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

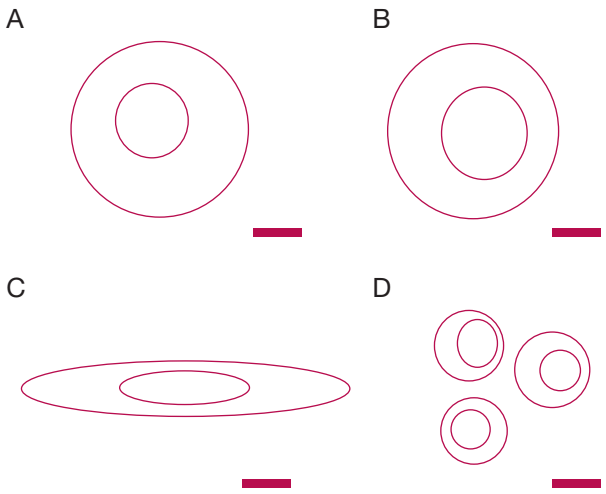

Figure 1 – *A-D, Circulating endometrial cell (CEC)-subtypes present in blood of patients with pneumothorax, identified by size-based separation and subsequent in vitro culture. Four main CEC-subtypes can be found in blood samples of patients with pneumothorax, including (A) epithelial, (B) stem cell-like, (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.

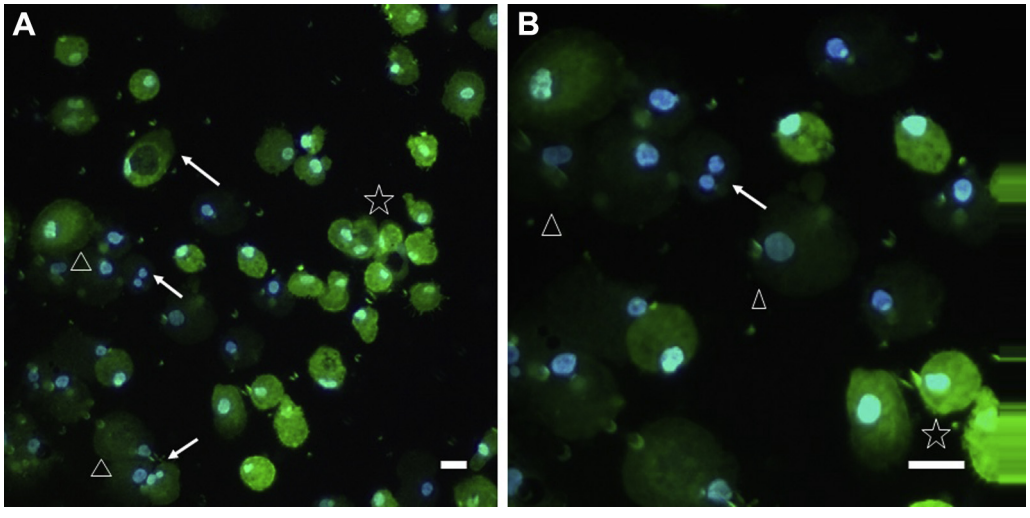

Figure 2 – *A-B, CEC-subtypes present in blood, identified by size-based separation and subsequent in vitro culture after vital fluorescent staining in spontaneous pneumothorax. The most abundant CEC-subtype found in blood samples of patients with pneumothorax is epithelial (A and B, assigned with a ☆) 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.*

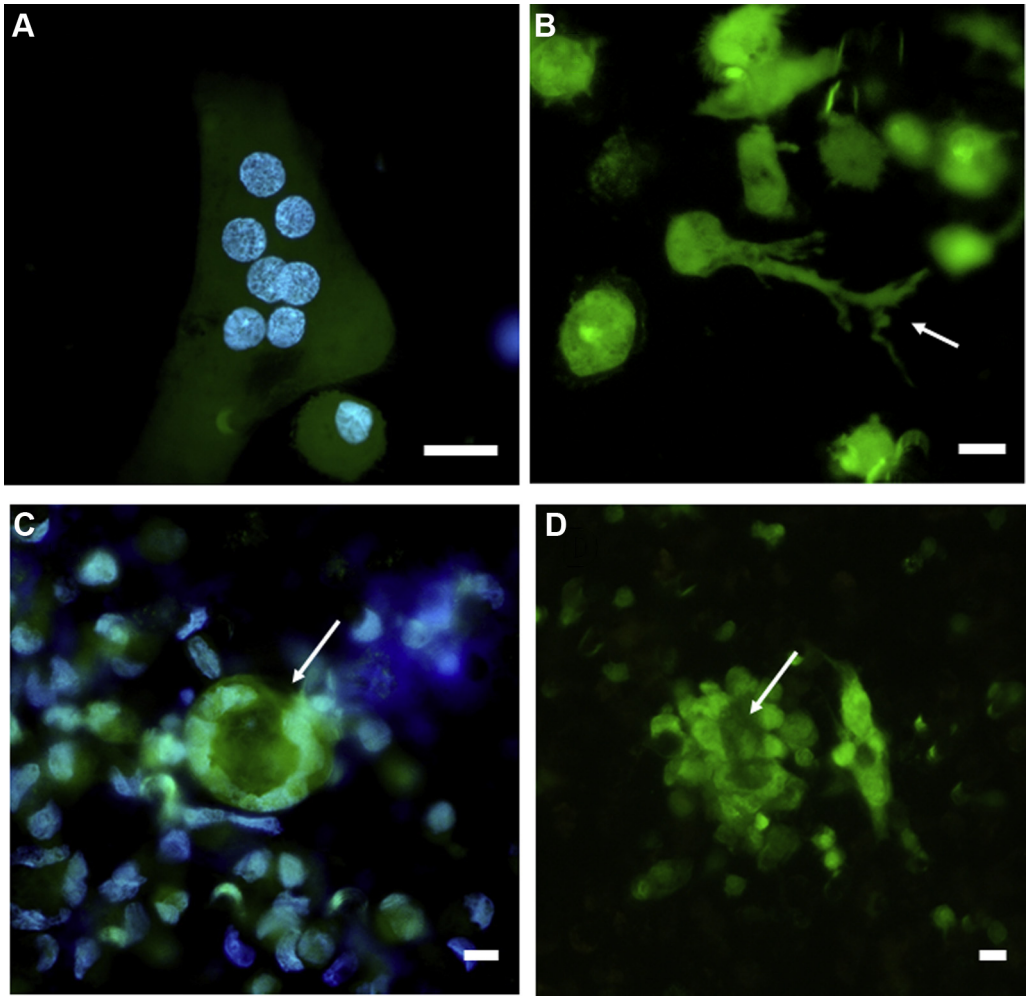

Figure 3 – *A-D, CECs present in pleural washings of women admitted to the hospital with pneumothorax, shown after vital fluorescent staining. CECs separated out of pleural effusion samples in pneumothorax cases exhibit mainly stromal-like character, where multinuclear cells can be identified (A) and 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

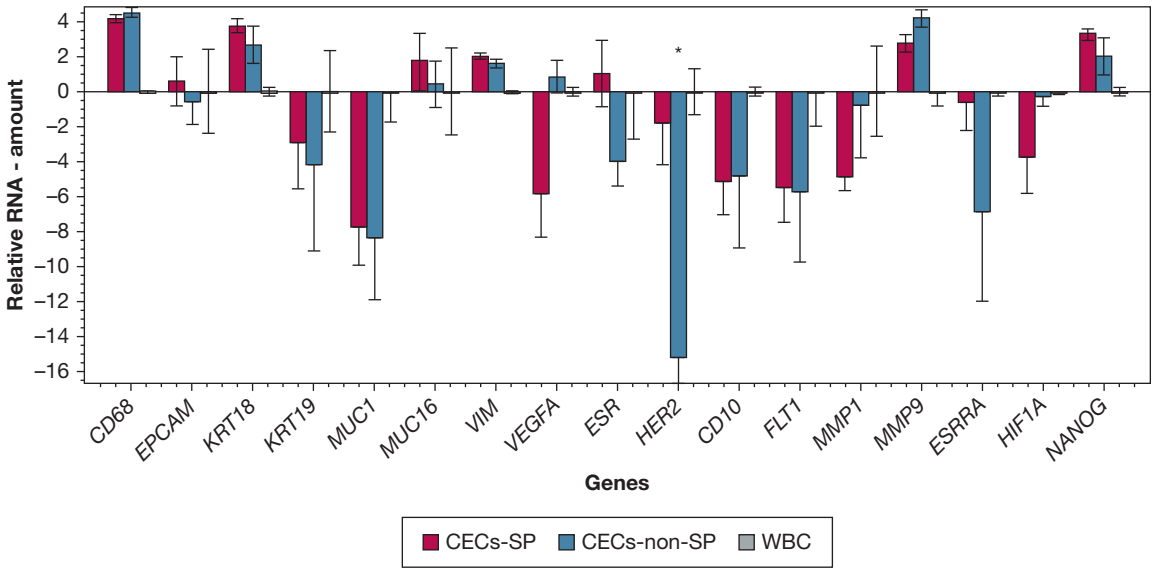

Figure 4 – *Comparison of gene expression in CECs-SP (red) with CECs-non-SP (blue) and WBC fraction (gray). *There was a significant difference for the following gene if CECs-SP and CECs-non-SP were compared: HER2 gene ($P \leq .05$). KRT18, and NANOG were elevated in CECs-SP nonsignificantly. CECs-non-SP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

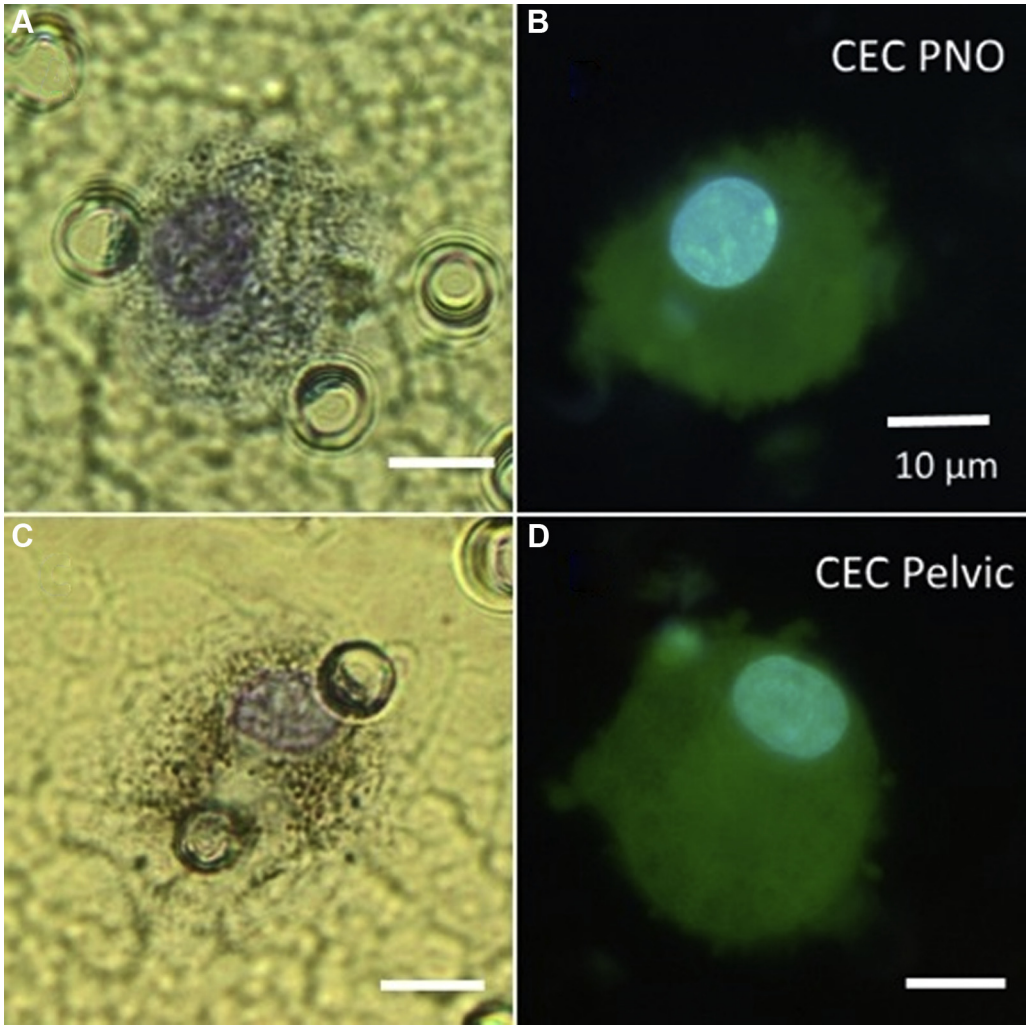

Figure 5 – *A-D, CECs present in blood, enriched by size-based separation cultured in vitro, displayed by fluorescent microscopy (B and D), and fixed and stained by immunohistochemistry, confirming vimentin presence (A and C). There was a higher expression of vimentin in CECs isolated from the patients 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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