

Circulating tumor cells help differentiate benign ovarian lesions from cancer before surgery: A literature review and proof of concept study using flow cytometry with fluorescence imaging

YUNG-CHIA KUO^{1-3*}, CHI-HSI CHUANG^{4*}, HSUAN-CHIH KUO¹⁻³, CHENG-TAO LIN^{3,5,6}, ANGEL CHAO^{3,5,6}, HUEI-JEAN HUANG^{3,5,6}, HUNG-MING WANG^{2,3}, JASON CHIA-HSUN HSIEH¹⁻³ and HUNG-HSUEH CHOU⁵⁻⁷

¹Division of Hematology-Oncology, Department of Internal Medicine, New Taipei Municipal Tucheng Hospital, New Taipei City 236; ²Division of Hematology-Oncology, Department of Internal Medicine, Chang Gung Memorial Hospital at Linkou; ³Department and College of Medicine, Chang Gung University, Taoyuan 333; ⁴Department of Pediatrics, New Taipei Municipal TuCheng Hospital, New Taipei City 236; ⁵Department of Obstetrics and Gynecology; ⁶Gynecologic Cancer Research Center, Chang Gung Memorial Hospital at Linkou, Taoyuan 333; ⁷Department and School of Medicine, National Tsing Hua University, Hsinchu 300044, Taiwan, R.O.C.

Received October 26, 2023; Accepted February 19, 2024

DOI: 10.3892/ol.2024.14367

Abstract. Current tools are insufficient for distinguishing patients with ovarian cancer from those with benign ovarian lesions before extensive surgery. The present study utilized a readily accessible platform employing a negative selection strategy, followed by flow cytometry, to enumerate circulating tumor cells (CTCs) in patients with ovarian cancer. These counts were compared with those from patients with benign ovarian lesions. CTC counts at baseline, before and after anti-cancer therapy, and across various clinical scenarios involving ovarian lesions were assessed. A negative-selection protocol we proposed was applied to patients with suspected ovarian cancer and prospectively utilized in those subsequently confirmed to have malignancy. The protocol was implemented before anticancer therapy and at months 3, 6, 9 and 12 post-treatment. A cut-off value for CTC number at 4.75 cells/ml was established to distinguish ovarian malignancy from benign lesions, with an area under the curve of 0.900 ($P < 0.001$). In patients with ovarian cancer, multivariate Cox regression analysis revealed

that baseline CTC counts and the decline in CTCs within the first three months post-therapy were significant predictors of prolonged progression-free survival. Additionally, baseline CTC counts independently prognosticated overall survival. CTC counts obtained with the proposed platform, used in the present study, suggest that pre-operative CTC testing may be able to differentiate between malignant and benign tumors. Moreover, CTC counts may indicate oncologic outcomes in patients with ovarian cancer who have undergone cancer therapies.

Introduction

Ovarian cancer is the fifth most common cause of cancer-related mortality worldwide (1). In 2017, the incidence of epithelial ovarian cancer (EOC) in the USA was 9.4 per 100,000 (2) and in 2020, it was 9.19 per 100,000 in Taiwan (3). The primary treatment for advanced EOC involves optimal debulking surgery with the aim of no residual disease (R0), followed by platinum-paclitaxel combination chemotherapy (4). Maintenance therapy with bevacizumab or a poly(ADP-ribose) polymerase inhibitor has been reported to extend progression-free survival (PFS) following first-line chemotherapy (5,6). However, despite advancements in surgery and systemic chemotherapy, the majority (~80% according to stages) of patients experience recurrent disease, leading to a 5-year overall survival (OS) rate of <50% across all stages of EOC (7-9). Early detection through modern liquid biopsies for new or recurrent cancer remains one of the primary challenges in managing ovarian cancers.

The use of blood biomarkers for monitoring cancer status or recurrence, carcinoembryonic antigen (CEA) (10), carbohydrate antigen 19-9 (11), human epididymis secretory protein 4 (12), apolipoprotein A1 (13), transthyretin (14), transferrin (15) and β 2-microglobulin (16), is well documented. Although these markers could facilitate earlier detection of recurrence, their utility is limited by inadequate sensitivity or specificity (16,17). Considering the high recurrence rate and poor prognosis following EOC recurrence, identifying

Correspondence to: Professor Jason Chia-Hsun Hsieh, Division of Hematology and Oncology, Department of Internal Medicine, New Taipei Municipal Tucheng Hospital, 6 Section 2 Jincheng Road, Tucheng, New Taipei City 236, Taiwan, R.O.C.
E-mail: wisdom5000@gmail.com

Professor Hung-Hsueh Chou, Department of Obstetrics and Gynecology, Chang Gung Memorial Hospital at Linkou, 5 Fushing Street, Taoyuan 333, Taiwan, R.O.C.
E-mail: ma2012@cgmh.org.tw

*Contributed equally

Key words: circulating tumor cells, liquid biopsy, ovarian cancer, survival, dynamic changes, prognosis

effective methods to stratify patients at elevated risk of recurrence for further therapy following first line treatment and to enable earlier detection of recurrence is of importance (18).

Ashworth (19) first reported a biomarker, the circulating tumor cell (CTC), in the peripheral blood of a patient with metastatic disease. Studies have demonstrated that CTCs, shed by ovarian cancer, disseminate to distant organs through the bloodstream, notably contributing to ovarian cancer metastasis (20–22). Although CTCs in EOC have been assessed for their prognostic value, the results have been inconclusive (23), primarily due to technological limitations. Consequently, CTC enumeration remains a challenge because of the scarcity of CTCs in peripheral blood samples (24). The US Food and Drug Administration (FDA) has approved only the CellSearch system, which uses EpCAM antibodies to measure CTCs. However, its establishment in the clinical treatment of EOC has not occurred (25). The use of CellSearch is limited by the low availability of devices and a low positive detection rate (26). We have previously reported a protocol employing a negative selection strategy followed by flow cytometry to precisely identify CTCs in blood (27). This method has been effective for cancers of the head and neck, colon, lung and breast, and for neuroendocrine tumors. The benefits of negative selection-based CTC enumeration platforms include: i) Label-free characteristics, allowing for further molecular analysis; ii) preservation of the heterogeneity of CTCs that express atypical epithelial markers; and iii) improved recovery and positive detection rates (28–31). However, this CTC enumeration platform has not previously been evaluated in patients with EOC.

The present study employed a novel technique for CTC enumeration and analysis, and a novel platform for CTC testing in patients with benign ovarian tumors and those with EOC. The objectives were to evaluate: i) The accuracy of the technique in distinguishing malignancy from benign ovarian masses and ii) the feasibility of using baseline CTC counts and decreased CTC levels post-anticancer therapy as prognostic factors for oncologic outcomes, such as survival.

Materials and methods

Patient enrollment. A prospective study was performed at Chang Gung Memorial Hospital (Linkou, Taiwan), enrolling patients with ovarian cancer at various stages, including new diagnosis, surveillance, and recurrent/unresectable or metastatic disease. Additionally, healthy female subjects without ovarian lesions were enrolled as controls. The Institutional Review Board of Chang Gung Memorial Hospital approved the study protocols (approval nos. 201802203B0C502 and 201601461B0). All participants provided written informed consent. Inclusion criteria for eligible patients were as follows: i) Age, ≥ 20 years; ii) understood and consented to the study protocol voluntarily; iii) had suspected new ovarian cancer or histologically confirmed EOC; and iv) had adequate (within normal range) liver and renal function and white blood cell counts before undergoing surgery or anticancer therapies. Exclusion criteria included: i) Refusal of anticancer therapy; ii) non-consent to the blood drawing schedule; or iii) the presence of metachronous or synchronous double cancers. Physicians staged and managed the disease according to institutional and National Comprehensive Cancer Network guidelines (4). Results were reported following the Reporting Recommendations for Tumor Marker Prognostic Studies (32).

Treatment responses were evaluated using CA125 measurement and imaging studies, including computed tomography, magnetic resonance imaging and positron emission tomography scans, according to version 1.1 of the Response Evaluation Criteria in Solid Tumors. Responses were categorized as complete remission, partial response, stable disease or progressive disease (PD). Diagnoses and treatment plans were reviewed at a weekly multidisciplinary gynecologic cancer tumor board meeting at Chang Gung Memorial Hospital, with gynecologic oncologists, diagnostic radiologists, pathologists, nuclear medicine physicians and radiation oncologists in attendance.

Sample preparations for circulating tumor cell testing. Blood samples from patients with EOC (4 ml each for microscopy and flow cytometry) were collected at enrollment (before anticancer therapy) and at months 3, 6, 9 and 12 post-treatment, between August 2019 and May 2021. For patients with suspected ovarian malignancy (subsequently confirmed as benign by pathology), blood samples were collected only once before surgery. CTC enrichment was achieved using red blood cell (RBC) lysis (by mixing 155 mM NH_4Cl , 14 mM NaHCO_3 and 0.1 mM EDTA at a 10:1 ratio with whole blood samples) and CD45-positive leukocyte depletion using EasySep Human CD45 Depletion Kits (cat. no. 18259; Stemcell Technologies Inc.) according to the manufacturer's instructions. The methods used for CTC enrichment and counting have been previously described (27,33,34). CTCs were not collected from patients experiencing disease progression or death from cancer, as these were the predefined endpoints of the study for predicting survival events.

Identification of CTCs by microscopy. CTCs isolated from 4 ml of whole blood samples were fixed using 4% paraformaldehyde for 10 min at 25°C. Cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min at 25°C. Following a PBS wash, cells were blocked with 2% bovine serum albumin and a HuFcR binding inhibitor (cat. no. 14-9161-73; eBioscience; Thermo Fisher Scientific, Inc.) for 30 min at room temperature. To reduce autofluorescence, 0.0025% Trypan Blue (cat. no. 15250061; Thermo Fisher Scientific, Inc.) was added before the antibody reaction. Cells were then incubated with anti-EpCAM antibody conjugated to Alexa Fluor 488 (1:400 dilution; cat. no. 5198S; Cell Signaling Technology, Inc.) for 1 h at 25°C and anti-p16 antibody conjugated to Alexa Fluor 647 (1:200 dilution; cat. no. ab199819; Abcam) overnight at 25°C. Nuclei were stained with Hoechst (10 $\mu\text{g}/\text{ml}$; cat. no. 62249; Thermo Fisher Scientific, Inc.) for 10 min at 25°C. Fluorescence images were captured using a Zeiss Axioskop 2 Plus Fluorescence Microscope (Carl Zeiss AG) and a Leica TCS SP2 Confocal Laser Scanning Microscope (Leica Microsystems GmbH). CTCs were defined as cells that: i) Exhibited definite evidence of epithelial cell differentiation (EpCAM-positive); ii) lacked characteristics of normal white blood cells (CD45-negative); and iii) possessed a nucleus (Hoechst-positive, to exclude non-nucleated blood impurities such as red blood cells). Throughout the experiment, the HeLa cell line (purchased from the Bioresource Collection and Research Center Taiwan; human cervical cancer cell line expected to stain as Hoechst+CD45-EpCAM-) and the H1975 cell line (purchased from the Bioresource Collection and Research Center Taiwan; human colon cancer cell line expected to stain as Hoechst+CD45-EpCAM+), alongside white blood

cells from healthy subjects (Chang Gung Memorial Hospital IRB approval nos. 201802203B0C502 and 201601461B0; control healthy cells expected to stain as Hoechst+CD45+EpCAM-) as an internal control were utilized for microscopic observation of patient specimens.

Analysis and enumeration of CTCs using flow cytometry. Cells enriched through RBC lysis and CD45 depletion were fixed with Fix & Perm Cell Permeabilization Reagents (100 μ l both for Fix and Permeabilization reagents; cat. no. GAS003; Thermo Fisher Scientific, Inc.) for 20 min at 25°C. Subsequently, cells were incubated with an anti-EpCAM antibody conjugated to phycoerythrin (1:400 dilution; cat. no. FAB960P-100; R&D Systems, Inc.) for 1 h at 25°C. To further exclude residual CD45-positive leukocytes, a goat anti-mouse IgG H&L secondary antibody conjugated to Alexa Fluor 488 (1:2,000 dilution; cat. no. ab150113; Abcam) was applied for 30 min at 4°C to label CD45 antibodies from the aforementioned CD45 depletion kit. Isotype-control antibodies (1:400 dilution; cat. no. IC108P; R&D Systems, Inc.) applied for 1 h at 25°C served as the negative control. Following staining, the cell samples were assessed using a CytoFLEX Flow Cytometer (Beckman Coulter, Inc.). To conduct CTC counting using the flow cytometer, two-dimensional displays (dot plots) were used to quantify cells that met predefined criteria. Briefly, the gating strategy contained six steps. First, the Hoechst+ cells were gated in 2 ml samples from all events to avoid cell debris and fragmentations after the negative selection process (Fig. S1A). Then, singlet cells were gated to avoid false positive results due to cell aggregation (Fig. S1B). CD45+ cells were then excluded to avoid residual white blood cell contamination (Fig. S1C). Before CTC enumeration, EpCAM+ (and its isotype+) cells were independently gated (Fig. S1D and H). Finally, the CTC count was defined as the number of EpCAM+ cells minus the number of cells gated using its isotype.

Statistical analysis. Descriptive statistics were used to present the basic characteristics of the enrolled patients. One-way ANOVA with Bonferroni's correction was used to assess CTC count differences among groups (malignancy, benign lesion and healthy donors). The staging criteria utilized in this study adhere to the American Joint Committee on Cancer 8th edition, incorporating pathologic staging of tumor (pT), lymph node (pN) and distant metastasis (pM) (35). PFS was calculated as the time from the CTC sampling date to cancer-specific progression, recurrence or death from any cause. To demonstrate the importance of longitudinal follow-up for CTC counts, patients with post-treatment CTC counts lower than their baseline at their first (month 3) sampling were categorized as the 'CTC decline group'; all others were placed in the 'no CTC decline group'. OS was defined as the time from CTC sampling to death from any cause. Receiver operating characteristic (ROC) curves and the Youden index were used to evaluate the differentiating accuracy and cut-off values of CTC counts. Kaplan-Meier survival plots and the log-rank test were used to assess factors affecting survival. Patients without disease progression or death (no event for PFS or overall survival) were censored but still contributed to the final statistical analysis. After confirming assumed clinicopathological factors, univariate and multivariate Cox proportional hazard regression models identified independent prognostic factors for PFS and OS. The multivariate analysis included all factors from the univariate

Table I. Basic characteristics of enrolled patients with epithelial ovarian cancer (n=26).

Variable	Value
Age, years	52 (39-76)
Initial symptoms at diagnosis	
Yes	18 (69.2)
No	8 (30.8)
CA-125 at baseline, U/ml	
≥ 35	10 (38.5)
<35	16 (61.5)
Stage (FIGO)	
I-II	11 (42.3)
III-IV	15 (57.7)
Grade	
1	0 (0.0)
2	0 (0.0)
3	25 (96.2)
Not available	1 (3.8)
Histology	
Serous carcinoma	16 (61.5)
Clear cell carcinoma	5 (19.2)
Endometrioid carcinoma	1 (3.9)
Carcinosarcoma	2 (7.7)
Others	2 (7.7)
Lymph node status	
N1	8 (30.8)
N0	18 (69.2)
Surgery before CTC testing	
Yes	9 (34.6)
No	17 (65.4)
Chemotherapy before CTC testing	
Yes	11 (42.3)
No	15 (57.7)
Radiotherapy before CTC testing	
Yes	3 (11.5)
No	23 (88.5)

Values are expressed as the median (range) or n (%). The table does not include information on enrolled patients with benign lesions (n=9) and healthy donors (n=29), as there are no available pathological results for these individuals. FIGO, International Federation of Gynecology and Obstetrics; CTC, circulating tumor cells.

analysis. Statistical analysis was conducted using SPSS (version 18; SPSS Inc.). $P < 0.05$ or 95% CI of hazard ratio (HR) > 1 was considered to indicate a statistically significant difference.

Results

Patient enrollment. Patient enrollment, according to the prospective design, is illustrated in Fig. 1. The characteristics of 26 patients with EOC are presented in Table I, and nine patients with benign ovarian lesions are not listed because no cancer staging information was available. Information of the

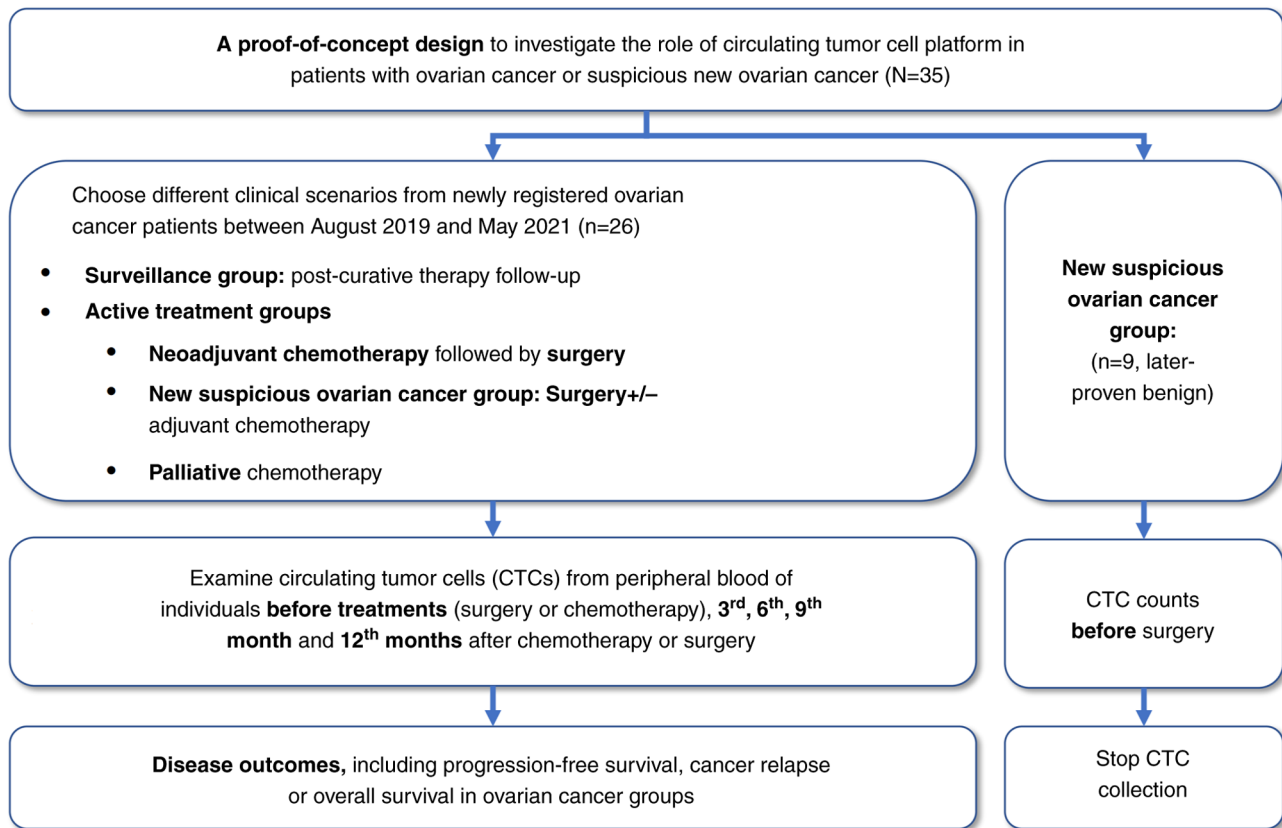


Figure 1. Study flowchart. CTC, circulating tumor cell.

29 healthy controls is not listed because they did not receive any surgery for cancer or suspicious lesion. Difference in age among the three groups were evaluated using ANOVA, resulting in a P-value of 0.110 (Table II). Notably, post-hoc comparisons revealed a difference between cancer [median: 52 (range: 39-76) years] and healthy donors [median: 45 (range: 27-53) years] with a P-value of 0.013. However, there was no significant difference between patients with cancer and benign lesions [median: 46 (range: 23-75) years], as well as between benign lesions and healthy donors (with P-values of 0.107 and 1.000, respectively), after applying Bonferroni correction for multiple tests.

Among 26 patients with cancer, 18 (69.2%) presented with initial symptoms at diagnosis, which included abdominal bloating, abdominal pain, constipation, urinary problems and loss of appetite. A baseline CA125 level ≥ 35 U/ml was observed in 10 (38.5%) patients. Advanced-stage disease [International Federation of Gynecology and Obstetrics (FIGO) stages III and IV] (36) was diagnosed in 15 patients (57.7%), and the majority (96.2%) exhibited grade 3 differentiation. Serous carcinoma was the most prevalent histology type (61.5%), followed by clear cell carcinoma (19.2%), carcinosarcoma (7.7%), other types (7.7%) and endometrioid carcinoma (3.9%). Lymph node involvement was noted in 8 (30.8%) patients. At the time of diagnosis and enrollment, a subset of patients had undergone operations (34.6%), radiotherapy (11.5%) and chemotherapy (42.3%).

Exploratory endpoint-CTC enumeration and identification. CTCs were captured and quantitatively measured using flow

cytometry, with verification using fluorescence microscopy. Fig. S1A-D illustrates the gating processes for counting CTC numbers from a real patient (study subject #006 with ovarian benign lesion). Fig. S1E-H demonstrates the processes of gating isotype control from the sample from the same patient (study subject #006). Fig. S2 demonstrates the images for confirmation of CTC identified. A few samples were excluded or not collected due to the following reasons: i) One patient withdrew from the trial, affecting three samples; ii) disease progression occurred in nine patients at various points during the trial, resulting in the death of five patients and the loss of 13 samples; and iii) eight samples were not collected due to patient-related issues, such as changes in the outpatient clinic schedule. Consequently, of the 89 samples expected, which included those from nine individuals with benign lesions, a total of 56 samples were analyzed. The analysis focused on the serial measurement of CTCs and the impact of CTC reduction in the first three months post-treatment, on survival.

CTC testing accurately differentiates between malignant and benign lesions. Table II demonstrates that CTC counts were significantly different among patients with ovarian cancer, those with benign ovarian lesions and healthy donors ($P < 0.0001$, malignant vs. benign groups; $P < 0.0001$, malignant vs. healthy group). No significant difference was demonstrated between patients with benign ovarian lesions and healthy donors ($P = 0.283$). The area under the curve (AUC) for the ROC curve for distinguishing patients with cancer ($n = 26$) from non-cancer individuals (benign ovarian lesions and healthy donors, $n = 38$) based on CTC number was 0.900, with $P < 0.001$

Table II. CTC counts among different groups.

Variable	Ovarian cancer (n=26)	Benign ovarian lesions (n=9)	Healthy donors (n=29)
Age median, years (range)	52 (39-76)	46 (23-75)	45 (27-53)
CTC counts, cells/ml			
Mean	6.8	1.1	2.4
Median	6.3	0.5	2.0
Standard deviation	3.9	1.5	1.5
Range (min-max)	(0.0-18.0)	(0.0-4.5)	(0.0-6.0)
95% CI	(4.9-8.6)	(0.0-2.3)	(1.8-3.0)

Bonferroni correction was used to adjust the significance values for multiple tests [P-values were both <0.0001 for ovarian cancer vs. benign lesions and ovarian cancer vs. healthy donors, respectively. No significance was observed between the benign ovarian lesions and healthy donors ($P=0.283$)]. The significance of age among the three groups was assessed using ANOVA, yielding a P-value of 0.11. Post-hoc comparisons revealed a P-value of 0.107 between cancer and benign lesions, a P-value of 0.013 between cancer and healthy donors and a P-value of 1.000 between benign lesions and healthy donors after Bonferroni correction for multiple tests. CTC, circulating tumor cell; CI, confidence interval.

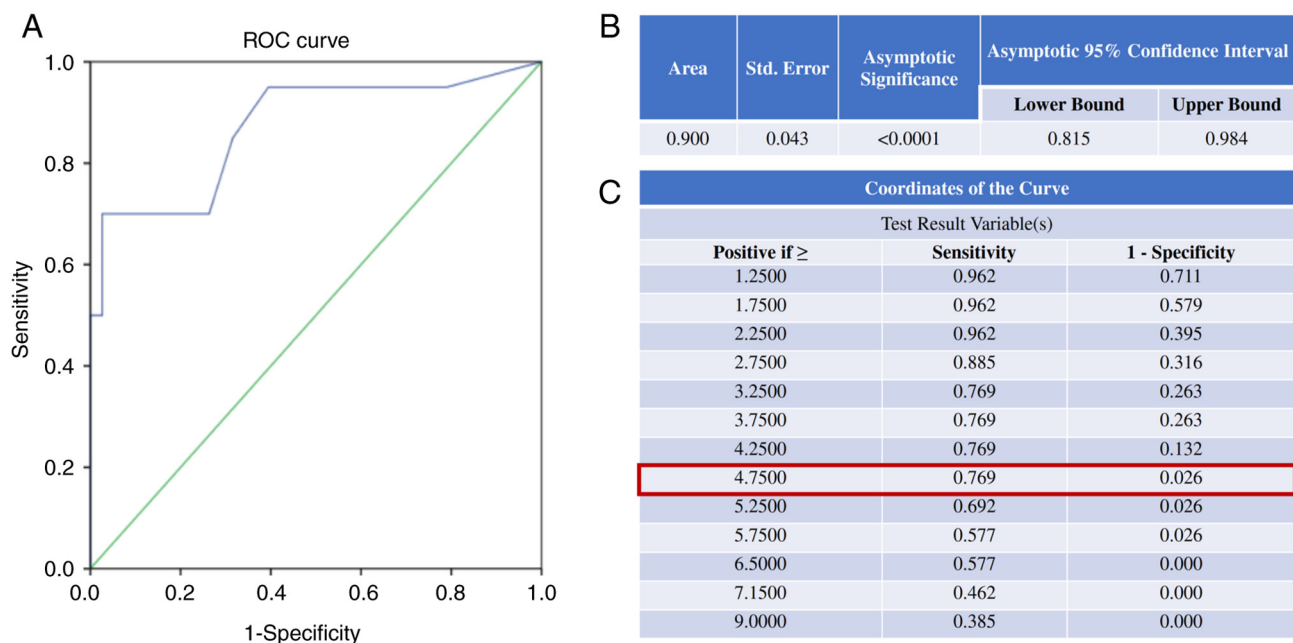


Figure 2. ROC curve and cutoff of CTCs to differentiate between malignant and benign lesions. The (A) ROC curve demonstrates the accuracy of CTC measurements in differentiating between ovarian cancer and benign ovarian lesions, with (B) an area under the curve of 0.900 and $P<0.0001$. (C) Cutoff value was defined as 4.75 cells/ml with a sensitivity of 76.9% and a specificity of 97.4%, which were calculated using the Youden index. ROC, receiver operating characteristic; CTC, circulating tumor cells; Std., standard.

(Fig. 2A and B). The optimal cut-off for CTC number in this cohort, determined using the Youden index, was 4.75 cells/ml, yielding a sensitivity of 76.9% and a specificity of 97.4% (Fig. 2C). Using 29 healthy donors as controls, the accuracy, positive predictive value and negative predictive value were 0.879, 0.933 and 0.860, respectively.

Baseline CTCs and serial CTC testing predict survival. During the study follow-up period, nine patients experienced PD, and five died from the disease after a median follow-up of 10.6 months (range, 0.4-19.0 months). The median PFS for the CTCs ≤ 4.75 cells/ml was not reached, and it was 7.2 months (95% CI: 5.4-9.0) for patients with baseline CTC counts >4.75 cells/ml. The median OS for the entire population was not reached. Baseline

CTC counts (cut-off value at 4.75 cells/ml) may have a significant effect on OS rather than PFS with $P=0.152$ and $P=0.025$ for PFS and OS, respectively (Fig. 3A and B). Conversely, a decline in CTC counts during chemotherapy appears to have a significant effect on PFS but not OS with $P=0.015$ and $P=0.119$ for PFS and OS, respectively (Fig. 3C and D). Median OS was not reached for the entire group after a median follow-up of 29.8 months (range, 0.4 to 49.9 months) until the cut-off date of October 2023.

CTC count represents an independent negative prognostic factor in the multivariate analysis. Univariate and multivariate Cox regression analyses were used to elucidate the prognostic role of CTCs, considering all known potential prognostic

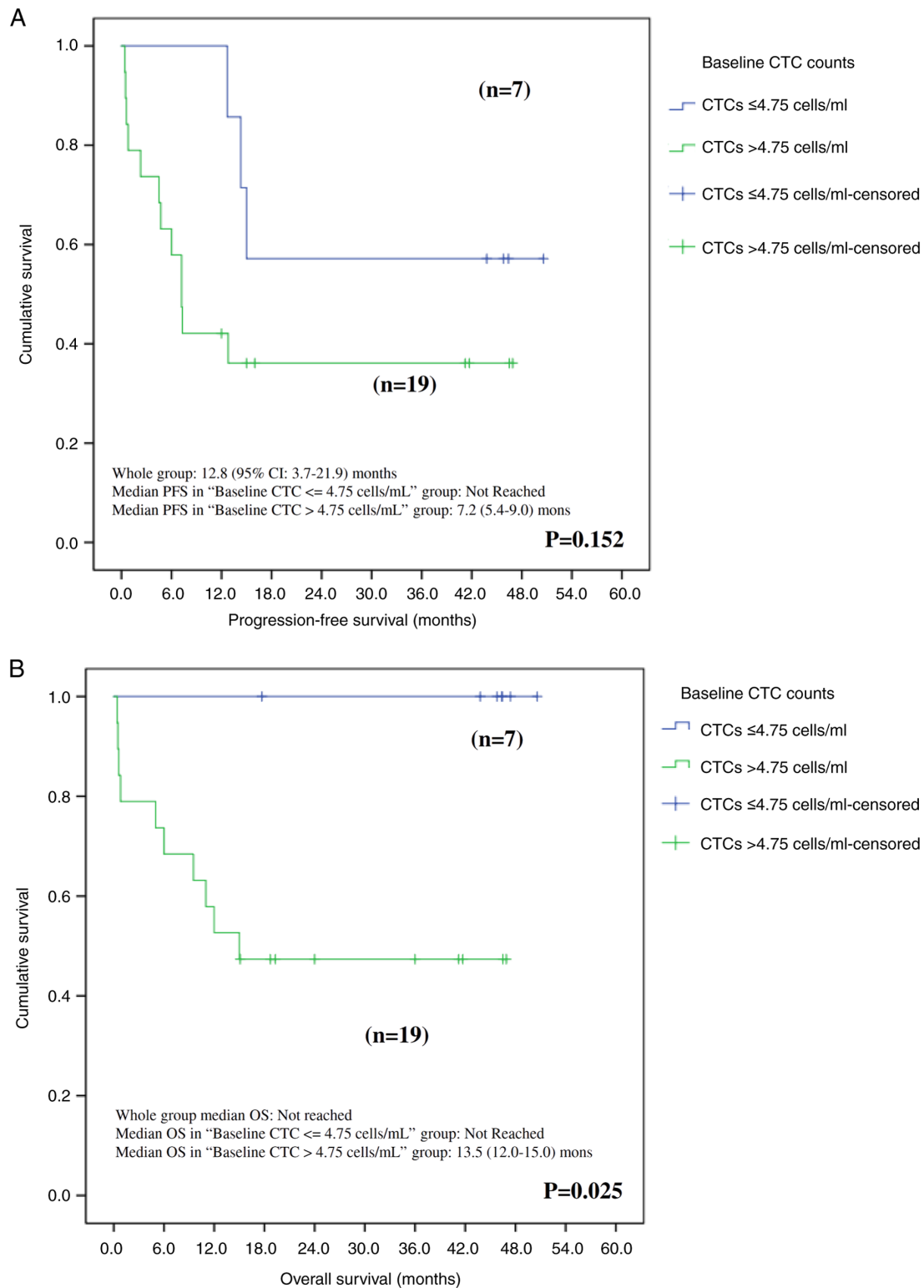


Figure 3. Continued.

factors. In the univariate analysis, age at diagnosis ($P=0.023$), FIGO staging ($P=0.018$), baseline CTC counts ($P=0.030$) and CTC decline within the first three months ($P=0.002$) were identified as prognostic factors for disease progression. In the multivariate analysis assessing the risk of cancer progression, CTC decline ($P=0.024$) and baseline CTC counts ($P=0.011$) remained independent prognostic factors. Regarding cancer mortality, FIGO staging ($P=0.05$) and baseline CTC counts

($P<0.0001$) showed prognostic significance. In the multivariate analysis for the risk of death, the baseline CTC count was the sole independent prognostic factor ($P=0.005$) (Table III).

Discussion

A review and summation of previous studies on CTCs in ovarian cancer as performed (Table IV). PCR-based

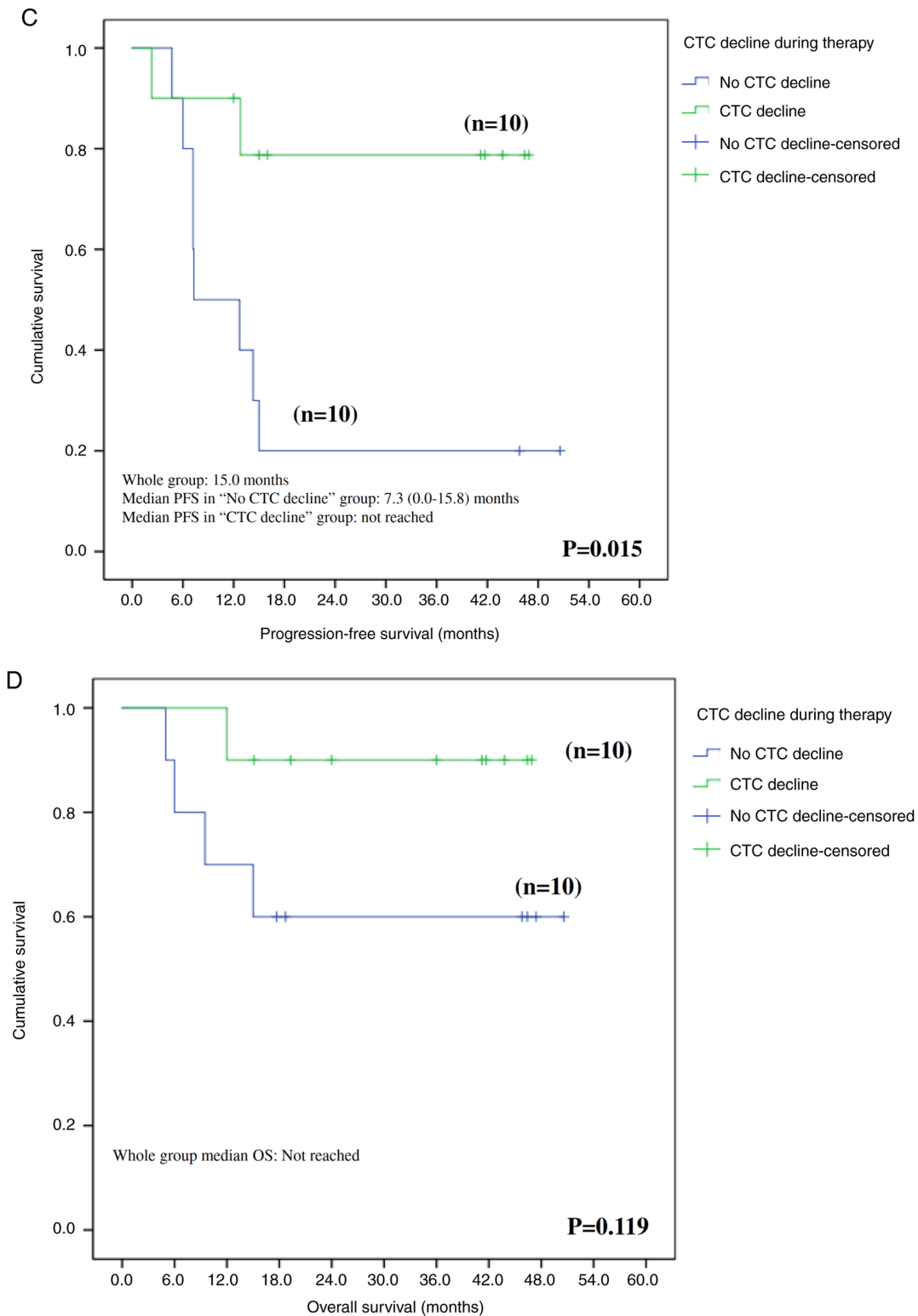


Figure 3. Differences in survival between groups by baseline and changes in CTCs. Patients with lower baseline CTC counts had (A) a longer PFS and (B) significantly longer OS. The decline of CTC numbers after therapy may predict (C) significantly longer PFS and (D) markedly longer. The red box in C highlights the optimal cutoff value determined through the Youden index method. CTC, circulating tumor cell; PFS, progression-free survival; OS, overall survival.

methodologies have been previously used to identify the presence of CTCs (37-39), these studies provided molecular proof of the existence of CTCs, though they did not capture CTCs directly. Other studies have reported the use of physical isolation/capture methods, such as filtration systems like

MetaCell (40), polydimethylsiloxane microchannels (41), tapered-slit membrane filters with immunocytochemistry staining (42), optimized tapered-slit filter platforms (43) and fluid-assisted separation technology discs (44). The major concerns with these methods stem from the variety of devices

Table III. Univariate and multivariate analysis of progression-free and overall survival.

A, Progression-free survival

Variable	Univariate			Multivariate		
	HR	95% CI	P-value	HR	95% CI	P-value
Age (continuous)	1.052	(1.007-1.100)	0.023			
FIGO stage (IV vs. III vs. II vs. I)	2.173	(1.140-4.141)	0.018			
Pathology (serous vs. non-serous)	1.530	(0.809-2.893)	0.191			
pN1 or M1 vs. pN0M0	2.459	(0.883-6.845)	0.085			
Baseline CA125 level (continuous)	1.000	(1.000-1.000)	0.929			
CTC decline in the first 3rd month (continuous)	0.178	(0.037-0.849)	0.030	0.154	(0.030-0.784)	0.024
Baseline CTC counts (continuous)	1.182	(1.063-1.315)	0.002	1.188	(1.040-1.357)	0.011

B, Overall survival

Variable	Univariate			Multivariate		
	HR	95% CI	P-value	HR	95% CI	P-value
Age (continuous)	1.029	(0.978-1.083)	0.269			
FIGO stage (IV vs. III vs. II vs. I)	2.059	(1.000-42.54)	0.050			
Pathology (serous vs. non-serous)	1.626	(0.890-2.972)	0.114			
pN1 or M1 vs. pN0M0	2.351	(0.678-8.144)	0.178			
Baseline CA125 level (continuous)	1.000	(0.999-1.001)	0.715			
CTC decline in the first 3rd month (continuous)	0.206	(0.023-1.851)	0.159			
Baseline CTC counts (continuous)	1.291	(1.120-1.489)	<0.0001	1.480	(1.129-1.941)	0.005

FIGO, International Federation of Gynecology and Obstetrics; HR, hazard ratio; CI, confidence interval; CA125, cancer antigen 125.

and the lack of sufficient external validation, which casts doubt on their clinical applicability. The most prevalent CTC enumeration/isolation methodologies are immunomagnetic beads with staining, exemplified by the CellSearch platform (45,46), and other widely used devices or technologies, such as flow cytometry (47,48) or immunocytochemistry staining (49). The present study advocates for the use of a commonly available platform over specific CTC testing innovations and provides evidence of its clinical value. It is crucial to emphasize that the goal was not to replace standard diagnostic and treatment methods but to complement them, offering a less invasive yet discriminative avenue for understanding and managing tumor behavior.

Criteria for positive CTC presence, including cut-off values, varied across the studies reviewed (Table IV). These differences primarily stemmed from the varying detection limits of different CTC isolation platforms (30,40). In EOC, detection limits ranged from 1 CTC/25 ml to 5 CTCs/ml. Using flow cytometry technology, the present study identified positive CTC presence as 4.75 cells/ml, nearing the upper limit of 5 cells/ml. Efforts were made to avoid incorrectly labeling cells in human circulation obtained under predefined conditions (i.e., EpCAM+CD45-) from healthy individuals as

CTCs, it would be inappropriate to call them CTCs in subjects without cancer. However, a consensus within the academic community is lacking, as these numbers may merely signify the background values of a detection tool, not necessarily indicating the presence of cancer. This scenario is similar to tumor markers, such as CEA and AFP, where distinctions exist between reference (or background) and abnormal values, and the mere presence of these markers does not definitively signify cancer (50). Furthermore, cell-free (cf)DNA can sometimes harbor clonal hematopoiesis of indeterminate potential in individuals without cancer. Extensive research is required to identify DNA abnormalities that are not cancer-related, similar to those observed in healthy individuals (51). In the future, extensive studies may help differentiate these cells in cancer patients or assign alternative names, such as the historical term-circulating epithelial cells (52). Furthermore, the presence of false positives, where certain cells expressing EpCAM are detected in healthy subjects, does not support a cancer diagnosis. Conversely, false negatives, where cells do not express typical epithelial markers but instead express vimentin markers, may introduce a potential bias in the utilization of CTCs. In the present proof-of-concept study, a negative selection and immunofluorescence identification

Table IV. Literature review for CTCs addressing clinical correlation.

A, PCR based						
First author, year	Country	n	CTC platform	Healthy control	Times/time points of CTC collection	CTC positivity threshold/detection rate (%)
Zuo <i>et al</i> , 2021	China	30	EpCAM liposome magnetic	Yes (n=30)	NA/NA	≥1 CTCs/7.5 ml/80.0%
Obermayr <i>et al</i> , 2021	Austria	215	qPCR and immuno-fluorescent staining	No	2/At baseline and six months after adjuvant treatment	≥1 CTCs/9 ml/50.5% (baseline)
Obermayr <i>et al</i> , 2021	Austria	185	qPCR	No	1/Before treatment	≥1 CTCs/25 ml/19.6%
Main findings						
						miR-181a detection in CTCs can help cancer diagnosis and prognosis.
						CTCs were associated with elevated risk of recurrence and death.
						PPIC-positive CTCs were significantly associated with a high CCES.
B, Microchannel or filter systems						
Kolostova <i>et al</i> , 2016	Czech Republic	40	MetaCell®, MetaCell s.r.o., Ostrava, Czech Republic	No	NA/NA	≥1 CTCs/8 ml/58.0%
Lee <i>et al</i> , 2017	South Korea	54	Polydimethylsiloxane microchannels	No	1/Before surgery or adjuvant therapy	≥1 CTCs/10 ml/98.1%
Suh <i>et al</i> , 2017	South Korea	31	Tapered-slit membrane filters + ICC	Yes (n=22)	1/Before surgery	≥1 CTCs/5 ml/77.4%
Kim <i>et al</i> , 2019	South Korea	30	Optimized tapered-slit filter platform	No	2/Before and after surgery	≥1 CTCs/5 ml/76.7%
						KRT7, WT1, EPCAM, MUC16, MUC1, KRT18, and KRT19 detection can indicate CTC presence.
						PFS decrement and platinum resistance are correlated with CTCs ≥3 cells, and positive CTC-cluster, respectively.
						CTCs before surgery could discriminate early ovarian cancer from benign ovarian tumors.
						No significant correlation was noted between CTCs and clinical outcomes.

Table IV. Continued.

B, Microchannel or filter systems							
First author, year	Country	n	CTC platform	Healthy control	Times/time points of CTC collection	CTC positivity threshold/detection rate (%)	Main findings (Refs.)
Kim <i>et al.</i> , 2020	South Korea	13	Fluid-assisted separation technology disc	No	>3 (varies)/At diagnosis, before and after treatment	≥1 CTCs/3 ml/ 84.6%	CTC counts was better associated with treatment response and recurrence than CA125 levels. Change in CTCs correlates to clinical disease status. (44)
C, Immune-fluorescent detection							
Pearl <i>et al.</i> , 2015	USA	31	CAM uptake-cell enrichment + flow cytometry	Yes (n=64)	9/Before treatment, follow-up at 1,3,6,9, 12,18, and 24 months after treatment	≥5 CTCs/ml/ 100.0%	Continuous invasive CTC measurements could be a predictor of chemotherapy efficacy. (47)
Lou <i>et al.</i> , 2018	USA	29	CellSearch	Yes (n=14)	1/Before treatment	≥1 CTCs/7.5 ml/ 17.0%	CTCs are more abundant in ovarian metastasis from other cancer (vs. primary ovarian cancer). (45)
Guo <i>et al.</i> , 2018	China	30	Size based microfluidic technique + ICC	Yes (n=25)	1/Before surgery	≥0.5 CTCs/1 ml/ 73.3%	Higher DAPI+/E&M+/CD45-/HE4+ CTC counts were found in EOC (vs. benign tumors). (49)
Banys-Paluchowski <i>et al.</i> , 2020	Germany	34	CellSearch	No	3/Prior to chemotherapy, after 3 and 6 cycles.	≥2 CTCs/7.5 ml/ 26.0%	Patients with ≥1 CTCs at baseline had significantly shorter OS and PFS than those with CTC-negative patients. (46)
Gening <i>et al.</i> , 2021	Russia	38	Negative selection + flow cytometry (Cytoflex S)	No	2/Before treatment and during first-line chemotherapy	NA/NA	CD133 + ALDH + CTCs have the greatest prognostic potential in ovarian cancer. (48)

Table IV. Continued.

C, Immune-fluorescent detection								
First author, year	Country	n	CTC platform	Healthy control	Times/time points of CTC collection	CTC positivity threshold/detection rate (%)	Main findings	(Refs.)
Kou <i>et al</i> , 2024	Taiwan	20	Negative selection + flow cytometry	Yes (n=38)	4/Baseline, at 6, 9, 12 months after treatment	≥5 CTCs/1 ml/ 100.0% (for CTC >0 cells/ml)	Post-treatment CTC decline rather than baseline CTC counts could serve as an independent prognostic factor.	Pre-sent study
RNA, not available; EOC, epithelial ovarian cancer; ICC, immunocytochemistry; CTC, circulating tumor cell; OS, overall survival; PFS, progression-free survival; CAM, cell adhesion matrix; EpCAM, epithelial cell adhesion molecule; CA125, cancer antigen 125; HE4, human epididymis protein 4; MUC1, mucin 1; miR-181a, microRNA-181a; CCES, a combined score for cancer exhaustion; KR17, keratin 7; WT1, Wilms' tumor suppressor gene 1; MUC16, mucin 16; E&M, epithelial and mesenchymal.								

NA, not available; EOC, epithelial ovarian cancer; ICC, immunocytochemistry; CTC, circulating tumor cell; OS, overall survival; PFS, progression-free survival; CAM, cell adhesion matrix; EpCAM, epithelial cell adhesion molecule; CA125, cancer antigen 125; HE4, human epididymis protein 4; MUC1, mucin 1; miR-181a, microRNA-181a; CCEs, a combined score for cancer exhaustion; KR17, keratin 7; WT1, Wilms' tumor suppressor gene 1; MUC16, mucin 16; E&M, epithelial and mesenchymal.

platform was used to enumerate CTCs. It was demonstrated that baseline CTC counts could be used to differentiate between patients with ovarian cancer and those with benign ovarian diseases, achieving an AUC of 0.900 ($P<0.001$). While an age imbalance was observed during case enrollment between the cancer group and healthy donors ($P=0.013$), no difference was noted between the EOC and benign lesion groups ($P=0.107$), suggesting that the ability to differentiate EOC from benign lesions is reliable. The results indicated that a decline in CTCs during the first three months of first-line treatment (HR, 0.154; $P=0.024$) and low baseline CTC counts (<4.75 cells/ml; HR, 1.188; $P=0.011$) were both significantly associated with longer PFS. Additionally, patients with low baseline CTC counts might experience prolonged OS (HR, 1.480; 95% CI, 1.129-1.941; Table III). However, due to the limited number of events (deaths) in this cohort, a model using CTCs to predict OS remains unreliable. While numerous studies have reported CTCs to be closely related to OS and PFS (36,37,39,42,44), this result is not universal (43). To the best of our knowledge, the present study is the first to suggest an independent prognostic role for baseline CTC counts and the decline in CTCs within the first three months after treatment, in predicting clinical outcomes for patients with EOC.

Few previous studies have addressed the value of changes in CTC counts through serial measurements (44,47). Pearl *et al* (47) conducted nine serial CTC measurements in 31 patients with EOC and reported that continuous invasive CTC measurements more accurately predicted chemotherapy efficacy than CA125 levels. In a small-scale study, Kim *et al* (44) reported positive predictive ability for clinical survival in 47 serial CTC measurements across 13 patients with EOC. Banys-Paluchowski *et al* (46) suggested that chemotherapy rapidly reduced CTC counts within the first three months following cancer therapy, with CTCs correlating with clinical scenarios. While the present study demonstrated that changes in CTC counts were associated with survival outcomes (Fig. 3).

In academic research on liquid biopsy, ctDNA is often compared with CTCs, both being important and rapidly evolving tools (53). Although considered to be liquid biopsies, they differ markedly in their biology, applications (i.e. finding targeted drugs or xenografts for *ex vivo* testing), and respective advantages and disadvantages. Detecting or capturing CTCs typically involves analyzing living cancer cells, while ctDNA reflects cancer-specific genes regardless of the cancer cells' viability. Consequently, CTCs are beneficial for studies that require living cells, such as CTC culture, CTC-derived xenografts and *ex-vivo* CTC drug testing (54). However, the advantage of CTCs is offset by the challenge of capturing cells, as the unstable expression of surface markers can lead to difficulties in identifying a small subset of cells. These issues include atypical CTCs that lack EpCAM expression and CTC subgroup heterogeneity (55). When choosing between CTCs and ctDNA as a liquid biopsy tool, it is crucial to carefully consider the research characteristics, acknowledging the coexistence of both benefits and challenges associated with CTCs.

The present study had certain limitations. Firstly, as a pilot and proof-of-concept study, only a small number of cases were considered. In future experiments, it is advisable

to compare patients with different types of malignancies or peritoneal metastases, this approach would support assessment of the specificity of the CTC enumeration method specifically for ovarian cancer rather than malignancy in general. Secondly, the FDA has not approved the CTC enumeration methodology. Nevertheless, the flow cytometer, a device commonly used for the quantification of labelled cell populations, has been employed in similar applications to detect minimal evidence of malignancy in circulation, particularly in hematologic malignancies such as leukemia (56). Consequently, we suggest that this methodology could be broadly applicable in clinical settings, particularly for patients with EOC. Thirdly, it is recommended that future experiments incorporate the tracking of long-term survival rates to comprehensively elucidate the correlation between the initial decline in CTC and overall survival. The absence of extended survival rate data is a limitation of the current study. In addition, the definition of CTCs in the present study does not consider interstitial CTC, which are EpCAM negative. The prospect has been extensively discussed in the literature (57,58). It is commonly held that incorporating more cancer-specific surface markers, such as Her2, may enhance the detection rate of particular cancers. It was found that augmenting the panel with markers such as CSV antibodies could reveal the stemness of CTCs. However, the challenge of tumor heterogeneity was also encountered, as not all cancers exhibit differentiation towards the same surface marker (58). Therefore, while the present study refrained from employing additional surface markers, their utilization to aid in the identification of EpCAM-positive CTCs with greater accuracy should be considered.

In conclusion, this proof-of-concept study utilized a negative selection and immunofluorescence identification platform to enumerate CTCs. The results demonstrated that baseline CTC counts could differentiate between patients with ovarian cancer and those with benign disease. Furthermore, longitudinal follow-up of CTC changes independently predicted PFS with a greater significance than baseline CTC counts. Furthermore, a decline in CTC counts may contribute to prolonged OS. While these results are promising for predicting survival in patients with EOC, further research with a larger sample size is necessary to independently validate the findings in this study.

Acknowledgements

Not applicable.

Funding

The study was partially funded by Chang Gung Memorial Hospital grants (grant nos. CMRPVVK0093, CMRPVVL0262 and CMRPG3M0931) and a National Science and Technology Council, R.O.C. grant (grant no. NSC 112-2314-B-182A-028-).

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

CHC was responsible for conception and design, analysis and drafting the manuscript. YCK was responsible for conception and design, analysis and drafting the manuscript. HCK was responsible for the collection of data from medical records. CTL, AC, HJH and HMW were responsible for conception, patient enrollment and supervision of the protocol and study. JCHH and HHC were responsible for conception, design, acquisition of funding, patient enrollment, data collection and analysis, writing the manuscript and they confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the Chang Gung Memorial Hospital institutional and national research committee (approval nos. 201802203B0C502 and 201601461B0) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Written informed consent was obtained from all individual participants involved in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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