 pointed to an association with stemness, while the expression of CTNNB1, STS, GDF15, RELA, RUNX1, BRAF and PIK3CA suggests potential therapeutic targets. The significance to clinical practice could be the identification of patients in need of additional systemic therapies after primary surgery to avoid metastasis and to eliminate the risk of recurrence in the future [54].

Cervical cancer

According to a recently published study by the GLOBOCAN, cervical cancer (CxCa) is the third most common cancer after breast and lung cancer worldwide and is also third in cancer-related deaths in female population [43]. Cervical cancer is the most frequently diagnosed cancer in more than half of the countries in Africa and accounting for about 30% of total cancer cases and deaths in the region [55]. In the USA, an estimated 13,240 cases of invasive cervical cancer are expected to be diagnosed with 4,170 deaths in 2018 [44]. In the European Union, there were about 34,000 new cases of cervical cancer and more than 13,000 deaths in 2012 [56].

The etiology of cervical cancer is the infection of cell by Human Papilloma Virus (HPV) and belongs to the so-called virus-induced cancers [57]. The cancers express viral oncogene transcripts specific for infected cells [58]. Over 99% of CxCa are high-risk HPV positive, while the oncogenic properties are mediated by the viral oncogenes E6 and E7 which are responsible for the inactivation of p53 and pRb tumour suppressor proteins [59,60]. The tumour is active only if E6 and E7 are expressed, otherwise cancer cells apoptosis is initiated by the restored p53 and pRb proteins [60]. Therefore, viral oncogene transcripts E6/E7 are the ideal markers for the detection of tumour cells in cancer patients. On this basis it was established a method by Pfitzner et al for detection and quantification of CTCs by digital RT-PCR [61]. She describes a CTC detection rate of 66% in patients witch systemic spread and the Digital-Direct-RT-PCR method as a highly sensitive method in separating HPV16/18-E6 expressing cells from a large number of HPV negative cells. This method could be applied in other tumour types where tumour specific transcripts are already discovered.

The presence of the integrated HPV virus in cervical cancer lesions alongside with cancer cell characteristics could be used in additional methods. Telomerase activity is responsible for the restoration of chromosomes length after cell division, which gives the cancer cells their immortality and its expression could be used as a potential biomarker [62]. The expression of hTERT has been identified as a determinant of telomerase activity and is transcriptionally regulated by its promoter [63,64]. Telomerase-specific replication-selective adenoviruses were designed from adenovirus vectors by inserting the hTERT promoter, restricting their proliferation to telomerase activity only, thus could be used in both *in vivo* and *in vitro* cancer cell detection and even in oncolytic virotherapy [65-67]. Takura et al used a modified adenoviral vector OBP-1101 which expresses GFP in infected cells. CTCs were identified in 6 of 23 samples (26% positivity), with no correlation with distant metastasis, overall survival or progression-free survival [68].

On the other hand, Wen et al published that elevated CTCs and SCC-Ag levels were associated with poor disease-free survival. They collected blood samples from 99 patients with locally advanced cervical cancer (FIGO stage IIB-IVA) and CTC were enriched and magnetically separated by anti-CD45 monoclonal antibody coated in magnetic beads and identified by negative enrichment and immune fluorescence *in situ* hybridization (Neim-FISH). The CTC-positive rate was 45.5% and CTC and SCC-Ag alone showed as strong predictors of DFS. The combination of these 2 biomarkers in a new risk model significantly improved their predictive efficiency for survival than CTC or SCC-Ag level alone [69].

Ovarian cancer

Ovarian cancer (OC) is the deadliest gynecological malignancy, with a 5-year survival rate approximately 47% - a number which remained constant over the past two decades. It is the fifth leading cause of cancer death among women in Europe and the United States and the second most common gynecological malignancy [70]. The annual estimates are 295,400 of new ovarian carcinoma cases and 184,800 deaths worldwide [43]. The

highest rates (11.4 per 100,000 and 6.0 per 100,000, respectively) are reported in Eastern and Central Europe [71]. Although China has a relatively low incidence rate of 4.1 per 100,000 due to its large population, the overall estimates are 52,100 new cases and 22,500 related deaths in 2015 [72]. The same year 21,290 new cases and 14,180 were estimated in the USA [73].

Early diagnosis improves survival, but unfortunately only 15% of ovarian cancers are diagnosed at an early localized stage. Most ovarian cancers are epithelial in origin and treatment prioritizes cytoreductive surgery followed by cytotoxic platinum and taxane chemotherapy. While most tumours initially respond to treatment, unfortunately recurrence is likely to occur within a median of 16 months in advanced-stage disease [74]. Postoperative residual tumour is one of the most important prognostic factors in advanced ovarian cancer [75]. Despite new therapeutic concepts are being used as antiangiogenic therapy or PARP inhibitors, more than half of all patients experience recurrence resulting in poor overall prognosis [76].

There are many studies evaluating the possible prognostic significance of CTCs in OC. Despite the early studies in which detection of tumour cells in the bone marrow and/or blood was not associated with poor prognosis [77], just CTC-positive patients had statistically more grade 3 tumours [78], and later studies proved their profitable use. In a large systematic review conducted by Cui et al on 10 relevant studies with 1164 patients showed a strong association of CTCs (disseminated tumour cells as well) with advanced staging (stage III-IV), poor prognosis (low OS, shortened PFS, DFS), and treatment response (platinum resistance). On the other hand, no association was found with tumor histology, lymph node metastasis and optimal or suboptimal surgery [79]. In a novel electronically conductive and nanoroughened microfluidic platform-based chip was introduced by Lee et al with 98.1% detection rate of CTCs in 54 OC participants. Additionally, reduced OS in patients with recurrent disease and chemoresistance correlated with CTC-cluster positive samples [80]. High detection rate of CTCs (90%) was published by Zhang et al, when from 109 newly diagnosed OC 98 were CTC-positive. The number of CTCs was significantly lower in stage I patients than in advanced stages. High diagnostic significance could be a 100% detection rate in 7 "occult" patients without epithelial ovarian carcinoma symptoms, while CA-125 was elevated only in 4 patients (57%). Elevated expression of EpCAM and HER2 in CTCs were associated with chemoresistance and shorter overall survival [81].

Not only OC is often diagnosed in later stages, but preoperative differential diagnosis of existing adnexal masses is also a challenge. Many studies have examined various modalities (biomarkers like CA-125 and HE4 levels, imaging studies like ultrasound, CT, MRI, PET and their combinations), while Suh et al studied CTCs as a new platform in the evaluation of findings on the ovaries [82]. From a total number of 87 patients, at least one CTC was found preoperatively in 49 (56.3%): 19/43 (44.2%) were benign, 10/10 (100%) early-stage and 14/21 (66.7%) advanced-stage cancer. Only 1 healthy control from 22 (4.5%) was positive for CTCs. In further analysis, preoperative CTC detection was more sensitive in benign vs. early stage (stage I and II) cancer compared with benign vs all-stage cancer and remained even in benign vs stage I cancer. Other diagnostic modalities showed a reversed pattern: modest performance in early-stage cancer and significant in all-stage cancer including borderline tumours. CTCs showed no association with CA-125 levels or ROMA index and could reflect early hematogeneous metastasis before even peritoneal spread. Another study assessed CTCs in 49 women with newly diagnosed complex pelvic masses. No CTCs were found in benign histology cases (0/14) while malignancy was associated with CTCs in 9/35 (25.7%). CTCs were detected only in 5/29 (17.2%) patients diagnosed with OC (all 5 patients had stage III or IV), and of the rest 5 patients 4 were CTC-positive (80%) and diagnosed with non-ovarian origin tumor that metastasized to the ovaries (2 Krukenberg tumour, 1 metastatic endometrial cancer, 1 abdominal soft-tissue sarcoma with peritoneal carcinomatosis) [83].

Another potential benefit of CTCs is that they could have a role in indicating invisible cancer lesions after complete or minimal-residual cytoreductive operations. CTCs present before surgery or neoadjuvant chemotherapy indicate a higher risk of death even after optimal debulking surgery (R0) [84].

In the majority of human malignancies PIK3K/AKT/mTOR signalling pathway is aberrantly activated stimulating proliferation and cell survival [85]. This pathway has also been reported in OC, while EMT is responsible for chemoresistance [86]. Chebouti et al analysed the incidence of epithelial (EpCAM, MUC-1) and EMT-like (PI3Ka, AKT-2, Twist) CTC at primary diagnosis of ovarian cancer (91 patients) and how their detection was altered by platinum-based chemotherapy. Higher number of EMT-like CTCs (30%) were detected than epithelial subtype of CTCs (18%) prior to surgery, which further increased in EMT-like CTCs even after chemotherapy (52%), but decreased in the epithelial subtype of CTCs (14%). Epithelial and EMT-like

CTCs exhibit a low phenotypic overlap as only a minor fraction of CTC-positive patients showed dual positivity for both phenotypes (18% before surgery and 12% after surgery). After chemotherapy a shift towards PIK3Ka and Twist expression was found, which could have a clinical interest as these signalling pathways could be responsible for the recurrence of OC [87].

Further studies of CTC characteristics showed that the presence of ERCC1-positive CTCs at primary diagnosis is and independent predictor of platinum resistance [88]. Auxiliary assessment of ERCC1 transcripts increase the CTC detection rate and presence of ERCC1-positive CTCs reduce progression-free survival and overall survival, while their persistence indicates poor post-therapeutic outcome [89].

Many authors published articles on molecular characterization of CTCs in OC patients. One of the largest studies was conducted by Obermayr et al, in which 11 gene markers (PPIC, GPX8, CDH3, TUSC3, COL3A1, LAMB1, MAM, ESRP2, AGR2, BAIAP2L1, TFF1, EPCAM) were studied in a cohort of 200 patients before therapy (surgery or neoadjuvant chemotherapy) and during follow-up. PPIC gene (Cyclophilin C) was overexpressed in 34 cases (17%) and PPIC positivity during follow up period (13 cases 14% positivity) showed significantly shorter disease-free survival, overall survival and platinum resistance [90]. Another large study of 118 OC patients was conducted by Kolostova et al, successful isolation of CTCs in 77 patients showed 65.2% positivity, from which further 20 patients were tested for gene expression. CTCs overexpressed MUC1 and EPCAM in more than 90% cases, KRT18 and KRT19 was also elevated, while MUC16 (CA125) was detected only in 30% [91]. In another study from the same authors 40 patients with OC were enrolled in a gene expression study. Statistically significant difference was confirmed for the following genes ($p<0.02$): KRT7, WT1, EPCAM, MUC16, MUC1, KRT18 and KRT19. The results suggest that the combination of the above listed genes could confirm CTCs presence in OC patients with higher specificity than when gene analysis tests are performed for one marker only [92].

Concluding remarks

Cancer cells in gynaecological malignancies are present in the circulation of patients and can be isolated and detected by numerous methods. The presence of CTCs seems to be associated by adverse clinicopathological features and worse

clinical outcomes. CTCs have their prognostic value and in times of personal medicine could help in therapy management and its effectiveness control. Recurrences could be detected earlier and reacted more precisely to them.

Conflict of interests

The authors declare no conflict of interests.

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Correlation Between Disease Stage and the Presence of Viable Circulating Tumor Cells in Endometrial Cancer

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Abstract. *Background/Aim: The presence of circulating tumor cells (CTCs) in the peripheral blood of patients with solid tumors is associated with a poor prognosis. However, there are limited data concerning the detection of CTCs in endometrial cancer (EC). The aim of this study was to evaluate the presence of CTCs in the peripheral blood of patients with EC. Materials and Methods: Peripheral blood samples from 92 patients who underwent a surgical procedure were evaluated using MetaCell® separation technology for CTCs. Results: CTCs were detected in 69 (75%) patients with EC. Conclusion: CTCs were detected in a higher percentage of patients than in other studies. The results showed that the technology applied in this study can efficiently capture viable tumor cells in the blood that can be cultured while maintaining their original phenotype. This paper discusses the first successful culturing of human circulating endometrial cancer cells for further downstream functional and molecular characterization.*

Endometrial cancer (EC), ovarian cancer and cervical carcinoma are the most common gynecological cancers. EC is a treatable cancer with a good prognosis because in 75% of women the disease is confined to the uterus, while women with metastatic disease have between 7 and 12 months median survival (1). Therefore, EC requires a more effective individualized therapy at a cellular and molecular level.

One way to better understand the invasion and metastatic process in cancer is to isolate and analyze circulating tumor cells (CTCs). CTCs are tumor cells present in the circulatory system of patients with solid tumors. Detection and quantification of CTCs provide additional information on the stage of cancer and response to therapy. CTCs' molecular characterization offers a possibility to control better the metastatic process.

The presence of CTCs in the blood of patients and their clinical correlation has been described in various cancers (2-5). Regarding gynecological cancers, most reports on CTC research are related to ovarian cancer patients (6). Currently, there is scarcity of information about CTCs in patients with EC. The aim of this study, was to capture viable CTCs, culture them *in vitro* and compare the ability of captured cells to grow *in vitro* at different disease stages.

Materials and Methods

Patients. A total of 92 patients diagnosed with EC have been enrolled in the study in accordance with the Declaration of Helsinki. The study was approved by the Ethical Committee of Wroclaw Medical University, Wrocław, Poland (EK 800/2012). All patients signed the consent to be enrolled in the study. All patients were candidates for surgery treatment. Based on their informed consent, clinical data were collected from all participating patients. The patient characteristics are shown in Table I and Figures 1-3. For each patient, approximately

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Key Words: CTCs, endometrial cancer, circulating tumor cells, culturing.

Table I. *Patient characteristics*.

	n	CTC+	CTC-	%	*p*-Value
Total number of patients	92	69	23	75	
Histology					
Endometrial adenocarcinoma	81	65	16	80	
Papillary serous adenocarcinoma	9	5	4	56	
Endometrial stromal sarcoma	1	0	1	0	
Clear cell adenocarcinoma	1	1	0	100	
Grade					
Grade 1	29	23	6	79	0.648
Grade 2	46	33	13	72	
Grade 3	16	13	3	81	
FIGO					
IA	49	37	12	76	0.966
IB	22	15	7	68	
II	13	10	3	77	
IIIA	1	1	0	100	
IIIC1	3	3	0	100	
IIIC2	3	2	1	67	
Further staging					
Lymph node involvment YES	6	5	1	83	0.616
Lymph node involvment NO	85	63	22	74	
Peritoneal carcinomatosis	1	1	0	100	N/A
Ascites	0	0	0	N/A	N/A
Residual disease	0	0	0	N/A	N/A

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

CTCs enrichment and culture. A size-based separation method for viable CTC-enrichment from peripheral blood was used (MetaCell®, MetaCell s.r.o., Ostrava, Czech Republic) (7-9). The size-based enrichment process is based on the filtration of peripheral blood through a porous polycarbonate membrane (pores of 8 μM in diameter). The standard 8 ml of peripheral blood from patients suffering with EC was transferred to the filtration tube. The peripheral blood flow is supported by capillary action of the absorbent touching the membrane filter. The captured CTCs were observed immediately after filtration on the membrane. CTC presence was controlled immediately after isolation steps to avoid false negative results of examination. The membrane filter, which is kept in a plastic ring, was transferred directly into a 6-well culture plate and 4 ml RPMI media containing 10% FBS was added to the membrane top and CTCs were cultured on the membrane *in vitro* under standard cell culture conditions (37˚C, 5% CO_2 atmosphere) for a period of minimum 14 days on the membrane. The cultured cells were analyzed by histochemistry (May-Grünwald staining) and unspecific DAPI staining (Sigma, Munich, Germany) (Figure 4). Alternatively, the enriched CTC fraction was transferred from the membrane and cultured directly on any plastic surface or microscopic slide. Microscopic slide culture was preferred if immunohistochemistry/immunofluorescence analysis was planned. If an intermediate CTC analysis was awaited, the CTC fraction was transferred in PBS (1.5 ml) to a cytospin slide. The slide was then dried for 24 h and analyzed immunohistochemically.

Cytomorphological analysis. The cells fixed and stained on the membrane were examined using light microscopy in two steps: (i) screening at ×20 magnification to locate the cells and (ii) observation at ×40 magnification for detailed cytomorphological analysis. Isolated cells and/or clusters of cells of interest (immunostained or not) were selected, digitized and examined by an experienced researcher and/or pathologist. CTCs were defined as cells presenting the following characteristics: (i) cell size equal or larger than 15 μm; (ii) nuclear size equal to or larger than 10 μm); (iii) irregularity of the nuclear contour; (iv) presence of a visible cytoplasm; (v) prominent nucleoli; (vi) high nuclear-cytoplasmic ratio; (vii) cluster presence; (viii) mitosis presence.

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

Results

In our study, 92 patients with EC were examined for circulating tumor cells (CTCs). Patient characteristics are presented in Table I. CTCs were successfully isolated in 69 out of 92 patients (75% positivity).

Here, a successful isolation method of CTCs with proliferation potential in patients with EC is described. The cells captured by a size-based filtration method showed a viable character. The viability of CTCs was only minimally

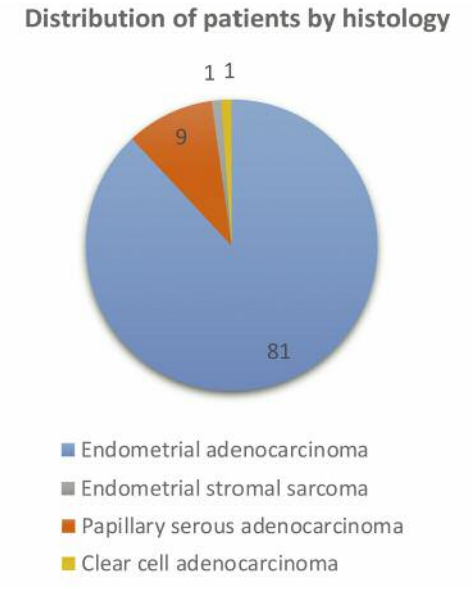

Figure 1. *Distribution of patients by histology.*

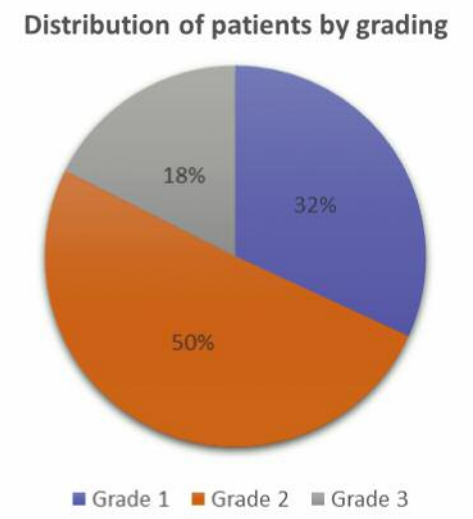

Figure 2. *Distribution of patients by grading.*

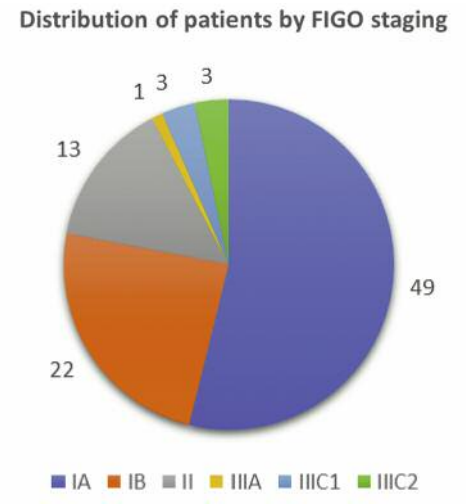

Figure 3. *Distribution of patients by FIGO stating.*

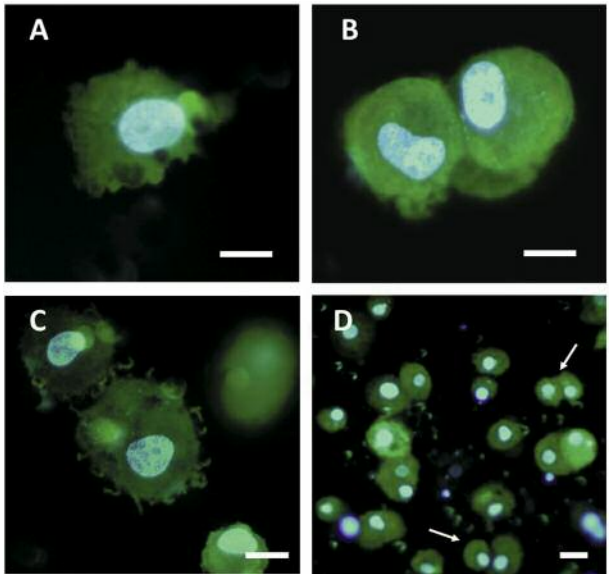

Figure 4. *Isolated CTCs of endometrial cancer visualized by vital fluorescent staining. The arrows indicate mitosis presence. Bars represent 10 microns.*

affected by the isolation procedure and completely unaffected by the used cultivation media or bounded antibodies. CTCs were cultured *in vitro* and were grown *in vitro* in several cases for as long as 6 months as a standard cell culture (Figure 4).

The distribution of EC histological subtypes and CTC detection is shown in Table I and Figures 5-7. The majority of patients (n=81) had endometrial adenocarcinoma with 80% CTC positivity, whereas the group of patients with papillary serous adenocarcinoma (n=9) had only 56% positivity. No CTCs were found in the sample with endometrial stromal carcinoma (n=1), CTCs were detected in the peripheral blood of a patient with clear cell adenocarcinoma (n=1). Characterization by disease grade and CTC detection is shown in Figure 6. There was no significant difference between CTC presence and differentiation level (grade) of the cancer (p=0.648). The stratification of patients into stages by FIGO is presented in Figure 7. There is no correlation between the stage of the disease and CTC positivity (p=0.966) or lymph node involvement and CTC detection (p=0.616). Captured CTCs grow independently of disease stage. All these results

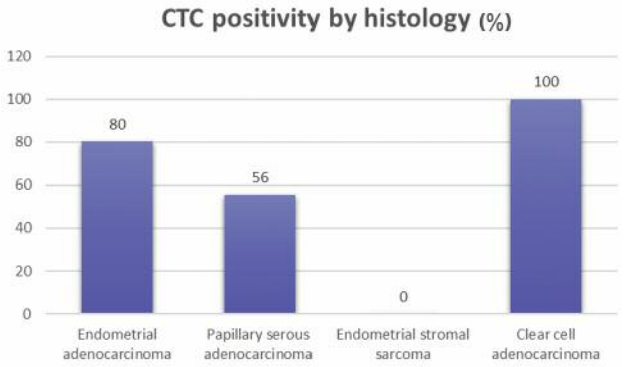

Figure 5. *CTC positivity by histology.*

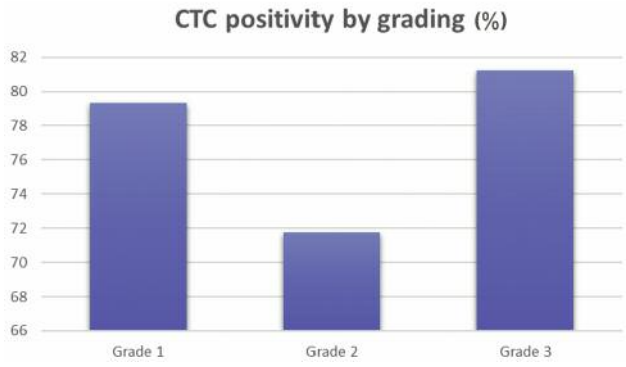

Figure 6. *CTC positivity by grading.*

indicate that the detection of CTCs in the peripheral blood is independent of the grade and stage of the cancer and of lymph node involvement.

Discussion

The aim of this study was to isolate viable CTCs from the peripheral blood of patients with EC, culture them *in vitro* and compare the cultured cells with disease stage. Only limited data are presented regarding the presence of CTCs or disseminated tumor cells (DTCs) in EC patients. DTCs in the bone marrow of patients were observed using immunocytochemistry in 17% of patients with EC (10). RT-PCR method was used in the first studies describing detection of CTCs in EC patients (11, 12). RT-PCR was used to determine the expression of cytokeratins (CK), namely CK-19 or CK-20 in the peripheral blood. The results were very ambiguous and ranged from 10 to 51% positive patients.

Overall, 28 patients were included in the study that evaluated CTC presence in patients with high-risk endometrial cancer (13). Two of 28 (7%) patients were positive for CTCs. The presence of positive CTCs was significantly associated with myometrial invasion and lymph node positivity. Only patients with endometrioid histology had positive CTCs. CTC detection rate was very low in this study. The authors used CellSearch technology which is dependent on the expression of epithelial cell adhesion molecule (EpCAM) by CTCs. It seems that using methods that are dependent on EpCAM can result in false negative detection of CTCs (14). This aspect was confirmed in another study which again used the CellSearch method (15). This study included 40 patients and only 15 percent of patients had one or more CTCs. EpCAM and cytokeratins are widely expressed in most epithelial malignancies but CTCs undergo epithelial-mesenchymal transition and during this process they lose expression of specific epithelial markers such as EpCAM (16).

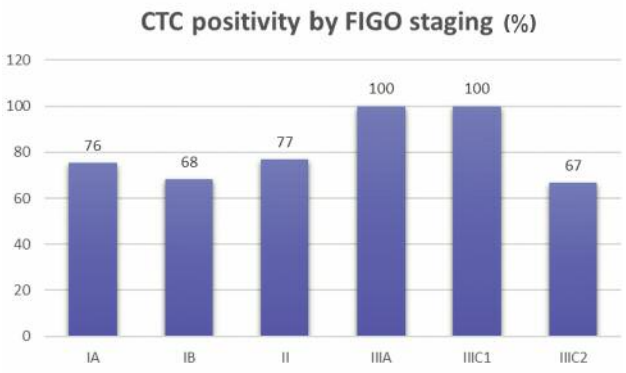

Figure 7. *CTC positivity by FIGO stating.*

In the reported study, a size-based method for separation of CTCs was used which is very efficient in detecting malignant cells with down-regulated epithelial markers. In the antigen-independent mode, the system can isolate CTCs from cancers that have lost or never had the epithelial marker characteristics. So, the fact that MetaCell® technology is independent of specific epithelial markers is the main reason for a higher detection of positive CTCs than that reported in the previous studies mentioned above.

Moreover, since the tumor cells are gently captured on the membrane, they may be used for further analysis at the cellular or molecular level. Separation of viable and intact CTCs provides a possibility of morphological investigation in addition to immunohistochemistry and RNA and/or DNA based PCR. We also believe that successful CTC cultures *in vitro* will provide important and necessary insight into the metastatic process.

Interesting results were obtained by comparing the presence of CTCs in patients who were subjected only to surgery and those who were given adjuvant radiotherapy. In the latter group the elimination of CTCs was significantly

higher, but the patients had already undergone surgery which means that the primary site of cancer that disseminated cancer cells to the circulatory system had already been removed. Regarding the surgery group, the blood for examination was drawn before a radical surgery. We suggest that surgical eradication in EC significantly reduces the number of CTCs, which may be useful for follow-up since recurrence of CTCs might indicate cancer recurrence.

More information will surely be obtained in the future by correlating the presence of CTCs and disease-free survival (DFS) and 5-year overall survival (5-y-OS).

However, more studies concerning the role of CTCs in endometrial cancer patients are needed before this method is introduced into everyday clinical practice.

In conclusion, CTCs were present in 75% of patients with endometrial cancer. The high percentage of CTC positivity in the peripheral blood of patients with well differentiated tumors and early-stage carcinomas shows the metastatic potential of the disease. Moreover, CTCs show no correlation with the grade, stage or lymph node involvement and thus could be used as an independent diagnostic and treatment effectivity marker. The results show that the technology applied in this study can efficiently capture viable tumor cells from the blood which can then be cultured while maintaining the original phenotype. Viable CTCs can be efficiently isolated and *in vitro* cultures of endometrial cancer can be successfully established for downstream functional and molecular characterization.

Conflicts of Interest

VB and KK are inventors of the patent EP3008162B1 for the device used in this study for CTC separation.

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Circulating Endometrial Cells: A New Source of Information on Endometriosis Dynamics

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Abstract: The focus of the presented work was to isolate and characterize circulating endometrial cells (CECs) enriched from peripheral blood (PB) of patients with diagnosed endometriosis. The molecular characteristics of CECs could be supportive for an understanding of endometriosis pathogenesis and treatment decisions in the future. Material and Methods: Blood samples (n = 423) were tested for CECs presence. Subsequently, gene expression analysis (GEA) was carried out for CECs. In parallel, CECs presence and characteristics were tested during menstrual cycle (MC) phases in 11 patients. CECs were enriched by size-based separation. Results: CECs were present in 78.4% of the tested blood samples. In line with the revised American Fertility Society (rAFS) classification, CECs presence was confirmed in all the acknowledged endometriosis stages: minimal, mild, moderate, and severe. Surprisingly, CECs negativity rate was also reported for severe disease in 21.1% of cases. The CECs captured during MC phases displayed different cytomorphology, including epithelial, stromal, and stem cell-like characteristics. The highest CECs numbers were detected in the mid-secretory phase of MC, which corresponds to uterine lining decidualization. CECs captured during mid-secretory periods expressed genes *KRT18*, *NANOG*, and *VIM* in higher amounts when compared to the proliferative phase of MC, where genes *KRT19* and *ESR1* were mostly elevated. GEA of the super-positive CECs samples (1000 CECs/8 mL PB) revealed high expression of genes *KRT18*, *VIM*, *NANOG*, and *FLT1*. The expression of these genes was also elevated in the endometriosis tissue samples and endometrioma. Conclusion: The panel of the identified CEC genes could be tested in a prospective manner to confirm the role of CECs in endometriosis pathogenesis and diagnostics.

Keywords: circulating endometrial cells; endometriosis; rare cells; menstrual cycle; liquid biopsy

1. Introduction

Endometriosis is a common disease among women of reproductive age and a major contributor to pelvic pain and subfertility causing disability and significantly compromised quality of life [1]. It affects up to 10% of women of reproductive age, 50–60% of women and two-thirds of teenage girls with pelvic pain and dysmenorrhea, and up to 50% of women with infertility [2,3]. Because of its wide and non-specific clinical symptoms and difficult diagnosis, endometriosis is frequently underdiagnosed or diagnosed in later, more severe stages [4]. Diagnostic lead marks could be put together from the patient's history, gynecological examination containing ultrasound, and a few specific laboratory markers such as CA125 (cancer antigen 125, known as MUC16). Until now, there is no biomarker from the endometrium, blood, or urine or combined non-invasive tests specific enough to be used in clinical practice. Therefore, laparoscopy remains the gold standard for the diagnosis of endometriosis, and using non-invasive tests should only be undertaken in a research setting [1,5–8].

As the etiology and pathophysiology of endometriosis is still not fully understood, and more theories are being studied, it is challenging to discover a highly specific and sensitive preoperative diagnostic tool. Furthermore, it is necessary to validate the diagnostic accuracy of every promising test prospectively in an independent symptomatic patient population with subfertility and/or pain without clear ultrasound evidence of endometriosis and with a clinical indication for surgery, divided into cases with laparoscopically and histologically confirmed endometriosis and controls with laparoscopically confirmed absence of endometriosis [9].

To ensure a full understanding of the hypothesis of circulating endometrial cells (CECs), the lymphovascular spread (also called embolization, metastasis, transplantation) theory must be introduced first. It was first published by Halban in 1925 [10], who detected endometrial cells in the lymphatic system of the uterus in patients with endometriosis. Meanwhile, Sampson studied the volume and shape of the uterine cavity in normal and pathologic conditions. When injecting the uterus with a suspension, he found the injected mass to escape from uterine veins, which led him to believe that the endometrial cells would enter circulation in the same way [11]. This theory was further studied in an experiment in 1940, when Hobbs and Bortnick injected endometrial cells into the circulation of rabbits. He found endometrial lesions in the lungs and pleura of these animals later during dissection [12]. In 1952, Javert followed up on Sampson's work and detected endometrial cells in the pelvic veins of patients with endometriosis [13].

More than half a century later, CECs were described by Bobek et al. [14] in accordance with the same vascular spread theory. Endometrial cells from peripheral blood (PB) and peritoneal washings (PW) in patients with endometriosis were successfully isolated by a size-based separation method (Metacell®). The endometrial origin of the captured cells was proven by immunohistochemistry [14]. Later, Chan et al. used immunofluorescence staining and separation via microfluidic chips for CEC detection. The results indicated that CECs could be a promising biomarker with great potential in the diagnosis of endometriosis [15].

The aim of this study was to isolate CECs in patients with different types of endometriosis and clinical symptoms and to characterize these cells by molecular analysis. In agreement with the innovative stem cell-based concept of endometriosis origin [16], CECs molecular analysis might fulfil the puzzle of endometriosis pathogenesis.

The results of CECs cytomorphological analysis and gene expression profiling were correlated with patients' clinical data. Further analysis was conducted in patients with multiple sampling throughout the menstrual cycle (MC) to understand the characteristics of CECs in each phase of MC. New information on the possible pathophysiology and development of endometriosis was brought

through comparisons of molecular profiles of endometrial lesions obtained during gynecological surgeries and CECs.

2. Material and Methods

A multi-center prospective study was initiated to collect blood samples from women with endometriosis. The inclusion criterion was that all the patients had a histologically proven history of endometriosis. A form was filled for each patient containing her detailed data, i.e., information about the menstruation cycle, hormonal therapy if any, type of endometriosis (ovarian, peritoneal, recto-vaginal septum, adenomyosis, extragenital), classification by revised American Fertility Society (rAFS), symptoms, and signs (pelvic pain, dysmenorrhea, dyspareunia, metrorrhagia, hypermenorrhea, sterility, infertility, gastrointestinal problems). A supplementary table reporting the clinical data is available. Samples were obtained from 423 patients. The protocol for this study was approved by the Ethical Committee of University hospital Kralovske Vinohrady in Prague, Czech Republic (EK-VP/20/0/2015) and the Ethical Committee of Medical University Wroclaw, Poland (Nr.KB-242/2015 part of study and grant SUB.C280.19.050).

As the samples were collected from various centers nationally and internationally, a possible bias in results due to different transport conditions was considered. The sample was marked CEC-positive if endometrial-like cells were detected. Subsequently, we divided the positive samples into categories based on CEC quantity (low positivity—up to 10 cells, medium positivity—up to 100 cells, high positivity—more than 100 cells). Samples with high positivity (n = 13) were subjected to molecular analysis. To be able to analyze the molecular character of CECs during the MC, we obtained multiple samples from 11 patients during their menstrual cycle (2 × 48 samples in total). A minimum of four samples were taken for every patient to correspond to different phases of the cycle (menstruation, proliferative phase, ovulation, secretory phase). The phase was calculated from the last menstrual bleeding and verified by ultrasound examination of the endometrium.

To enrich CECs, approximately 2 × 8 mL of PB was drawn from the antecubital veins and placed into S-Monovette tubes (Sarstedt AG & Co., Numbrecht, Germany) containing 1.6 mg EDTA/mL blood as an anticoagulant. The samples were processed at room temperature using an isolation procedure completed within 36 h of the blood draw. The ethics committees of the participating universities and hospitals approved the study protocol according to the Declaration of Helsinki. Size- based filtration and an in vitro culture method (MetaCell®, Ostrava, Czech Republic) were used to enrich CECs. [14]. The captured cells grew in the fetal bovine serum -enriched RPMI medium (10%) (Merck KGaD, Darmstadt, Germany) for the period of a minimum of 7–14 days on the separation membrane. The cultured cells were analyzed by vital fluorescent microscopy using unspecific nuclear (NucBlue™, ThermoFisherScientific, Waltham, U.S.) and cytoplasmatic (Celltracker™, ThermoFisherScientific, Waltham, U.S.) staining. Cells on the membrane were later put into RLT buffer lysis (Qiagen, Hilden, Germany) and kept in the freezer for further analysis.

As mentioned earlier, further molecular analysis was initiated in the single site sample group with the highest number of CECs (2 × 13 samples in total). To confirm the origin of the cells on the separation membrane, CECs gene expression analysis was performed. Gene expression analysis (GEA) allowed up to 20 endometriosis-associated markers in RNA from different cell fractions to be tested within a single quantitative polymerase chain reaction (qPCR) run. Differential diagnostic markers for the qPCR test were chosen in concordance with the expected diagnosis. The key purpose of GEA was to compare gene expression of endometriosis-associated markers in the CECs enriched fractions to that in the whole blood.

Soon after, RNA was isolated from the whole blood's white blood cell fraction (WBC) and CEC-enriched fraction on the membrane. Finally, the CECs gene expression analysis allowed identification of the relative amount of endometriosis-associated markers in the whole blood and in CEC-enriched fractions. The RNA from the whole blood was isolated with a modified procedure, and the quality/concentration of RNA was measured by NanoDrop (ThermoFisherScientific, Waltham,

U.S.). As there were only a few hundred cells on the membrane, the median concentration of RNA was quite low (5–10 ng/μL). A High-Capacity complementary DNA (cDNA) Reverse Transcription Kit (ThermoFisherScientific, Waltham, U.S.) was used for cDNA production. qPCR analysis was performed using Taqman chemistry with hydrolysis probes for all the tested genes (ThermoFisherScientific, Waltham, U.S.). The tested genes which were thought to be endometrial-associated were *CD68*, *EpCAM*, *KRT7*, *KRT18*, *KRT19*, *MUC1*, *MUC16*, *VIM*, *VEGFA*, *WT1*, *ESR1*, *PGR*, *HER2*, *CD10*, *FLT1*, *MMP1*, *MMP9*, *TP63*, *ESSRA*, *ESSRB*, *HIF1A*, and *NANOG*.

To ectopically analyze growing endometrial cells in tissues, we obtained several layers of histologically proven endometrioma (n = 11) from two patients during gynecological surgeries. Both patients had procedures planned because of pelvic pain and had a cystic adnexal tumor diagnosed during ultrasound examination. The perioperative findings in the first patient were bilateral massive endometriomas of the ovaries with no peritoneal or other lesions. The second patient had one-sided endometrioma forming a convolute consisting of the ovary and fallopian tube, severe peritoneal lesions of the urinary bladder, sacrouterine ligaments, and Douglas pouch. Eutopic endometrial tissue during menstruation bleeding was acquired from a healthy control. All tissues were further analyzed using the same qPCR protocol as for the CECs samples.

Gene expression analysis was conducted using Genex v. 6 (MultiD, Sweden) software to enable normalization and statistical analysis (cluster analysis, Mann–Whitney tests) for qPCR-generated data. The relative RNA amounts are reported for tested groups in comparisons to white blood cell fractions (WBC) or endometriosis tissue.

3. Results

3.1. CECs Presence in Endometriosis

CECs were detected by cytomorphological evaluation in 78.3% (331/423) of the tested samples. Four main CEC subtypes can be found in blood sample of patients with endometriosis: epithelial, stem cell-like, stromal, and glandular. CEC positivity did not vary significantly in different patient cohorts from Italy (n = 20), Poland (n = 82), or Czech Republic (n = 321) (75% vs. 66% vs. 81%) (see Figure 1A).

In line with the rAFS classification, CECs presence was confirmed for all of the acknowledged endometriosis stages: minimal, mild, moderate, and severe. Surprisingly, there was a significant portion of CEC-negative samples reported for severe disease (21.1%) (see Figure 1B).

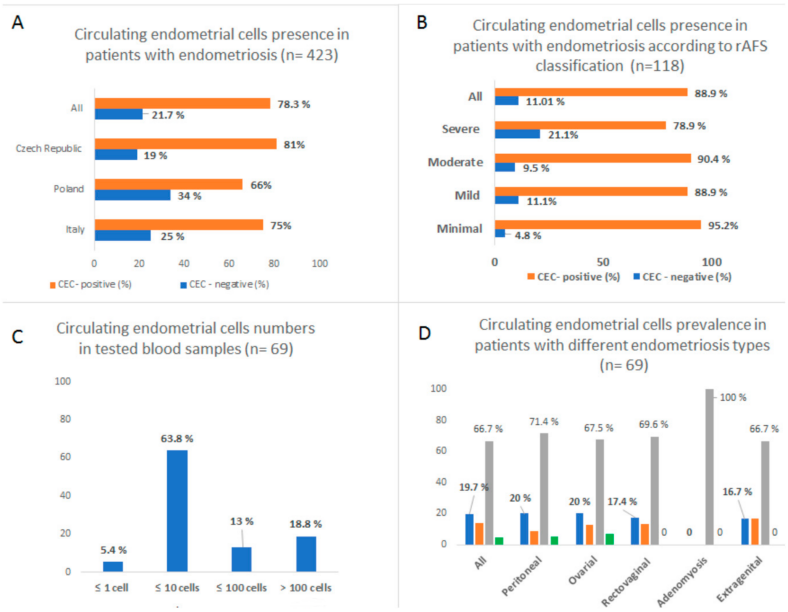

Figure 1. Circulating endometrial cells (CECs) presence in patients with endometriosis. (**A**) CEC-positive/negative sample frequency is shown for all of the tested patients. (**B**) CEC-positive/ negative sample frequency is shown in connection to the revised American Fertility Society (rAFS) classification. (**C**) CEC prevalence/load in tested samples is shown as numbers counted. The numbers of CECs were then ascribed to the following categories: CEC-negative (≤1 cell detected) and CEC-positive: 1–10 cells (low positivity), 10–100 cells (medium positivity), and >100 cells (high positivity). The distribution of CECs load in the tested samples reflected normal distribution in the tested cohorts. (**D**) CECs load is shown for different endometriosis types.

3.2. CECs Load in Patients with Different Endometriosis Types

If accessible, CECs numbers were counted and then ascribed to the following categories: CEC-negative (≤1 cell detected) and CEC-positive: 1–10 cells (low positivity), 10–100 cells (medium positivity), and >100 cells (high positivity) (see Figure 1C–D). The distribution of CEC load in the tested samples reflected normal distribution in the tested cohorts. The conclusion was that, in 20% of cases, there were patients with very high CEC numbers, and, in 10–20% of patients with endometriosis, there were no CECs present in PB.

The highest CEC numbers were detected in the after-ovulation periods (day 14–17, i.e., secretory phase), which corresponds to estrogen decrease and slow subsequent progesterone increase associated with uterine lining decidualization. We succeeded in setting up in vitro cultures of isolated cells.

For ovarian, peritoneal, rectovaginal, and extragenital endometriosis, CECs were found in 90–95% of samples and mostly in numbers of 1–10 cells (low positivity) for 8 mL of PB (see Figure 1D). It was confirmed that CEC presence is most probably independent of the different extrauterine (ectopic) locations of endometriosis tissue. Finally, it was shown that there is a subgroup of patients in all of the mentioned endometriosis subgroups with very high numbers of CECs (up to 20% of patients) in the blood.

3.3. Gene Expression Profiling of Endometriosis Tissue and Related CECs Samples

To confirm the origin of CECs in PB evaluated by cytomorphology, additional molecular testing was provided, analyzing gene expression of endometriosis tissue samples (TS) and blood samples from the same patient (WBC, CECs). Up to 20 markers were analyzed in total. The cluster analysis of normalized qPCR results enabled identifying a group of "endometriosis" genes that could be used as confirmatory for CECs.

There were several genes strongly expressed in endometriosis tissue: *EPCAM*, *KRT18*, *WT1*, *MUC16*, *MUC1*, and *ESR1*, if compared to the endometrial cells from healthy controls. In some tissue samples, *MMP1* was also present. In the CEC fraction of these patients, only *KRT18*, *KRT19*, *VIM*, and *NANOG* were detected in a relatively high amount compared to the WBC profile. Interestingly, in CEC samples, *ESR1* was expressed very rarely. The genes with increased expression (*KRT18*, *KRT19*, *VIM*, and *NANOG*) were then used in subsequent analyses as "endometriosis confirmatory genes". The cluster analysis of qPCR results for a sample collection of one patient is shown in Figure 2A.

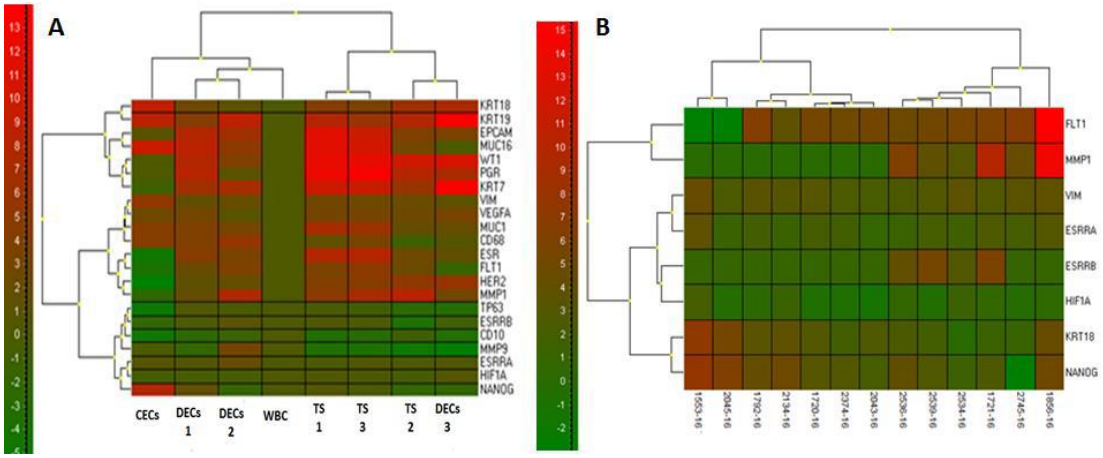

Figure 2. Relative RNA quantity for CECs and endometriosis tissue samples shown after cluster analysis. (**A**) Samples for one patient's CEC tissue sample (TS) collection are presented with eight samples in total. Tissue samples TS 1–3 were obtained during surgery, as well as peritoneal washing (PW; a source for disseminated endometrial cells (DEC)). DEC 1 and DEC 2 were cultured after size-based filtration. DEC 3 was cultured without preliminary enrichment of PW. In the left subcluster, there are gene expression profiles of loose endometrial cells from the blood and DECs isolated by size-based separation. The second cluster is represented by endometriosis tissue biopsy samples TS 1, TS 2, and TS 3 and in vitro cultured PW. Two tissue samples (TS 1 and TS 3) are clustered together and show a very high level of similarity. For this patient with an endometriotic cyst, *MUC16* was also detected on CECs. (**B**) Cluster analysis for high-positive CECs samples is shown. The analysis revealed that there were at least two different sample types of high-positive CECs (two clusters). The first one is represented by the cluster showing a high expression of *FLT1*, *MMP1*, and *ESRRB* (cluster on the right). The second group of samples shows elevation of *NANOG*, *KRT18*, and *VIM* expression. *KRT19* was relatively highly expressed in both clusters (not shown).

3.4. Gene Expression Analysis of Positive CECs Samples with Significant Cellularity

The genes confirmed to be expressed in the endometriosis tissue and CECs, as presented in the first part of the results (Figure 2A), were then analyzed in the group of patients (n = 13) in whom CECs were detected in relatively high numbers (up to 1000 CECs/8 mL PB). In this CEC cohort, the following genes were confirmed to be elevated: *VIM* (elevated in 13 out of 13 CECs samples—13/13), *FLT1* (12/13), *KRT18* (8/13), *KRT19* (8/13), *MMP9* (12/13), *NANOG* (8/13), and *ESR1* (7/13). The statistical significance of differential expression values was confirmed for *VIM*, *MMP9*, *FLT1*, and *KRT19*. As expected, CD68 was elevated in all of the tested samples (13/13), which suggests that some of the frequently observed genes could be found because of the presence of the captured and in vitro cultured macrophages, which have a very similar cytomorphology to the endometrial cells. However, correlation analysis revealed that *KRT18*, *FLT1*, and *NANOG* expression was *CD68*-independent. On the other hand, in this specific patient's cohort, there was a correlation between *CD68* and *VIM*.

The cluster analysis of these positive CEC samples revealed that there were at least two different cell types of CECs in the analyzed samples. The first one was represented by the cluster showing a high expression of *FLT1*, *MMP1*, and *ESRRB* (see Figure 2B—cluster on the right). The second group of

samples showed an elevation of *NANOG*, *KRT18*, and *VIM* expression. *KRT19* was relatively highly expressed in both clusters (not shown). Significantly elevated *FLT1* did not correlate with any other of the tested genes. Interestingly, *ESR1* expression was present in parallel with *ESSRA* and *ESSRB* in two super-positive samples only. Very high expression of *ESSRB* was observed in samples with elevated *VIM* expression.

3.5. Gene Expression Profiling Data of CEC—Samples with Average Cellularity

The analysis of CEC samples (n = 52) showed that messenger RNA (mRNA) expression of the following genes was significantly elevated in the CECs fractions when compared to the WBC fractions: *KRT18*, *VIM*, *NANOG*, and *MMP9*. *KRT19* was at the limit of significance (see Figure 3A). The following genes were significantly decreased in CEC samples: *HIF1A*, *CD10*, and *MUC1*. There was a significant difference between these CEC samples and the CEC samples with high cellularity found for *FLT1* expression (see Figure 3B—white arrow).

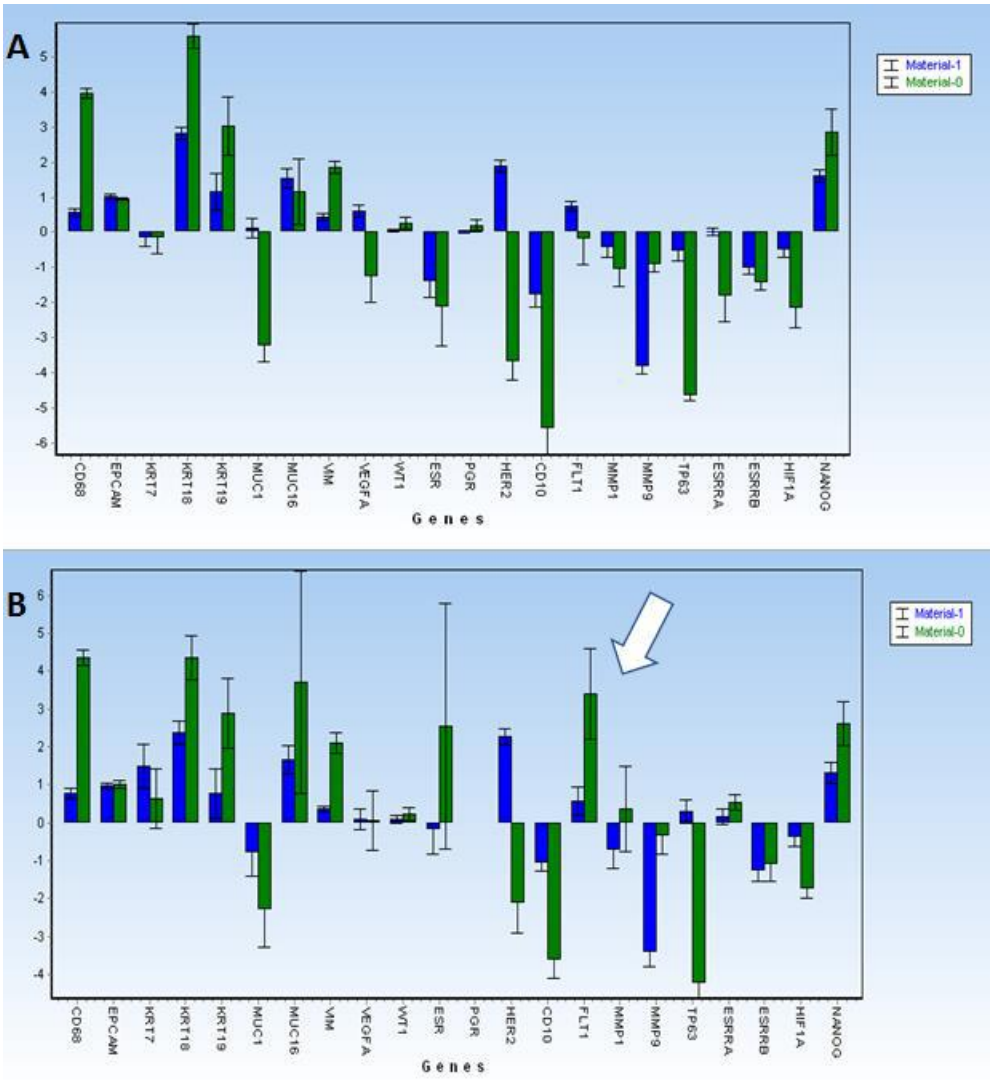

Figure 3. Gene expression profiling data displayed for CECs when compared to white blood cells (WBC). (**A**) Gene expression profiling data displayed for all CECs tested in the study (n = 52) (**B**) Gene expression profiling data for high-positive CEC (n = 13) samples when compared to the white blood cell fraction (WBC). In super-positive CEC samples, *FLT1* was significantly elevated (see white arrow).

3.6. CEC Prevalence and Characteristics during Menstrual Cycle (MC)

Based on the simple presumption that CECs should be present in PB during all phases of MC if they are to be used as a biomarker of the disease, blood samples from 11 patients were collected throughout all MC phases. The highest CEC numbers were detected in the after-ovulatory periods (mid-secretory phase) of MC. CECs were present throughout the MC phases but their characteristics varied. The characteristics of CECs during MC reflected the physiological cycle of endometrium decidualization. The cytomorphology of CECs captured during MC changed between epithelial, stromal, and stem cell-like.

In line with this finding, gene expression changes during MC phases in CECs were analyzed, and it was observed that the structural genes like *KRT18*, *VIM*, and *NANOG* were expressed in a relatively stable manner in all four MC phases, but their expression was significantly elevated in the middle of the MC (early/mid secretory phase) (see Figure 4). In this period, the expression of *KRT18* and *VIM* increased. This could possibly represent the more frequent presence of a stem and/or mesenchymal cell population in this period. Additionally, in the late secretory and early proliferative phase, the elevation of *FLT1* and *MMP1* expression was observed.

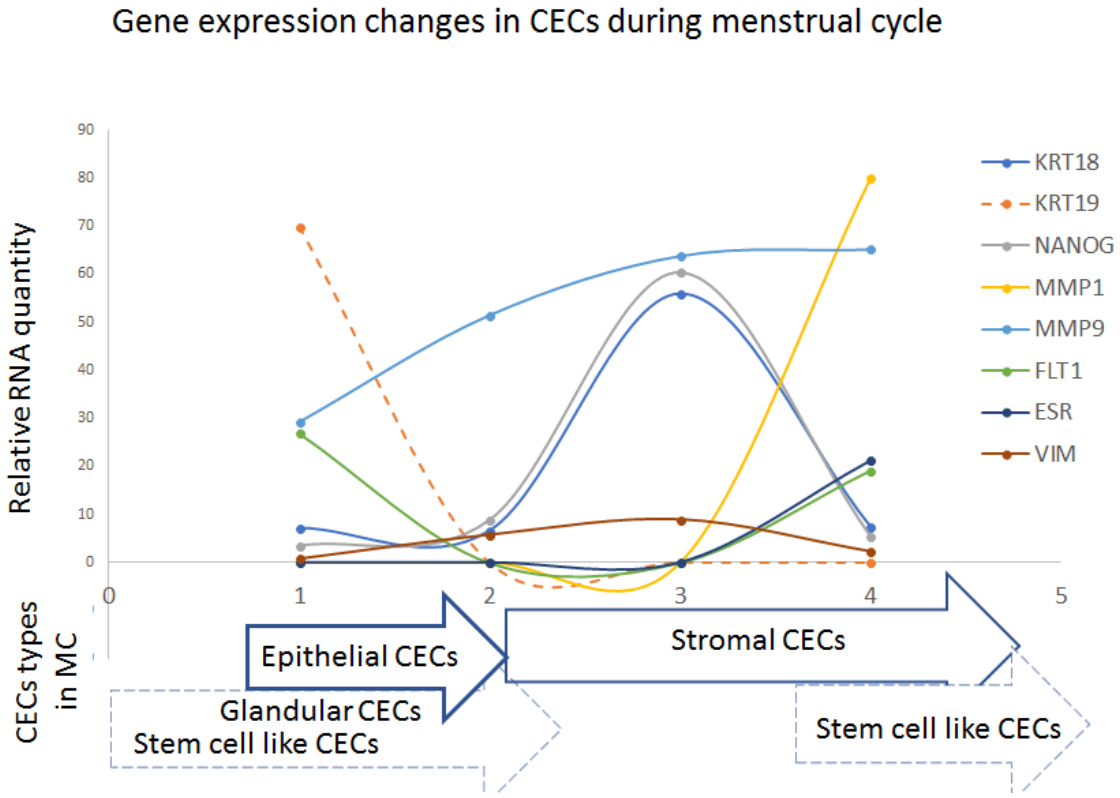

Figure 4. Gene expression changes in CECs during the menstrual cycle (MC; four examinations) are shown in relation to CECs cytomorphology type: epithelial, stromal, stem-cell like, glandular. Examination No. 1 (day 24 of MC), No. 2 (day seven of MC), No. 3 (day 14 of MC), No. 4 (day 21 of MC). The values are presented as relative RNA amount.

Cells shed into circulation during the decidualization process were mostly stromal-like endometrial cells, as shown by their cytomorphology and gene expression profile. These cells were most probably estrogen or progesterone non-responsive but they did express *ESRRB*. The highest expression of *ESSRB* was found after ovulation in the secretory phase between days 20 and 26.

The *ESR1*-positive cells were regularly shed to the blood during the proliferative phases of MC (days 1–14). Epithelial *KRT19*$^{+}$ cells which were *ESR1*-positive were typically found during this phase of MC.

4. Discussion

Our study confirmed the presence of CECs in most patients with histologically proven endometriosis. The CECs occurrence was confirmed during all phases of the menstrual cycle, but the CECs cytomorphology differed depending on the changing hormone levels. The cytomorphological changes were accompanied by differences in the gene expression profile, as shown by the presented data.

Subsequently, the gene expression profiling of the endometriosis lesions and of the parallel CECs samples from PB identified a range of potential biomarkers which could be used to identify CECs in patients with undiagnosed endometriosis. Early detection of CECs in women with pelvic pain or other symptoms, in addition to objective gynecological examination suspecting endometriosis, could accelerate and improve diagnosis.

The shedding of CECs into PB could be ascribed to the well-known physiology of decidualization. The process of decidualization of the uterine lining denotes the transformation of endometrial stromal fibroblasts into specialized secretory decidual cells that provide a nutritive and immune-privileged matrix essential for embryo implantation and placental development. Decidualization of the human endometrium is driven by the postovulatory rise in progesterone levels and increasing local cAMP production. In response to falling progesterone levels, spontaneous decidualization causes menstrual shedding and cyclic regeneration of the endometrium. Under endometriosis conditions, the decidualizing cells tend to be progesterone non-responsive, which results in the need for a different energy [17].

Endometrial-derived stem-cell vascular metastasis, as described in the study of Li F et al. [18], might provide a valuable explanation for cases of distant, deep infiltration and recurrent endometriosis. It was shown that circulating endometriosis stem cells propagate endometriosis through vascular dissemination and may also serve as biomarkers of active lesion establishment. Furthermore, endometriosis-derived circulating cells were consistently found in the blood of animals with endometriosis, and their number increased during new lesion establishment in the mouse endometriosis model [18]. A similar study using a mouse endometriosis model showed that donor bone marrow-derived circulating endothelial progenitor cells were found to be elevated acutely after endometriosis induction [19]. The abovementioned circulating cell types with different expression profiles may be involved in endometriosis establishment and could serve as biomarkers of active disease.

Implementing the theory of somatic stem cells, endometriosis may be regarded as a stem-cell disease [20,21]; these endometrial stem cells differentiate into local tissue types, but cells may also differentiate into the epithelium, glands, and stroma to form functional ectopic endometrial tissue [22,23]. All of the mentioned cell types were detected in different frequencies in enriched CEC samples in our study.

However, the following questions still remain: What are the characteristics of CECs causing endometriosis? What markers might be used to identify CECs with some level of certainty? The CEC-positive samples displayed elevated gene expression of *KRT18*, *KRT19*, *NANOG*, and *VIM* in most of the tested samples. The CEC cells characterized in our cohort did not express PGR (progesterone receptor) and, in at least half of the cases, *ESR1* was also not present. Does this mean that mostly hormone non-responsive CECs are shed into circulation?

The histological appearance of the endometrium was referred to as predecidua in several previous publications [18,24]. In parallel with the predecidua changes, various CEC types isolated from patients with endometriosis in our cohort showed different gene expression profiles, represented by typically elevated gene expression of *KRT18*, *NANOG*, and *VIM* or of *KRT19* and *ESR1*.

Interestingly, *KRT18*, *VIM*, and *NANOG* were elevated in the secretory phases of the menstrual cycle, while *KRT19* and *ESR1* were observed in the proliferative MC phases. Angiogenesis might be driven by elevated *FLT1* and *MMP1* in the late secretory phases. Our results mirror data reviewed by Wang et al. [16], summarizing the probable pathogenesis of endometriosis. In short, the proliferative phases of MC are presented by relatively high keratin expression, ascribed to the quickly proliferating epithelial cells. Upon epithelization, the process of lining decidualization is supported by a stem

cell and/or mesenchymal cell supply. It was shown that somatic stem cells may originate from different tissue stem-cell reservoirs and/or directly from bone marrow. As shown by the distinct gene expression profiles, there were also cells with mesenchymal characteristics (*VIM*-positive) on the way to the endometrium. Similarly, circulating stromal cells ($CD10^+$ cells) but no epithelial cells in the circulating blood of endometriosis patients were detected using another size-based separation approach (ScreenCell®) [25], who reported the presence of only circulating stromal cells. One of their explanations for the absence of epithelial circulating cells in their study was that cells smaller than 8 µm could have been missed using this filtration technique.

Our data showed that, during the MC, there is often elevated *KRT19* and *ESR1* detected in CECs in the proliferative phase, and that, during the whole MC, *KRT18*, *NANOG* (a stem-cell marker), and *VIM* (mesenchymal marker) are present in different levels in enriched CECs.

The results discussed in this paper offer a chance to identify CEC subtypes circulating in PB and may facilitate the management of preoperative and postoperative endometriosis therapy in the future, using the CEC characteristics and their hormone non-responsiveness. Further studies are necessary to fully understand the advantages of CEC application and its use in clinical practice.

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CHEST

Circulating Endometrial Cells in Women With Spontaneous Pneumothorax

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BACKGROUND: The occurrence of catamenial pneumothorax (CP) is rare, and the awareness of this diagnosis among physicians is insufficient. CP is highly correlated with pelvic endometriosis and remains the most common form of thoracic endometriosis syndrome. Circulating endometrial cells (CECs) have been previously detected in patients with pelvic endometriosis. Could CECs bring new insights into pneumothorax management?

METHODS: This study aims to describe the occurrence and molecular characteristics of CECs in women with spontaneous pneumothorax (SP) (N = 20) with high suspicion of its catamenial character. CECs were enriched from peripheral blood by size-based separation (MetaCell). In addition to cytomorphology, gene expression profiling of captured cells was performed for 24 endometriosis-associated genes.

RESULTS: CECs were present in all 20 patients with SP. Enriched CECs exhibited four character features: epithelial, stem cell-like, stroma-like, and glandular. However, not all of them were present in every sampling. Gene expression profiling revealed two distinct phenotypes of CECs in SP and/or CP: one of them refers to the diaphragm openings syndrome and the other to endometrial tissue pleural implantations. Comparisons of the gene expression profiles of CECs in pneumothorax (CECs-SP group) with CECs in pelvic endometriosis (CECs-non-SP group) have revealed significantly higher expression of *HER2* in the CECs-SP group compared with the CECs-non-SP group.

CONCLUSIONS: This proof-of-concept study demonstrates successful isolation and characterization of CECs in patients with SP. Identification of CECs in SP could alert endometriosis involvement and help early referral to gynecologic consultation for further examination and treatment.

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KEY WORDS: biomarker; catamenial pneumothorax; circulating endometrial cells; culturing; endometriosis; gene expression profiling; in vitro; liquid biopsy; MetaCell

FOR EDITORIAL COMMENT, SEE PAGE 245

ABBREVIATIONS: CD = cluster of differentiation; CEC = circulating endometrial cell; CP = catamenial pneumothorax; ESR = estrogen receptor; GEA = gene expression analysis; PCR = polymerase chain reaction; SP = spontaneous pneumothorax; VIM = vimentin

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Endometriosis is defined as the presence of ectopic endometrial tissue consisting mainly of stromal and epithelial cells. Endometriotic lesions are considered to be benign inflammatory lesions but can have cancer-like features such as local invasion and resistance to apoptosis. Endometriosis affects approximately 10% of women of reproductive age and its treatment is a serious issue for health-care systems worldwide.[1]

Endometriosis typically occurs in the pelvis but is also known to occur in extrapelvic organs and tissues. Development of extrapelvic endometriosis is typically rare (8.9%). The most common locations include the GI tract (32.3%) and the urinary tract (5.9%). Other sites can include the lungs, umbilicus, abdominal scars, liver, gall bladder, pancreas, breasts, and the extremities.[2-6] Thoracic endometriosis or thoracic endometriosis syndrome can present with pneumothorax, hemothorax, hemoptysis, lung nodules, isolated chest pain, or pneumomediastinum. The symptoms are synchronized with the menstrual cycle.[4]

Catamenial pneumothorax (CP) is defined as recurrent accumulation of air in the pleural cavity in women of reproductive age in the perimenstrual period.[7,8] This period according to different studies ranges from 72 h before and up to 7 days after menstrual bleeding.[9-12] Additional criteria for CP include pleural lesions, right-sided occurrence, and coexistence of endometriosis, especially within the pelvis in 32% of CP cases.[13] Pelvic endometriosis seems to be an important aspect of CP. When present, there is a significantly higher rate of recurrence, endometrial thoracic implants, and histologically confirmed endometriosis lesions than in patients with a healthy pelvis.[14] The study by Tulandi et al[15] described the presence of pelvic endometriosis in 93.7% of patients with CP mainly in stage 3 and 4, whereas thoracic endometriosis was present in 60%. The mean age of patients is 32 to 35 years. About 3% to 6% of spontaneous pneumothorax (SP) ends up diagnosed as catamenial.[16] This low incidence rate was contradicted in a study by Bobbio et al,[17] in which 42,595 patients with SP were analyzed based on their age, sex, and primary and secondary characteristics. The study found that there was a higher incidence in men than women (ratio, 3.3:1), and there was also a difference in the age at first diagnosis. In men, the first peak of incidence occurred before the age of 20 years and progressively decreased until 50 years. In women, the first peak appears to be delayed and the incidence remains stable up to 40 years. Of those diagnosed in the 30- to 50-year-old age group, women had a significantly higher surgery and rehospitalization rate. The authors hypothesized that a significant contributing factor in women of this age is related to thoracic endometriosis syndrome. This was confirmed in pathologic studies where CP and endometriosis-related pneumothorax were responsible for approximately one-half of pneumothorax episodes in patients of childbearing age indicated for surgery.[18-21]

The diagnosis of CP is associated with the following: single or multiple fenestrations in the tendinous part of the diaphragm and red and/or brown spots or nodules located on the diaphragm or visceral pleura.[22,23] Histopathologic analysis of the nodules reveals glandular cells, endometrial stroma, and macrophages filled with hemosiderin. Immunohistochemistry may demonstrate the presence of cluster of differentiation (CD) 10, estrogen, and progesterone receptors.[24,25] Symptoms of pelvic endometriosis, secondary or primary infertility, and previous gynecologic procedures may help to diagnose CP.[26] About one-third of CP cases require surgery (wedge lung resection, pleurectomy, chemical or mechanical pleurodesis, diaphragm reconstruction).[27] The recurrence rate in patients with CP after surgery ranges from 8% to 40%.[28] Postsurgical hormonal therapy can be provided to reduce recurrence rate.[29]

The etiology of CP is still unknown. The four main theories are as follows: physiological (alveolar rupture because of high concentration of prostaglandin F2), migrational (endometrial tissue travels via fenestrations in the diaphragm), coelomic metaplasia, and transformation of pleural epithelium. Additionally, it is thought that endometrial dissemination may occur through lymphatic and/or vascular embolization.[1]

Circulating endometrial cells (CECs) refer to the rare cells and have been previously isolated from peripheral blood and cultured with success via the size-based separation method (MetaCell; MetaCell s.r.o.) in pelvic endometriosis.[30] These sporadic cells of mostly epithelial origin could be used in the process of CP diagnostics in the future.

The focus of this study was to isolate and characterize CECs in patients with SP to understand the catamenial

character of pneumothorax. The characterization of pneumothorax could prevent later SP recurrence.

The following questions need to be answered: (1) could CEC detection help to identify patients with CP in the SP group; (2) how do we characterize CECs in SP and/or CP to confirm their endometrial origin?; and (3) how do we best manage patients with positive CECs in CP?

We hypothesize that CEC characterization could expedite the diagnostic processes of CP at thoracic units and could support personalized therapy for endometriosis in the future.

Methods

Patients

Women with SP (N = 20) were admitted to the thoracic unit during 2016 to 2019. For every patient, two blood samples were evaluated for CEC presence (N = 40). Out of these CEC-SP samples, 35 were included into the gene expression studies. Clinical data are summarized in Table 1. (More details on study subjects can be found in e-Tables 1 and 2.)

In summary, all women with SP were of reproductive age (age range, 23-52 years; average age, 39.3 years). Of the 20 patients with SP, nine (45%) had a recurrence of SP (age range, 29-52 years; average age, 40.7 years). The previous SP was managed either conservatively or with surgery in the past (1999-2019). This was the first SP episode in 11 patients (55%) (age range, 23-49 years; average age, 31.7 years). Seven patients (35%) had partial pneumothorax, and the other 13 (65%) had total pneumothorax. In the recurrent pneumothorax group, total pneumothorax represented 89% of cases.

The most common symptoms reported at admission to the hospital were dyspnea, indefinite thoracic pain, and irritating dry cough. All patients had their difficulties starting 1 week before or after the onset of menses. In the history of three patients, similar but less severe cyclic symptoms were reported. Interestingly, one patient with recurrent pneumothorax was in her 35th week of pregnancy. Radiologic findings showed right-sided pneumothorax in 17 patients; three patients were diagnosed with left-sided pneumothorax. CT scans revealed small bullas and nodules in the lung parenchyma and pleura in 19 patients. Endometriosis affecting the diaphragm was diagnosed in one patient.

In our thoracic department, less invasive treatment consisting of puncture and drainage was provided in 10 patients (50%). The other 10 patients (50%) required more complex surgeries. These included the following: thoracotomy or thoracoscopy, resection of bullas, pleural abrasion, lung resection, talc pleurodesis, and adhesiolysis. Tissue obtained by pneumothorax surgery was evaluated by histologic examination in seven patients. Four tissue samples tested positive for extragenital endometriosis by immunohistochemistry (CD10+, vimentin [VIM] +, and estrogen receptor [ESR] +). Additionally, gene expression profiling (24 genes in total) was conducted for the collected pneumothorax tissue samples (n = 2) by quantitative polymerase chain reaction (PCR).

Three of the 20 patients had previous laparoscopic surgery for infertility, which could be a consequence of pelvic endometriosis. Another patient had surgery for extrauterine pregnancy, and the third patient was diagnosed with uterine fibroids. Eleven patients were actively smoking or had admitted smoking in the past. One patient had been in the course of a sex change (woman to man), had already underwent bilateral mastectomy, and was being provided testosterone therapy. SP occurrence was diagnosed during a pause in the testosterone therapy.

As a control group, blood samples from patients (n = 18) with pelvic endometriosis and no signs or symptoms of SP were collected and analyzed for CECs (CECs-non-SP group). Additionally, cells from menstrual flow were analyzed in healthy people (n = 3) assigned as being endometriosis negative. Tissue from pelvic (n = 8) and pneumothorax endometriosis lesions (n = 2) was also collected and compared by gene expression analysis (GEA).

This study obtained approval by the multicentric ethic committee of the Faculty Hospital Kralovske Vinohrady, Prague (Nos. EK – VP/56/02014, EK – VP/20/02015). All participants signed informed consent before participating in the study.

CEC Enrichment and Culture

A size-based separation method for CEC enrichment from peripheral blood (MetaCell) has been previously described.[30] In short, peripheral blood samples (2 × 8 mL) from a patient with SP are filtered through the porous membrane. Subsequently, the separation membrane with enriched CEC population is transferred into the six-well cultivation plate, cultivation medium is added, and CECs are cultured directly on the membrane under standard in vitro cell culture conditions (37°C, 5% atmospheric CO_2). The CECs were grown in vitro in fetal bovine serum-enriched RPMI medium (10%) with antibiotics for a minimum of 3 to 6 days.

CECs Microscopy Analysis

CECs grown in vitro on the separation membrane were stained by vital fluorescent stains (NucBlue, CellTracker, or MitoTracker; Thermo Fisher Scientific) and evaluated by means of vital fluorescence microscopy (Olympus X10; Olympus) in the following two steps: (1) screening at 10× and 20× magnification to locate viable cells; and (2) observation at 40× and 60× magnification for detailed cytomorphologic analysis of the cytoplasm, nucleus, and mitochondria. Enriched cells and/or cell clusters of interest were scanned and digitized, and the images were subsequently examined by an experienced researcher and/or pathologist. Each sample was evaluated by two different specialists. After completing vital fluorescence microscopy analysis of the cells, the separation membrane was fixed by drying, used later for immunohistochemistry, and/or stored in the RLT buffer for planned RNA GEA.

Immunohistochemistry analysis enables only one marker to be analyzed on one slide because of the type of available antibodies; therefore, the choice of the right marker is crucial. We have compared gene expression profiles by quantitative PCR analyzing endometriosis tissue samples from pelvic and pleural cavities, and in CECs enriched out of the blood. VIM showed relatively high messenger RNA expression in all tested sample groups (CECs, CP, and pelvic endometriosis) and was qualified to be evaluated on CECs enriched out of the blood on the membrane by immunohistochemistry (Dako Agilent Technologies). Along with CD10 and ESR, VIM is routinely used in the diagnosis of endometriosis.

GEA

GEA was conducted on the enriched CECs. The GEA using quantitative PCR allowed for testing of up to 24 genes in each sample. Genes possibly associated with endometriosis

TABLE 1] Patient Clinical Characteristics and CEC Examination

Patient		Pneumothorax		Previous Diagnosis of Endometriosis	CECs		Endometriosis-Related Data				
No.	Age (Years)	Diagnosis	Type		CEC Positivity	CEC No.	History	Syndromes	Imaging (Radiograph or CT Scan)	Therapy	Histology
1	28	SP	Total	No	Yes	< 100	December 2015: laparoscopy for infertility	Pain under left clavicula, dry irritating cough, dyspnea	Total left-sided pneumothorax, CT scan: bilateral lung parenchyma bulla up to 2 mm, subpleural nodules in the right middle lobe up to 5 mm	Thoracic puncture and drainage	X
					Yes	< 100					
					Yes	< 100					
2	43	Recurrent SP	Total	Yes	Yes	> 100	1999: right-sided pneumothorax with drainage, 2001: VATS revision, 2005: left-sided pneumothorax with drainage February 2016: thoracotomy bulla resection, middle and lower right-sided lung lobule resection, pleural abrasion	Cough, dyspnea, unspecified chest pain	Left-sided pneumothorax, fluidothorax	Thoracotomy bulla resection, parietal pleura abrasion	Extragenital endometriosis of pleural tissue
					Yes	< 100					
					Yes	< 100					
					Yes	< 100					
3	42	Recurrent SP	Total	YES	Yes	< 100	November 2014: Right-sided pneumothorax during menstruation—conservative therapy, after that recurrent dyspnea in the beginning of menstruation	Dyspnea, mild right thoracic pain	Right-sided pneumothorax, fluidothorax, CT scan: adhesions, minimal shift of central structures to the left	VATS, adhesiolysis, lung apex resection, abrasion, drainage	Endometriosis of visceral pleura and diaphragm
					Yes	< 100					
					Yes	< 100					

(Continued)

TABLE 1] *(Continued)*

Patient		Pneumothorax		Previous Diagnosis of Endometriosis	CECs		Endometriosis-Related Data				
No.	Age (Years)	Diagnosis	Type		CEC Positivity	CEC No.	History	Syndromes	Imaging (Radiograph or CT Scan)	Therapy	Histology
4	45	Recurrent SP	Total	No	Yes	< 100	July-August 2015: recurrent right-sided pneumothorax in relationship with menstruation (2× treated with drainage) August 2015: VATS revision, chlamydial lung infection 2 y ago	Heavy menstrual bleeding, right-sided chest pain	Residual bullas up to 3 mm, one bulla size 11 mm	Thoracic puncture and drainage	No endometriosis detected
					YES	< 100					
5	25	SP	Partial	No	Yes	< 100	Not significant, hormonal combined contraception	Sudden right-sided infraclavicular pain, progressive inspire pain, dyspnea	Subpleural bullas size 2-3 mm	Thoracic puncture and drainage	X
6	24	SP	Partial	No	Yes	< 100	Bronchial asthma in childhood, hormonal combined contraception	Sudden intensive right-sided chest pain, dyspnea	Small nodules 2 mm in size, small subpleural bullas	Thoracic puncture and drainage	X
7	31	SP	Total	No	No	< 100	Not significant	Sudden back pain between shoulder blades	Total right-sided pneumothorax	Thoracic puncture and drainage	X
8	48	Recurrent SP	Total	Yes	Yes	< 100	2011 and 2015: recurrent spontaneous right-sided pneumothorax—puncture and drainage, March 2016: VATS revision for recurrent right-sided pneumothorax—upper lobule apex resection, biopsy, abrasion, adhesiolysis—macroscopic endometrial lesions on the diaphragm and parietal pleura	Dyspnea, right-sided thoracic pain, cyclical in relationship with menstruation, heavy bleeding	Right-sided fluidopneu-mothorax	Thoracic puncture and drainage	Endometriosis of parietal pleura

(Continued)

TABLE 1] *(Continued)*

Patient		Pneumothorax		Previous Diagnosis of Endometriosis	CECs		Endometriosis-Related Data				
No.	Age (Years)	Diagnosis	Type		CEC Positivity	CEC No.	History	Syndromes	Imaging (Radiograph or CT Scan)	Therapy	Histology
9	26	SP	Partial	No	Yes	< 100	Laparoscopy for infertility, combined hormonal contraception	Dyspnea	Small subpleural nodules of the middle and upper lobule of right side of the lung	Thoracic puncture and drainage	X
10	30	Recurrent SP	Total	Yes	Yes	< 100	3× spontaneous right-sided pneumothorax with correlation to menstruation, always after pause in hormonal therapy	Not available	X	Apical pleurotomy, indicated for video-thoracoscopic pleurodesis	
11	27	SP	Total	No	Yes	< 100	Laparoscopy for infertility	Cough, dyspnea	Total right-sided pneumothorax	Thoracic puncture and drainage	X
12	30	SP	Partial	…	Yes	<100	…	Cough, dyspnea	X	Thoracic puncture and drainage	X
13	29	Recurrent SP	Total	No	Yes	100	2017: spontaneous tension pneumothorax with puncture and drainage	Dyspnea	Right-sided tension pneumothorax	Thoracic puncture and drainage	X
14	52	Recurrent SP	Total	No	Yes	100	2016: posttraumatic right-sided pneumothorax—conservative therapy, patient on Medroxyprogestron acetate (Depo Provera) since 2016	Right-sided thoracic pain, dyspnea	Right-sided apical pneumothorax and fluidothorax	VATS apical resection of the right side of the lung, abrasion, drainage	X
15	49	SP	Partial	No	Yes	100	Laparoscopy for extrauterine pregnancy	…	Right-sided apical pneumothorax and fluidothorax, bullas, emphysema	VATS apical resection of the right side of the lung, abrasion, drainage	X
16	45	Recurrent SP	Partial	Yes	Yes	100	August 2018: spontaneous right-sided pneumothorax—puncture and drainage	Dyspnea, back pain	Right-sided apical pneumothorax	VATS right-sided lung resection, abrasion, drainage	Endometriosis of parietal pleura
					Yes	5					
					Yes	50					
					Yes	200					

(Continued)

TABLE 1] *(Continued)*

Patient		Pneumothorax		Previous Diagnosis of Endometriosis	CECs		Endometriosis-Related Data				
No.	Age (Years)	Diagnosis	Type		CEC Positivity	CEC No.	History	Syndromes	Imaging (Radiograph or CT Scan)	Therapy	Histology
					Yes	100					
					Yes	20					
					Yes	50					
17	45	SP	Total	No	Yes	100	Uterine fibroids	Right-sided shoulder pain, dyspnea, cough	Right-sided pneumothorax	Thoracic puncture and drainage, VATS, diaphragm openings	Endometriosis of visceral pleura and diaphragm
18	41	SP	Total	No	Yes	50	…	Pain under left clavicula	Left-sided apical pneumothorax, emphysema	Thoracic puncture and drainage, VATS resection of the left side of the lung, abrasion	X
19	32	Recurrent SP	Total	No	Yes	100	March 2019: spontaneous right-sided pneumothorax—VATS, pleurectomy	Right-sided parasternal pain	Right-sided pneumothorax	VATS talc pleurodesis	No endometriosis detected
20	23	SP	Partial	No	Yes	50	2017: sex change (woman to man). Bilateral mastectomy, testosterone treatment, in testosterone pause a pneumothorax outbreak	Dyspnea, right-sided chest pain	Right-sided apical pneumothorax, solitaire bulla with partial atelectasis	Thoracic puncture and drainage after VATS: right-sided apical lung resection, abrasion of pleura	No endometriosis detected

Descriptions of patients with pneumothorax included in this study, including characteristic and clinical data in relation to the CEC positivity and molecular profile. The numbers of CECs are placed into groups as follows: CEC-negative (0 cells), CEC-positive (1-99 cells), and CEC-high positive (100-1,000 cells). CEC = circulating endometrial cell; SP = spontaneous pneumothorax; VATS = video-assisted thoracoscopic surgery; X = not obtained.

(subsequently described) were chosen to report on the origin of the cells captured on the separation membrane.

Gene expression profiles of CECs were compared with the WBC fraction to obtain relative RNA levels for every sampling. WBC fraction from every blood sample was obtained by erythrocyte lysis. Cells were stored at −4°C in RLT with beta-mercaptoethanol (RNA Blood Mini Kit; QIAGEN). After viable fluorescent microscopy analysis, CECs captured on the membrane were placed into RLT buffer and stored at −4°C until RNA analysis. RNA was isolated from the WBC and CEC-enriched fraction by the RNeasy Mini Kit (QIAGEN). RNA concentration was measured by NanoDrop (Thermo Fisher Scientific). Because there are only a few hundred cells on the membrane, the median concentration of RNA is quite low (5-10 ng/μL).

The High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) was used for complementary DNA production. GEA was performed using TaqMan Fast Advanced chemistry with TaqMan MGB probes for all tested genes (Thermo Fisher Scientific).

The following 24 endometriosis-associated genes were tested by quantitative PCR run (cobas 480; Roche Diagnostics): *ACTB;* *EPCAM*; keratins: *KRT7*, *KRT18*, and *KRT19*; mucins: *MUC1* and *MUC16*; *VIM*; *VEGFA*; *VEGFR* (*FLT1*); *WT1*; *ESR1*; *PGR*; *HER2*; *CD10*; matrix metalloproteinases: *MMP1* and *MMP9*; *TP63*; *ESRRA*; *ESRRB*; *FGF4*; *HIF1A*; *NANOG*; and *CD68*.

GEA was performed in two steps. First, each patient's WBC gene profile was compared with their CECs. Second, group comparisons for CEC subgroups (CECs-SP vs CECs-non-SP) were analyzed. The following five types of patient samples were included into gene expression comparisons: (1) CEC samples isolated from women with SP (n = 35), (2) CEC samples isolated from women with confirmed pelvic endometriosis diagnosis without SP (n = 18), (3) endometriosis-like tissue from pleural/lung parenchyma resection in patients with SP undergoing surgical intervention (n = 2), (4) endometriosis tissue from women with confirmed pelvic endometriosis (n = 8), and (5) cells sampled during the menstrual phase from menstrual flow in a healthy person (n = 3).

The GEA data were analyzed using GenEx version 6 software (MultiD) using calculations based on the ddCt method.[31] The gene expression comparisons made between different patient groups were made by Kruskal-Wallis and Mann-Whitney *U* test. $P \leq .05$ was considered significant.

Results

CEC Rates

In total, 40 blood samples from 20 women admitted to hospital because of SP were withdrawn and analyzed for presence of CECs. First, cytomorphologic evaluation after short in vitro culture of separated cells confirmed CEC presence in all tested patient samples (100%). In four of these patients, CEC presence was tested during the follow-up period (weekly during 4 weeks after pneumothorax diagnosis), therefore allowing for CEC testing in different menstrual cycle phases. After surgical treatment for pneumothorax in these four patients, significantly lower CEC numbers were reported after surgery. However, CECs, although in lower numbers, were present during the entire 4-week period in three patients.

Samples were placed in the following categories according to CEC quantification: (1) CEC-negative (0 cells/8 mL blood), (2) CEC-positive (1-99 cells/8 mL), and (3) CEC-high positive (100-1,000 cells/8 mL). In 35% of subjects (seven of 20) with SP, high positive CECs were detected (Table 1). In three of the seven patients (43%), endometriosis lesions were confirmed by pathologists, and five of them (72%) had recurrent pneumothorax. This could indicate that there is a correlation between high CEC numbers and pneumothorax susceptibility. Other clinical correlations were not found for the patients in the CEC-high positive group.

Cytomorphologic Evaluation of CECs

CECs cytomorphology analysis was based on vital fluorescent microscopy using vital fluorescent stains. The size-based captured CECs, cultured on the separation membrane, exhibited four main character types: epithelial, stem cell-like, stromal, and glandular. The main CEC features are described in Figure 1. Usually, a mixture of these cell phenotypes was observed in a given sample. In the tested samples, CECs were seen as follows: epithelial (55%), stem cell-like (30%), stromal-like (7%), and glandular (7%). Epithelial vs stem cell-like can be distinguished by size and fluorescent staining of cytoplasm (eg, CellTracker) (Fig 2).

The average size of captured CECs of the epithelial type was 20.0 ± 2.1 μm. These epithelial cells are relatively big and rounded, with a precisely rounded nucleus, relatively smooth nuclear structure, and identifiable transcriptionally active regions—nucleoli. Usually up to five nucleoli can be seen in one nucleus. The nuclear membrane contours are regular. The captured CECs of epithelial character are typically observed to be growing individually, but these epithelial cells are accompanied by stem cell-like cells as seen in Figure 2.

The average size of captured CECs of the stem cell-like type was 24.0 ± 1.2 μm. Stem cell-like cells are usually bigger and rather pale green in comparison with the bright green epithelial cells. Stem cell-like cells are characterized by having a bigger and smoother nucleus (no chromatin clumps). They usually proliferate very quickly (Fig 2, arrows) under the conditions of the

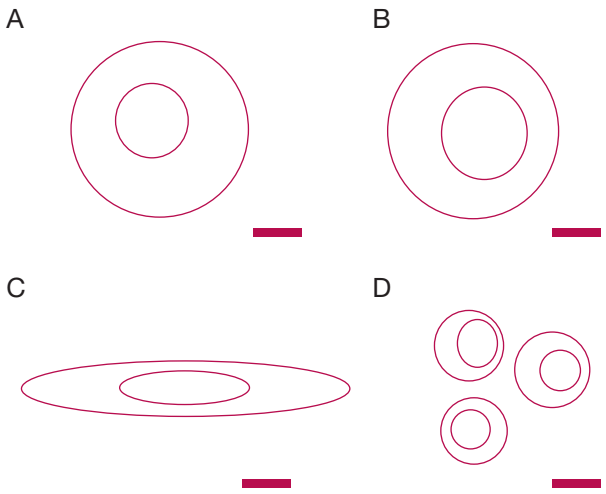

Figure 1 – *A-D, Circulating endometrial cell (CEC)-subtypes present in blood of patients with pneumothorax, identified by size-based separation and subsequent in vitro culture. Four main CEC-subtypes can be found in blood samples of patients with pneumothorax, including (A) epithelial, (B) stem cell-like, (C) stromal, and (D) glandular. Different size of the captured cells is a relatively reliable identification marker. The two most frequent cell subtypes (epithelial and stem cell-like) can be distinguished using fluorescent staining of cytoplasm (eg, CellTracker). Stem cell-like cells usually have rather pale green cytoplasm in comparison with epithelial cells and are usually a little bigger. Bar represents 10 μm.*

in vitro culture and can eventually be found under the microscope in actively proliferating cell clusters. Cytomorphologically similar stem cell-like cells were observed in healthy endometrium cultures.

The presence of CEC stromal cells was confirmed in blood and pleural washings as was expected. The stromal cells are known to be the direct supporters of growing epithelia in the endometrium. Sometimes the cells with stromal-like features are present in the multinuclear stage (Fig 3A). The presence of stromal cells likely supports growth of glandular epithelial cells. The average size of captured CECs of the stromal-like cell subtype was 40.0 ± 5.2 μm (Fig 3B). The glandular epithelial cells are usually found to form uniquely shaped cavities (Figs 3C, 3D). The average size of the glandular cells identified was 9.0 ± 1.2 μm. (More details on CEC cytomorphology can be seen in a CEC gallery published via the web link in e-Table 3.)

Molecular Character of CECs

Molecular analysis was performed to describe the characteristics of enriched CECs to confirm their epithelial and/or endometrial origin. The CEC cytomorphologic diversity as described in the cytomorphologic part of the results is mirrored in the GEA results. Both epithelial and nonepithelial marker expression were detected in the CEC samples by GEA. Detailed GEA data are described in e-Figures 1-11.

In short, the comparisons showed there is a significant difference between CEC pneumothorax samples (CECs-SP) and corresponding WBC fractions in expression of the following genes: *VIM*, *KRT18*, *NANOG*, *CD10*, and *ESRRA* ($P \leq .05$) (e-Fig 1). Genes with elevated expression in CEC-SP samples are listed for every patient in e-Table 4.

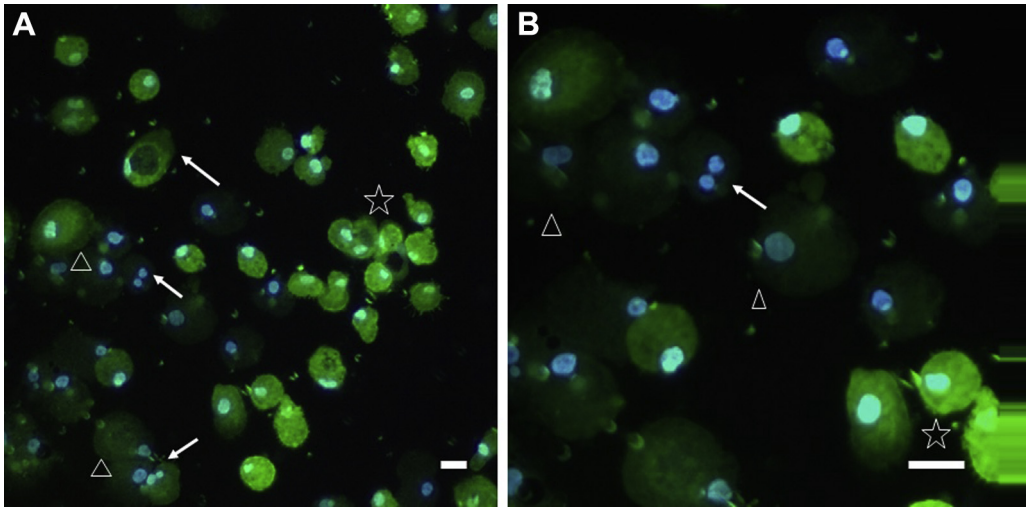

Figure 2 – *A-B, CEC-subtypes present in blood, identified by size-based separation and subsequent in vitro culture after vital fluorescent staining in spontaneous pneumothorax. The most abundant CEC-subtype found in blood samples of patients with pneumothorax is epithelial (A and B, assigned with a ☆) cells with bright green cytoplasm. The epithelial cells are usually accompanied by stem cell-like cells (A and B, assigned with a △), which are a little bigger than epithelial cells and have a bigger and smoother nucleus and pale green (almost not visible) cytoplasm. These stem cells usually proliferate very quickly (arrows) under the conditions of the in vitro culture. It may be of importance in endometriosis treatment to distinguish these CEC-subtypes properly. Bars represent 10 μm. See Figure 1 legend for expansion of abbreviation.*

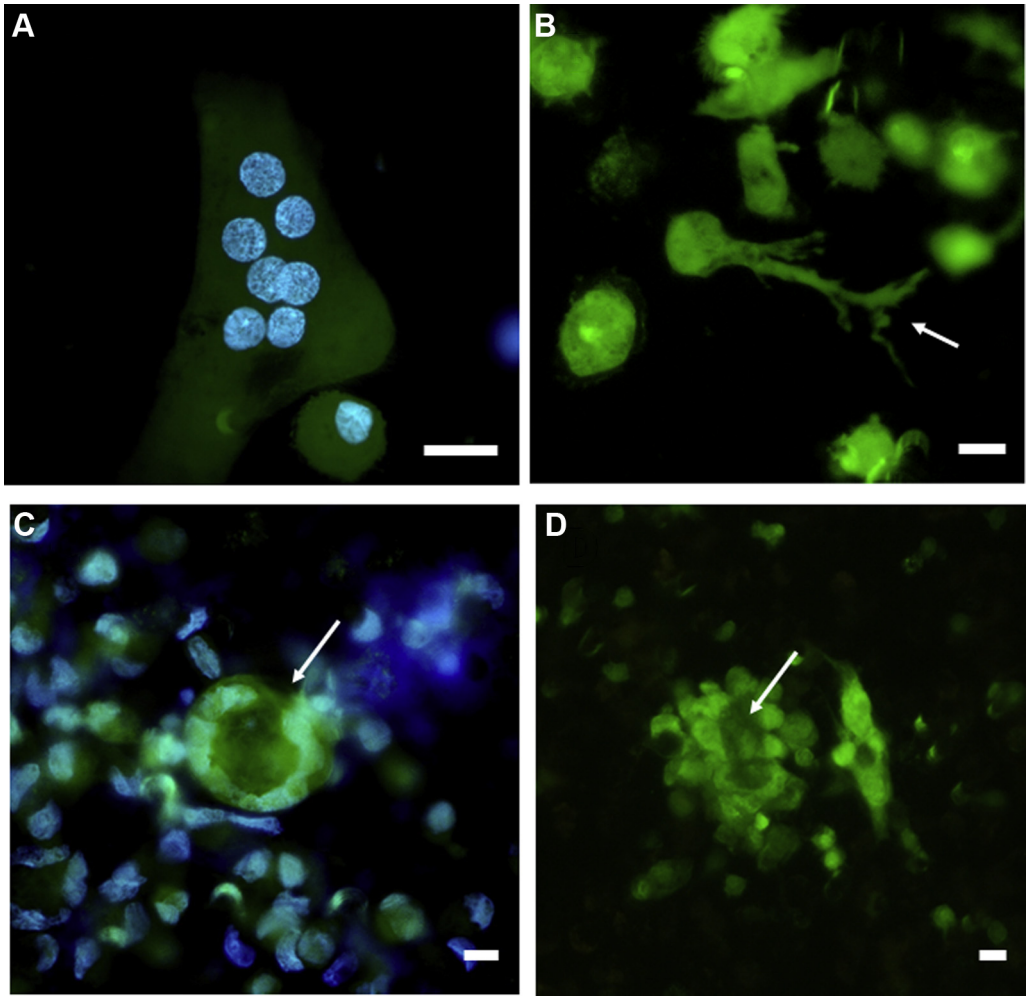

Figure 3 – *A-D, CECs present in pleural washings of women admitted to the hospital with pneumothorax, shown after vital fluorescent staining. CECs separated out of pleural effusion samples in pneumothorax cases exhibit mainly stromal-like character, where multinuclear cells can be identified (A) and stromal cells do have typical long pseudopodia-like structures (arrow) (B). The smaller cells most probably could be assigned as glandular; they do form unique structures (C and D) where the cells try to form a cavity (arrows). These cavity-like structures were observed in cell cultures grown from healthy endometrium tissue as well. Bars represent 10 µm. See Figure 1 legend for expansion of abbreviation.*

We have identified two distinct CECs-SP phenotypes comparing gene expression data: the first phenotype is related to the diaphragm endometriosis pneumothorax, and the second is related to the pleura pneumothorax episodes (e-Figs 2-4).

Elevated expression of ESR was observed in CECs-SP of new spontaneous pneumothorax cases when compared with the recurrent SP episodes (e-Fig 5).

Next, CECs-SP were compared with CECs from pelvic endometriosis samples (CECs-non-SP group). There was significantly higher expression of *HER2* in CECs-SP ($P \leq .05$) (e-Figs 6, 7; Fig 4). *HER2* in combination with *KRT18* could present a very specific identification tool for CECs connected to pneumothorax episodes. Interestingly, CECs-non-SP exhibited higher *VEGF* expression than CECs-SP.

Patients with high-positivity CEC rates had an elevated expression of *MUC1* and *MUC16*, which are thought to also be pelvic endometriosis-related markers. Differences could be seen among all tested groups by GEA applying cluster analysis.

The CECs in the compared groups (CECs-SP and CECs-non-SP) showed elevated *KRT18* and *VIM* expression when compared with healthy endometrium (e-Figs 8-11), impressing individual pathophysiologic path and diagnostic entity of CP.

Immunohistochemistry Analysis

VIM detection by immunohistochemistry confirmed possible endometrial origin of the captured cells (Fig 5). Significantly higher levels of VIM were detected among CECs in the group of patients with pelvic

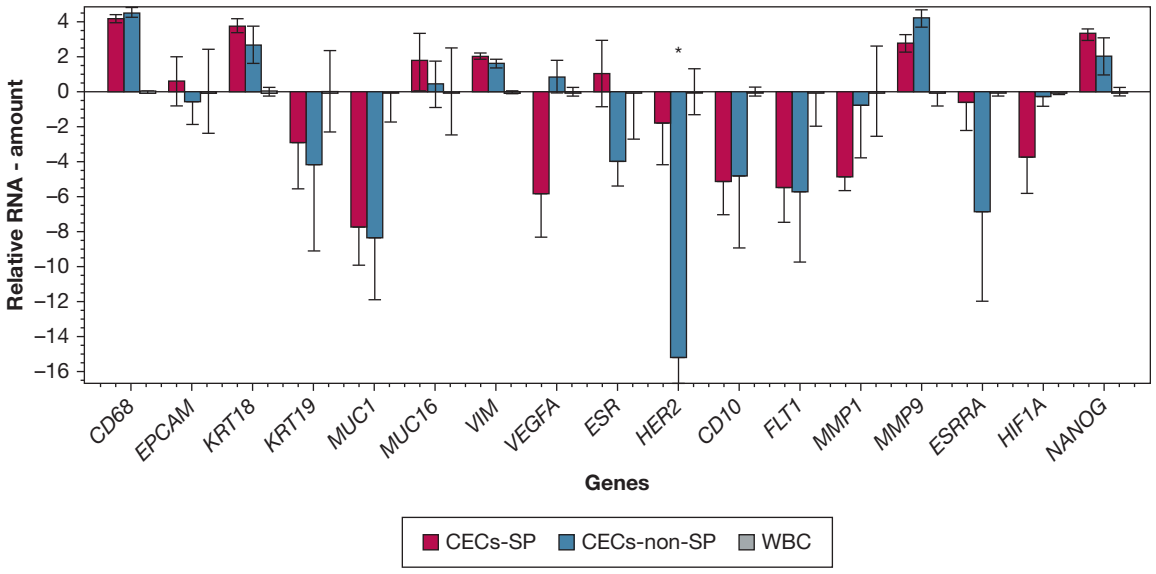

Figure 4 – *Comparison of gene expression in CECs-SP (red) with CECs-non-SP (blue) and WBC fraction (gray). *There was a significant difference for the following gene if CECs-SP and CECs-non-SP were compared: HER2 gene ($P \leq .05$). KRT18, and NANOG were elevated in CECs-SP nonsignificantly. CECs-non-SP = CECs in pelvic endometriosis; CECs-SP = CECs in pneumothorax. See Figure 1 legend for expansion of other abbreviation.*

endometriosis. However, VIM-positive CECs-SP were bigger than those isolated from patients with pelvic endometriosis (49 ± 12 vs 37 ± 9 μm, respectively). More details on CECs expressing VIM are shown in e-Figures 1-11 and e-Table 3.

Discussion

To our knowledge, this is the first study reporting on the presence and characterization of CECs in SP with catamenial character.

As shown several years ago, there is a measurable population of circulating endometrial-like cells (CECs) in the blood of patients with confirmed endometriosis.[31] The main hypothesis of the presented work was to show that CECs could be detected in SP cases. The identification of these cells could expedite diagnosis of CP and subsequently assist in recurrent CP episode prevention.

The data report on 20 cases of women with SP with catamenial character with CECs detected in all tested blood samples. The CECs were represented by four main cytomorphologic subtypes: epithelial, stem cell-like, stromal, and glandular. Most of the CECs in the pneumothorax were epithelial and stem cell-like. Based on previous transcriptomic data comparing healthy endometrium tissue and eutopic endometrium tissue,[32-40] it was proposed that specific gene expression profiles could be found in CECs, especially in CECs associated with SP and/or CP. Patients with CP in our study represent a rather homogenous group of patients, where the character of the CECs refers to the menstrual phase of the cycle because all CP episodes were diagnosed in the early menstrual phase.

The CECs in the compared groups (CECs-SP and CECs-non-SP) have shown elevated *KRT18* and *VIM* expression when compared with healthy endometrium. The high expression of *VIM* in the CECs in both groups in our study showed that the CECs may be more mesenchymal, which probably potentiates invasion and accelerates growth of endometriotic lesions. There is higher VIM expression in CECs associated with pelvic endometriosis compared with those found in pneumothorax, as shown by immunohistochemistry as well.

CECs in pelvic endometriosis (CECs-non-SP) exhibited higher *VEGF* expression than CECs-SP. *VEGF*, as a key mediator of angiogenesis having its specific place in endometrium cyclic life, is very tight connected to its two high-affinity receptors on the surface of microvascular endothelial cells (ie, *VEGFR-1*, *VEGFR-2*). Hull et al[41] were the first to report that treatment with *FLT-1*/*VEGFR1* or *VEGF* antibody could significantly inhibit the growth of endometriotic lesions in mice by disrupting immature microvasculature of endometriosis.

The answers to our original questions are as follows. First, relating to this study, all patients with catamenial character of SP had been positive for CECs. Early isolation of CECs during primary admission to hospital

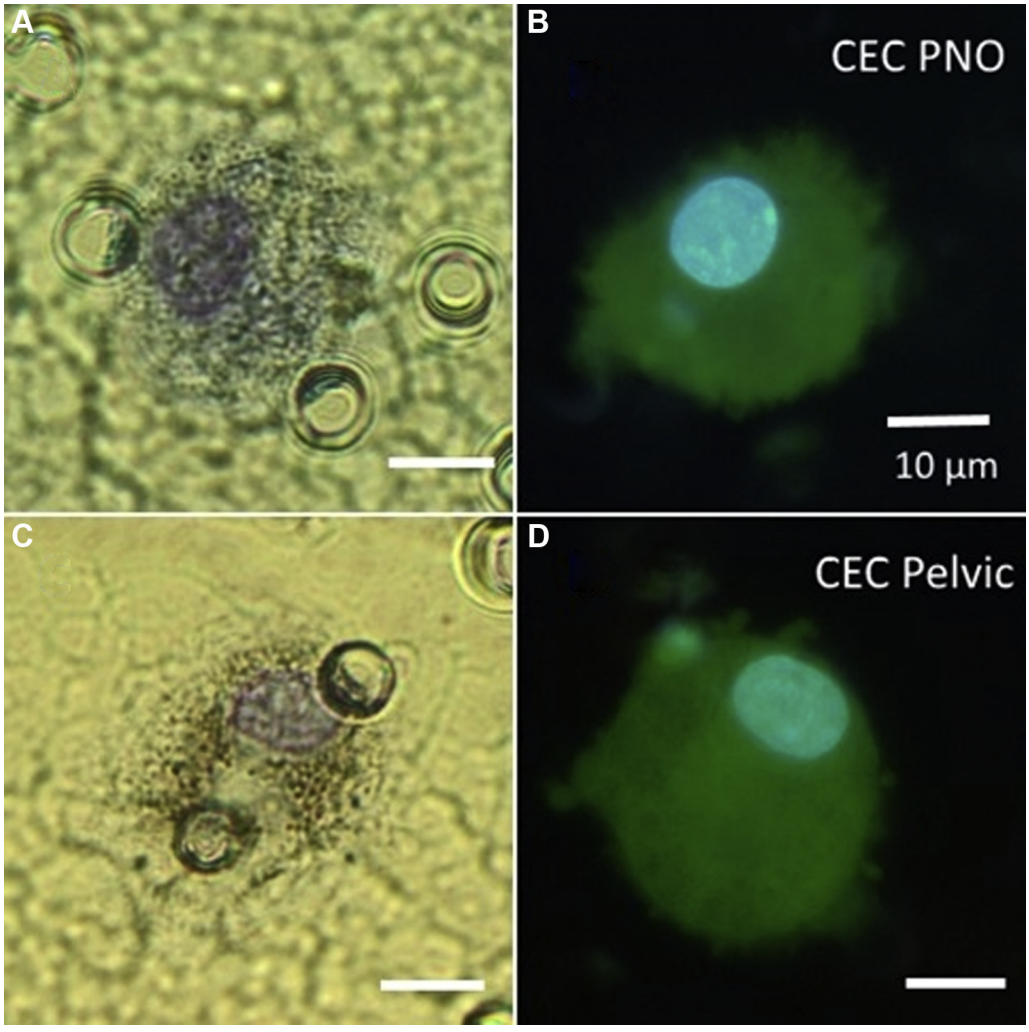

Figure 5 – *A-D, CECs present in blood, enriched by size-based separation cultured in vitro, displayed by fluorescent microscopy (B and D), and fixed and stained by immunohistochemistry, confirming vimentin presence (A and C). There was a higher expression of vimentin in CECs isolated from the patients with pelvic endometriosis. More figures are available in the website listed in e-Table 3. See Figure 1 legend for expansion of abbreviation.*

could identify patients with CP over SP occurring from other etiology. Second, detection of *VIM* by immunohistochemistry (identified as the marker being present in all CEC samples based on GEA) proved mesenchymal and/or endometriosis character. Cytomorphology and especially GEA of CECs, endometriosis tissue from patients with CP, pelvic endometriosis, and healthy control subjects showed some common features. On the other hand, differences could be seen among all groups by GEA applying cluster analysis (e-Fig 5), impressing individual pathophysiologic path and diagnostic entity of CP.

Third, because the clear etiology of CP is still unknown, all the CP causes based on the presented hypotheses could play a role in the CP process. Gene expression profiles of CEC samples from two patients with SP and CP of different types (diaphragm vs pleural) provide evidence that two distinct CEC phenotypes can distinguish two pathways of pneumothorax appearance. This assumption was endorsed from the surgery protocol, in which fenestrations of the diaphragm were found in the first patient, whereas intact diaphragm with no communication of the abdomen with the thoracic cavity was reported in the second case.

Finally, because CP is in relation with pelvic endometriosis, detection of CECs in SP cases should raise suspicion of endometriosis, and patients should be referred for further gynecologic examination.

The phenomena of CEC presence could be helpful. If based on their molecular character, it would be possible to stop the new CEC release out of primary endometriosis lesions. The studies of endometriosis tissue and endometrial cells in circulation will never be straightforward because of

difficulties in obtaining enough tissue suitable for the genetic studies (especially from peritoneal lesions), considering that the hormonal changes have a significant impact on the CEC behavior.

Before detection and evaluation of CECs become routine clinical practice, additional studies need to be conducted on patients with endometriosis and healthy control subjects.

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Additional information: The e-Figures and e-Tables can be found in the Supplemental Materials section of the online article.

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