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# Lymphocyte and macrophage infiltration in omental metastases indicates poor prognosis in advance stage epithelial ovarian cancer

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## Abstract

**Objective:** To investigate the prognostic value of immune cells within omental metastases originating from advanced epithelial ovarian cancer (EOC).

**Methods:** We performed immunohistochemical analysis to determine the levels of CD4+/CD8+ tumor-infiltrating lymphocytes (TILs) and CD68+ tumor-associated microphages (TAMs) in omental specimens from 100 patients with advanced EOC. Significant prognostic factors, including immune cells and clinical parameters, were assessed by Kaplan–Meier survival analysis and Cox models.

**Results:** Cox regression analysis showed that elevated levels of CD68+ TAMs and intra-islet CD4+ TILs in omental metastases were the main risk factors associated with worse survival outcomes for advanced EOC. Moreover, the survival analysis of relationships between omental immune cells and favorable clinical predictors revealed additional prognostic stratification information.

**Conclusion:** Omental immune cells (TAMs and TILs) provide alternative prognostic factors in advanced EOC. In contrast to markers of the EOC tumor microenvironment at the primary site, elevated CD68+ TAMs and intra-islet CD4+ TILs in omental metastases serve as negative prognostic markers in advanced EOC and imply an unfavorable outcome.

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Epithelial ovarian cancer, omental metastasis, tumor-infiltrating lymphocyte, tumor-associated macrophage, chemoresistance, prognostic factor

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## Introduction

Epithelial ovarian cancer (EOC) is the fifth leading cause of cancer death among women.[1] Only 25% of patients with EOC are diagnosed at an early International Federation of Gynecology and Obstetrics (FIGO) stage (stage I/II), and advanced-stage (stage III/IV) cases have a 5-year survival rate of 46%.[2] The high mortality rate is due to a lack of specific early clinical manifestations. The standard therapeutic management consists of surgery and platinum-based chemotherapy, but chemoresistance and recurrence develop within a short time in women with advanced disease.[3]

Tumor-infiltrating lymphocytes (TILs), a subgroup of white blood cells from the vasculature, localize to the tumor islet and stroma. CD4+ and CD8+ TILs are the two main subpopulations with different functions in the immune response to tumors. Briefly, cytotoxic CD8+ TILs directly kill cancer cells, and helper CD4+ TILs mobilize other immune cells involved in antitumoral immunity. Despite some controversy, multiple studies have supported that TILs, specifically CD4+ and CD8+ TILs, are associated with improved prognoses and survival among patients with ovarian cancer.[4,5] Similarly, the role of macrophages within tumor tissues, termed tumor-associated macrophages (TAMs), is controversial. For example, TAMs indicate worse survival outcomes in gastric[6] and esophageal[7] cancers but better prognoses in colon[8] and gastric[9] cancers. In addition, accumulative studies have reported that CD68+ TAMs are not associated with survival outcomes in ovarian cancer.[10,11]

The greater omentum is composed primarily of fatty tissue. It connects to the transverse colon and stomach at the top and extends to the bottom of the pelvis while covering the bowels in the abdominal cavity. The immune structures in the omentum, termed milky spots, are composed of lymphocytes and macrophages and function as conventional lymphoid tissue.[12] Thus, TILs and TAMs are also found in the omentum when ovarian cancer cells spread to the peritoneal cavity.

Omental metastasis has been observed in almost all patients with advanced ovarian cancer, but the role of the omental tumor microenvironment (TME) during the development of ovarian cancer is complex and poorly understood.[13] We assessed the value of CD4+/CD8+ TILs and CD68+ TAMs in omental tumors in predicting clinical outcomes in patients with advanced EOC.

## Materials and methods

### *Patients and specimens*

We conducted a retrospective analysis of patients with pathologically confirmed advanced EOC between 1 January 2002 and 30 September 2009 in the Women's Hospital, School of Medicine, Zhejiang University. The inclusion criteria were as follows: 1) debulking/tumor reductive

surgery as the initial therapy; 2) EOC with omental metastasis proven by pathological diagnosis; and 3) subsequent platinum-based chemotherapy and regular follow-up. The exclusion criteria were as follows: 1) radiotherapy/neoadjuvant chemotherapy before surgery; 2) potential omental lesions, such as cyst rupture or peritonitis; and 3) loss to follow-up before postoperative chemotherapy. Omental tumor specimens were obtained from all cases with archived paraffin tissue blocks. The reporting of this study conforms to REMARK guidelines.[14] Approval was obtained from the Human Ethics Committee of the Women's Hospital, Zhejiang University School (No: IRB-20200280-R). Each enrolled patient signed informed consent forms for the use of her samples and records for scientific research.

## Database

The relevant data and follow-up information collected from enrolled patients included the following: age, preoperative serum cancer antigen 125 (CA125) level, cytological examination of ascites/peritoneal washes during surgery (predebulking), residual disease status, pathologic stage and grade (based on the FIGO staging and grading system), newly classified EOC histotypes[15] (type I EOC defined as low-grade serous and endometrioid cancers and all clear cell/mucinous tumors, with all others defined as type II EOC), responses to chemotherapy (<6 months recurrence of EOC after postoperative chemotherapy was recognized as chemoresistance), and survival information. The clinical endpoints evaluated were progression-free survival (PFS; time from cancer diagnosis to recurrence) and overall survival (OS; time from cancer diagnosis to death). Survival was censored on 30 September 2019, the last date of follow-up.

### *Immunohistochemistry (IHC) and scoring*

TILs were identified by IHC labeling for CD4 (monoclonal, EP204, 1:50 dilution, Thermo Fisher, CA, USA) and CD8 (monoclonal, SP16, 1:100 dilution, Thermo Fisher, CA, USA). TAMs were characterized by IHC labeling for CD68 (monoclonal, KP1, 1:100 dilution, Abcam, Cambridge, UK).

For representative IHC, 3-μm-thick sections were cut from paraffin-embedded tumor tissue. Briefly, tissue sections were subjected to routine deparaffinization, rehydration, and antigen retrieval procedures. Then, each section was incubated with the primary antibody at room temperature for 1 hour, followed by the secondary antibody at room temperature for 30 minutes. All sections were reacted with diaminobenzidine (GeneTech, Co., Ltd., Shanghai, China), followed by counterstaining with hematoxylin (Maixin Biotech, Co., Ltd., Fujian, China), dehydration, and mounting. Two senior pathology physicians examined and evaluated all slides independently.

The histological score was counted as described previously[4,16] to analyze the CD4+ and CD8+ TILs in the area of interest (cancer islet/stroma) in each high-power field (HPF, 400×). The specimen was considered "positive" when the average immunolabeled cell counts were CD4 $\geq$10/HPF and CD8 $\geq$20/HPF and "negative" when the cell counts were CD4 $<$ 10/HPF and CD8 $<$ 20/HPF. CD68-positive TAMs were scored as the percentage of the tumor area. An average TAM density $\geq$5% of the tumor area was considered to indicate high expression of CD68, and <5% tumor area indicated low expression.

### *Statistical analysis*

For the description of clinical characteristics, continuous variables are expressed as the mean $\pm$ standard deviation (normally

distributed) or median and range (nonnormally distributed), and categorical values are reported as frequencies. All comparisons of IHC results with a nonnormal distribution were performed using the Mann–Whitney U-test or Kruskal–Wallis test. ANOVA was used to compare the median survival time among EOC subgroups of two histotype classification systems. Univariable and multivariable Cox regression models were used to analyze the hazard ratios (HRs) and 95% confidence intervals (Cls) of the clinical factors and selected biomarkers in the study population. Kaplan–Meier survival analysis and log-rank tests were used to assess the 10-year (120 months) survival rates of patient groups with different risk factors. A two-sided p-value of $<0.05$ was considered statistically significant. Statistical analyses were performed using IBM SPSS Statistics for Windows, Version 20 (IBM Corp., Armonk, NY, USA) and Prism 8 (GraphPad Software, San Diego, CA, USA).

## Results

### *Baseline and follow-up information*

The main clinical characteristics of the 100 patients are summarized in Table 1. The mean age of the enrolled patients was 50.7 years, the median serum CA125 value was 667 U/mL, the median PFS was 20 months, and the median OS was 56 months. All operative reports of enrolled cases described the surgical debulking status as complete resection (no residual disease).

Regarding pathology results, the percentage of patients was the highest for serous cancer (74%, 74/100) and the newly classified type II histotype (64%, 64/100), followed by grade 3 (58.9%, 56/95; five

**Table 1.** Clinical and pathological characteristics of the study population.

| Characteristic (unit) | |
|---|---|
| Age (years) | $50.7 \pm 9.3$ |
| CA125 (U/mL, 0–100th range) | 667 (5.3–100,000) |
| Classic histotype (n) | |
| Serous | 74 |
| Endometrioid | 12 |
| Mucinous | 4 |
| Clear cell | 5 |
| Mixed | 4 |
| Undifferentiated | 1 |
| Newly histotype (n) | |
| Type I | 36 |
| Type II | 64 |
| Serous cancer (n) | |
| Low-grade | 20 |
| High-grade | 54 |
| FIGO stage (n) | |
| IIIA | 25 |
| IIIB | 15 |
| IIIC | 56 |
| IV | 4 |
| FIGO grade (n) | |
| G1 | 10 |
| G2 | 29 |
| G3 | 56 |
| NG* | 5 |
| Ascites (n) | |
| Positive | 34 |
| Negative | 66 |
| Lymph node metastasis (n) | |
| Positive | 3 |
| Negative | 97 |
| Chemotherapy response (n) | |
| Sensitive | 38 |
| Resistant | 62 |
| Tumor residual disease (n) | |
| NRD | 100 |
| RD | 0 |
| PFS (months, 0–100th range) | 20 (4–95) |
| OS (months, 0–100th range) | 56 (7–197) |

CA125: carbohydrate antigen 125; FIGO: International Federation of Gynecology and Obstetrics; NG: no FIGO grade; PFS: progression-free survival; OS: overall survival; NRD: no residual disease; RD: residual disease.

cases of clear cell cancer with no FIGO grade) and stage IIIC (56%, 56/100). In addition, high-grade serous cancer cases accounted for 73.0% (54/74) of patients with serous cancer.

Regarding metastasis to other sites in these cases, the proportion of malignant ascites was 34%, and that of positive lymph nodes was 3%. Chemoresistance was observed in 62% of patients with short-term (<6 months) recurrence of EOC after postoperative chemotherapy (paclitaxel plus carboplatin).

At the last follow-up, only 19 patients were alive. Five patients withdrew after finishing postoperative chemotherapy, two patients were lost to follow-up after cancer recurrence, and only one patient had a second operation for recurrent cancer of the vaginal stump.

### *TIL and TAM infiltration in omental specimens and their relationship with clinicopathological characteristics*

To assess the infiltration of TILs and TAMs, omental metastatic samples were stained for CD4, CD8 and CD68 (Figure 1a–j). Comparisons of positive and negative expression for CD4, CD8, and CD68 in cancer islets or stroma revealed significant differences ($p \leq 0.005$) by the Mann–Whitney U-test (Figure 1k–m). CD4+ cells, which are most recognized for their role as helper lymphocytes, were observed predominately in the tumor stroma (47% of cases, 47/100) compared with tumor islets (31%, 31/100 cases), with average counts of 72.0 (19.8–252.0)/HPF and 42.2 (10.8–141.0)/HPF, respectively (Figure 1k). CD8+ cells showed a comparable distribution (in the stroma in 47% of cases; in the islets in 31% of cases) with similar counts of 42.0/HPF in the stroma and 35.0/HPF in the islets (Figure 1l). Furthermore, a high density of infiltrating CD68+ macrophages was found in omental metastatic samples in 61% of cases (61/100 cases), and they were distributed in both the tumor islets and stroma (6%–50% of the tumor area) (Figure 1m).

To further understand the role of the TIL and TAM infiltration in omental metastatic tumors, we investigated the relationship between the levels of omental immune cells and clinicopathological characteristics of 100 samples, as shown in Table S1. None of the clinicopathological subgroups showed positive expression of CD4 in the cancer islets of omental tissues, but CD4 expression was significantly different between the groups of serum CA125 levels ($\leq$667 U/mL vs >667 U/mL, $p = 0.018$), newly classified histotypes (type I vs type II, $p = 0.018$), and FIGO stages (stage IIIA/IIIB vs stage IIIC/IV, $p = 0.004$). Patients with type II EOC and a more advanced stage (IIIC/IV) showed an elevated level of CD4+ TILs in the cancer stroma of omental tissues ($p = 0.048$ and $p < 0.001$, respectively). Stromal CD4+ TILs tended to be enriched in subgroups, including age younger than 50 years, serum CA125 > 667 U/mL, cancer histotypes of serous, mucinous, mixed/undifferentiated, high-grade serous cancer, FIGO grade 3, malignant ascites, and chemoresistance.

Positive expression of CD8 tended to be detected in the cancer stroma of the following subgroups: age younger than 50 years, serum CA125 >667 U/mL, cancer histotypes of mucinous and mixed/undifferentiated, stage IIIC/IV ($p < 0.001$), malignant ascites, positive lymphatic metastasis ($p = 0.035$), and chemoresistance. Only the

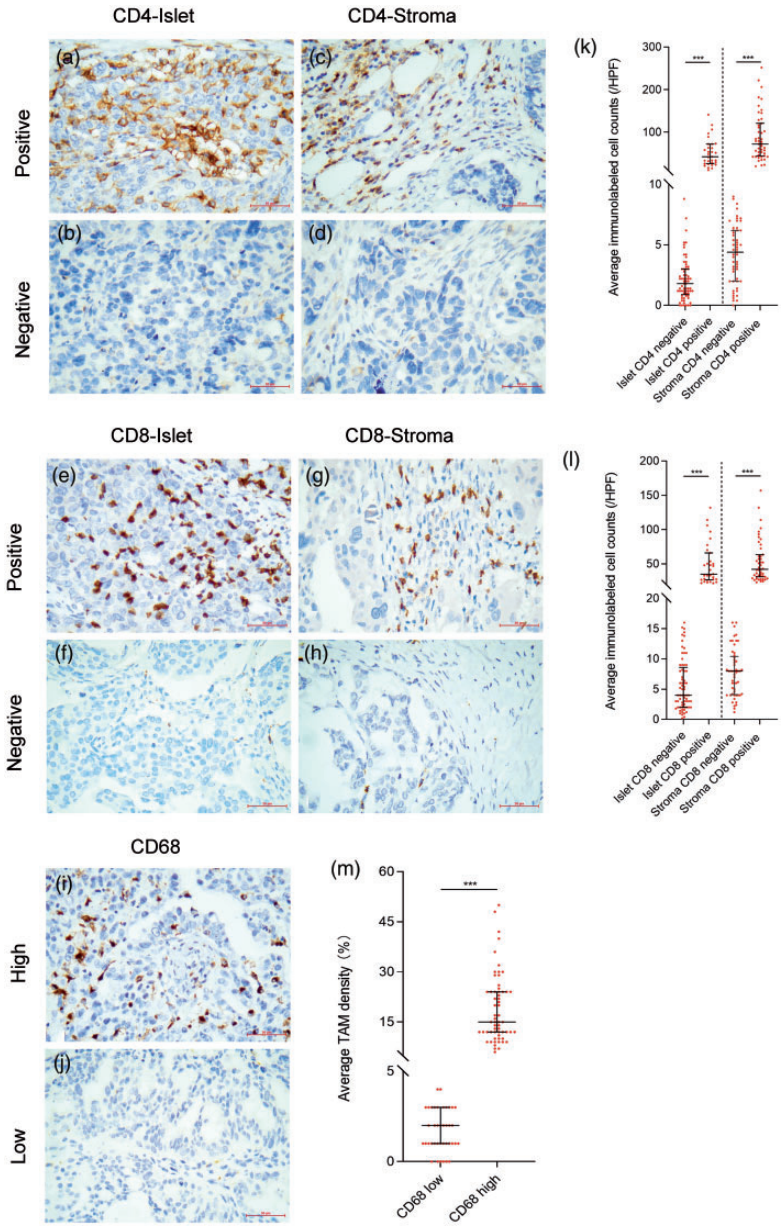


**Figure 1.** TIL and TAM immunostaining and levels in omental metastatic tumors from 100 patients. The number of CD4+ and CD8+ TILs in the cancer islet and stroma was counted in each HPF (400×; scale bar = 50 μm). The specimen was considered "positive" when the average immunolabeled cell counts were CD4 ≥10/HPF and CD8 ≥20/HPF and "negative" when the cell counts were CD4 < 10/HPF and CD8 <20/HPF. CD68-positive TAMs were scored as the percentage of the tumor area. An average TAM density ≥5% of the tumor area was considered to indicate high expression of CD68, and <5% tumor area indicated low expression. (a–d) CD4 expression. (e–h) CD8 expression. (i,j) CD68 expression. (k–m) Histological score of TILs and TAMs.

TIL: tumor-infiltrating lymphocyte; TAM: tumor-associated macrophage; HPF: high power field.

subgroup of positive lymphatic metastasis showed CD8+ TILs in cancer islets.

Omental metastatic tumors with a higher density of CD68+ TAMs tended to be observed in the following subgroups: age younger than 50 years, serum CA125 > 667 U/mL ($p < 0.001$), type II EOC, cancer histotypes of serous, mucinous, and mixed/undifferentiated, high-grade serous cancer, stage IIIC/IV ($p < 0.001$), grade 3, malignant ascites ($p < 0.001$), negative lymphatic metastasis, and chemoresistance ($p < 0.001$).

The results revealed a strong positive relationship between the infiltration of omental immune cells and advanced tumor stage. More significant correlations were found between the higher density of CD68+ TAMs and subgroups of clinicopathological characteristics in omental metastatic lesions (Table S1).

## *Association of clinical characteristics with immune cell infiltration of omental tumors and patient survival*

PFS and OS curves were generated using Kaplan–Meier analysis, and the difference in survival was compared with the log-rank test. As shown in Figure 2, higher levels of serum CA125 (>667 U/mL), the type II histotype, a more advanced stage (IIIC/IV), higher grade (G3), and chemotherapy resistance were significantly associated with shorter PFS and OS ($p < 0.001$). Peritoneal ascites were correlated with poorer OS but not PFS (Supplement Figure 1).

We also analyzed the relationship between the infiltration of immune cells in metastatic omental tumors and patient survival. A higher density of CD68+ TAMs in omental tumors indicated both poorer PFS ($p < 0.001$) and OS ($p < 0.001$). CD4+ TILs in cancer islets were associated with poorer PFS ($p = 0.008$) and OS ($p = 0.008$), and CD4+ TILs in the cancer stroma were associated with poorer PFS ($p = 0.010$). CD8+ TILs in cancer islets were correlated with poorer OS ($p = 0.012$), and CD8+ TILs in the cancer stroma were correlated with poorer PFS ($p = 0.004$) and OS ($p = 0.046$). (Figure 3 and Supplement Figure 1). Our data show that immune cell infiltration in metastatic tumors in the omentum is an indicator of poorer survival in advanced ovarian cancer.

## *Comparison between classic and newly classified histotypes by survival data*

To better understand classic and newly classified histotypes, we analyzed them separately to determine clinical outcomes by different histotype classification systems (Figure 4). Our data revealed that patients with serous ovarian cancer had shorter PFS times compared with mucinous ($p = 0.002$), clear cell cancer ($p = 0.033$), and other cancers in this study ($p = 0.017$) (Figure 4a left, e). Poorer OS was observed in serous cancer cases compared with mucinous cancer cases ($p = 0.022$), but there was no difference compared with all non-serous cancers (Figure 4b left, f). Of note, patients with mucinous ovarian cancer who were alive at the last follow-up and those lost to follow-up were excluded in the ANOVA model (Figure 4a, b). As a result, there were few significant survival differences among the subgroups of classic histotypes, particularly when the mucinous cancer cases were excluded. Newly classified histotypes displayed more significant differences in survival outcomes (Figure 4a right, b right, c, d) ($p < 0.001$) and were more suitable for inclusion in further models of survival determinants. In addition, the analysis of clinical outcomes revealed worse prognoses in the subgroup of high-grade serous ovarian cancer (Figure 4g, h) ($p < 0.001$).

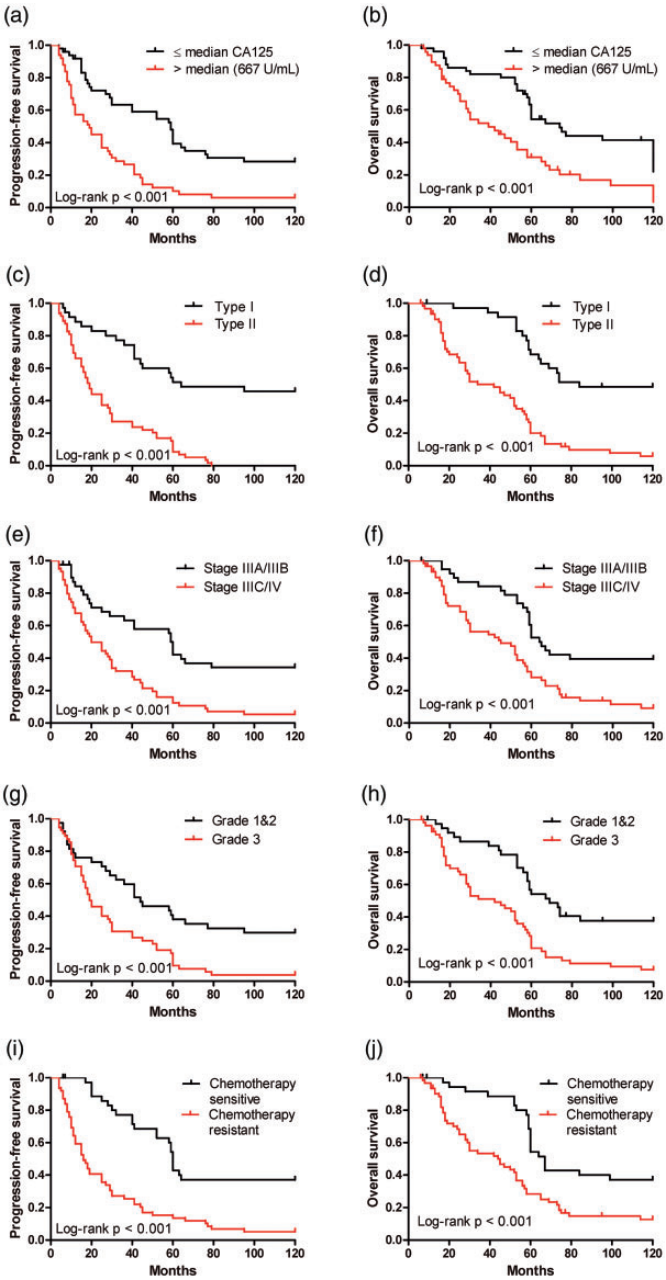


**Figure 2.** Kaplan–Meier survival analyses of main clinical characteristics in the study population. (a) Comparison of PFS by CA125 (≤median value, 667 U/mL vs >667 U/mL). (b) Comparison of OS by CA125. (c) Comparison of PFS by histotype (type I vs type II). (d) Comparison of OS by histotype. (e) Comparison of PFS by FIGO stage (stage IIIA/IIIB vs stage IIIC/IV). (f) Comparison of OS by FIGO stage. (g) Comparison of PFS by FIGO grade (grade 1 and 2 vs grade 3). (h) Comparison of OS by FIGO grade. (i) Comparison of PFS by response to chemotherapy (sensitive vs resistant). (j) Comparison of OS by response to chemotherapy.

CA125: cancer antigen 125; OS: overall survival; PFS: progression-free survival; FIGO: International Federation of Gynecology and Obstetrics.

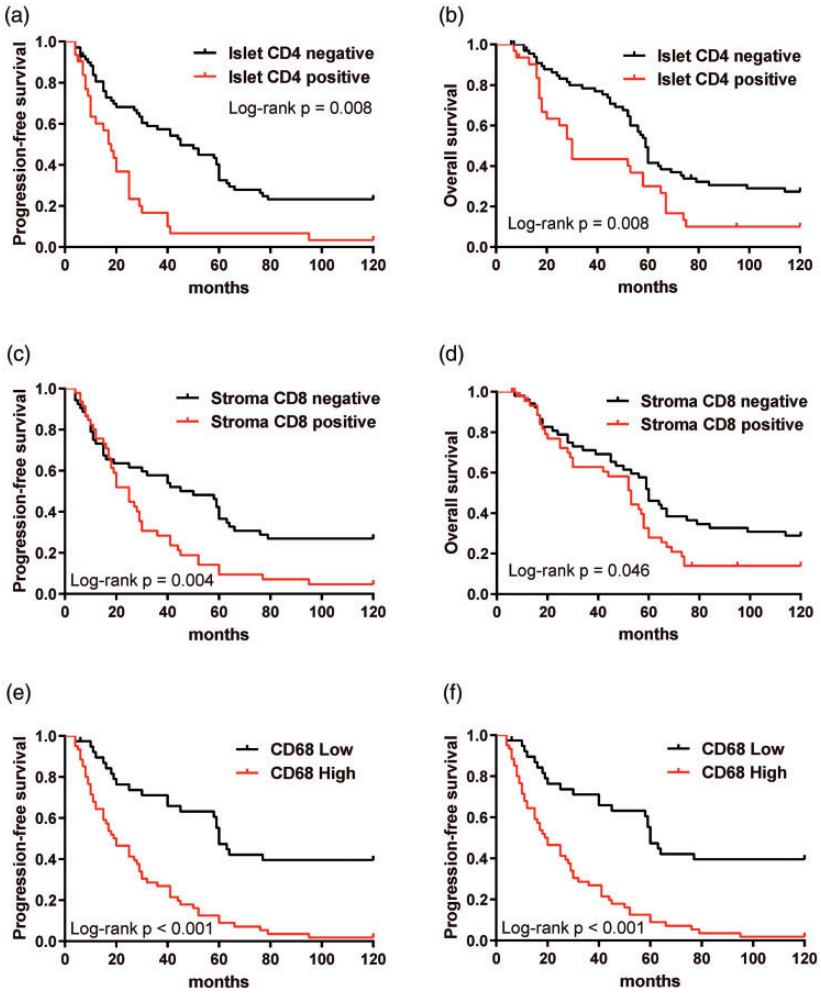


**Figure 3.** Kaplan–Meier survival analyses of significant immune cells in omental specimens. (a) Comparison of PFS by cancer islet CD4+ TILs (positive vs negative). (b) Comparison of OS by cancer islet CD4+ TILs. (c) Comparison of PFS by cancer stroma CD8+ TILs (positive vs negative). (d) Comparison of OS by cancer stroma CD8+ TILs. (e) Comparison of PFS by CD68+TAMs (low versus high). (f) Comparison of OS by CD68+TAMs.

TIL: tumor-infiltrating lymphocyte; TAM: tumor-associated macrophage; OS: overall survival; PFS: progression-free survival.

### *Univariate and multivariate Cox regression analysis of clinical risk parameters and immune cells in metastatic sites*

Through Kaplan–Meier survival analysis, we found that risk factors, including age, lymph node metastasis, ascites, stromal CD4+ TILs, and intra-islet CD8+ TILs, were weakly correlated with prognostic outcomes (Supplement Figure 1). To rule out the influence of confounding factors and identify key predictors, we performed Cox regression analysis to assess the value of each risk factor and adjusted models.

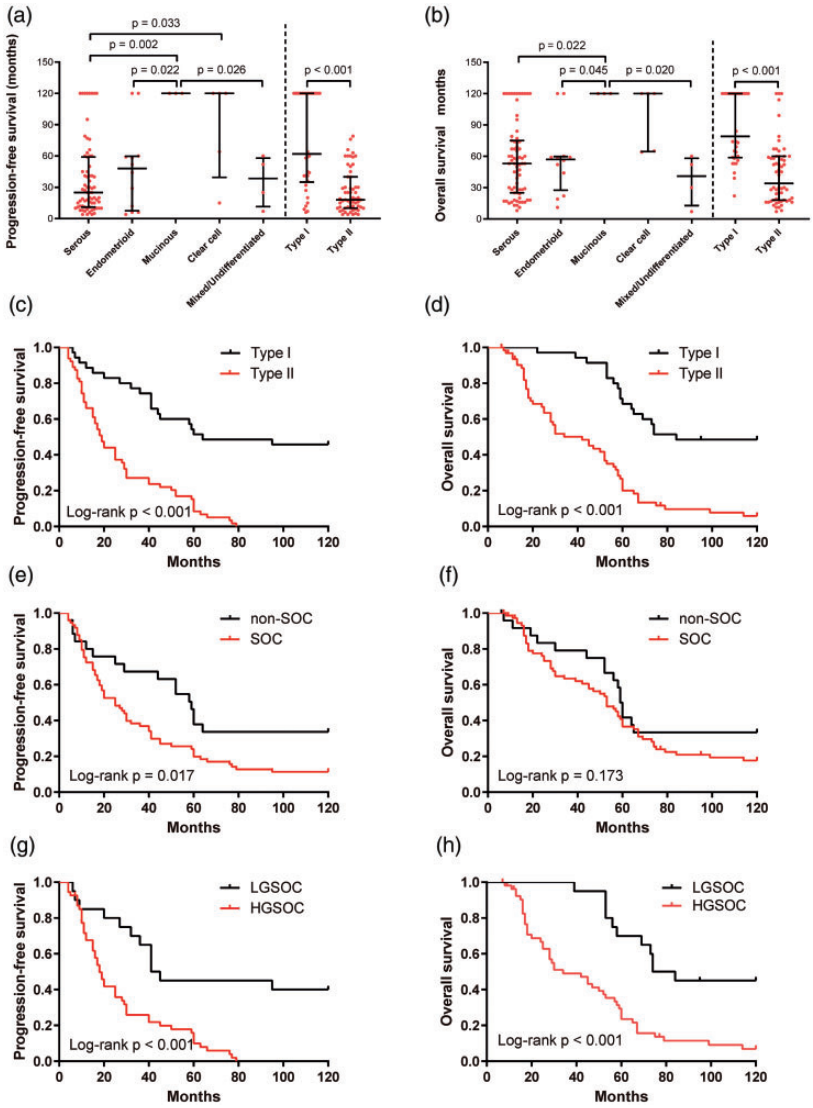


**Figure 4.** Comparison between classic and newly classified histotypes. (a, b) Patient survival information for the two histotypes. (c, d) Comparison of survival (PFS and OS) by histotype (type I vs type II). (e, f) Comparison of survival by classic histotypes (non-serous ovarian cancer vs serous ovarian cancer). (g, h) Comparison of survival by serous ovarian cancers (low-grade vs high-grade).
OS: overall survival; PFS: progression-free survival.

As shown in Table 2, there was no significant correlation between PFS and variables, such as age, ascites, lymph node metastasis, and intra-islet CD8+ TILs. There was no significant correlation between OS and variables, such as age, lymph node metastasis, stromal CD4+ TILs, and stromal CD8+ TILs. The univariate Cox model showed that worse PFS or OS was correlated with key factors, such as the type II histotype (PFS: HR 4.054, $p < 0.001$; OS: HR 3.992, $p < 0.001$),

**Table 2.** Univariate analysis of PFS and OS.

| | PFS | | OS | |
|---|---|---|---|---|
| Variables | HR (95% CI) | p-value | HR (95% CI) | p-value |
| Clinical characteristics | | | | |
| Age (≤50 vs >50 years) | 0.793 (0.509–1.235) | 0.304 | 0.438 (0.693–1.097) | 0.117 |
| CA125 (≤667 vs >667 U/mL) | 2.570 (1.621–4.073) | <0.001 | 2.702 (1.684–4.337) | <0.001 |
| Histotype (type I vs II) | 4.054 (2.329–7.085) | <0.001 | 3.992 (2.320–6.870) | <0.001 |
| Stage (IIIA/IIIB vs IIIC/IV) | 2.709 (1.642–4.468) | <0.001 | 2.400 (1.459–3.945) | 0.001 |
| Grade (G1/G2 vs G3) | 2.207 (1.346–3.618) | 0.002 | 2.598 (1.572–4.292) | <0.001 |
| Ascites (positive vs negative) | 1.531 (0.950–2.465) | 0.080 | 1.811 (1.121–2.925) | 0.015 |
| Lymph node metastasis (positive vs negative) | 0.891 (0.280–2.832) | 0.845 | 0.657 (0.161–2.682) | 0.558 |
| Chemotherapy response (sensitive vs resistant) | 3.213 (1.937–5.330) | <0.001 | 2.439 (1.474–4.036) | 0.001 |
| Immune cells | | | | |
| Islet CD4+ TILs (positive vs negative) | 2.656 (1.629–4.332) | <0.001 | 1.881 (1.167–3.032) | 0.009 |
| Stroma CD4+ TILs (positive vs negative) | 1.717 (1.089–2.707) | 0.020 | 1.552 (0.981–2.453) | 0.060 |
| Islet CD8+ TILs (positive vs negative) | 1.569 (0.977–2.520) | 0.062 | 1.843 (1.132–2.970) | 0.014 |
| Stroma CD8+ TILs (positive vs negative) | 1.881 (1.181–2.985) | 0.007 | 1.585 (0.999–2.513) | 0.050 |
| CD68+ TAMs (positive vs negative) | 3.527 (2.106–5.905) | <0.001 | 3.138 (1.879–5.242) | <0.001 |

PFS: progression-free survival; OS: overall survival; HR: hazard ratio; CI: confidence interval; CA125: carbohydrate antigen 125; TILs: tumor-infiltrating lymphocytes; TAM: tumor-associated macrophages.

chemoresistance (PFS: HR 3.213, $p < 0.001$; OS: HR 2.439, $p = 0.001$), and CD68+ TAMs (PFS: HR 3.527, $p < 0.001$; OS: HR 3.138, $p < 0.001$).

Next, we established three multivariate Cox models: A) a model adjusted for clinical variables, including CA125, histotype, stage, grade, ascites, and chemosensitivity; B) a model adjusted for parameters, including all immune cells; and C) a model adjusted for significant variables from model A and model B. To identify the determinants of survival, we generated the above multivariate Cox models with a forward approach (Table 3). The results were as follows: 1) model A revealed that higher serum CA125, type II histotype, and chemoresistance were strong clinical predictors of worse prognostic outcomes (all $p < 0.05$); 2) model B indicated that higher levels of CD68+ TAMs were associated with poorer survival ($p < 0.001$), and elevated intra-islet CD4+ TILs were correlated with poorer PFS ($p < 0.003$) but not poorer OS; 3) model C showed that higher serum CA125 and type II histotype were associated with poorer survival (all $p < 0.05$). Elevated islet CD4+ TILs ($p = 0.001$) and chemoresistance ($p = 0.001$) were closely associated with poorer PFS, and higher levels of CD68+ TAMs ($p = 0.013$) were an immune determinant of poorer OS (Table 3).

## *Relationship between omental immune cells and clinical parameters of survival in patients with advanced EOC*

We were interested in whether there was a relationship between clinical prognostic factors and immune predictors. To assess these interactions, we used Kaplan–Meier survival analysis. As described above, lower serum CA125 levels, type I histotype, and sensitivity to chemotherapy were associated

**Table 3.** Multivariate analyses of PFS and OS.

| | PFS | | OS | |
|---|---|---|---|---|
| Model/Variables | HR (95% CI) | p-value | HR (95% CI) | p-value |
| Model A: clinical variables (CA125, histotype, stage, grade, ascites, and chemotherapy response) | | | | |
| CA125 (≤667 vs >667 U/mL) | 1.834 (1.138–2.956) | 0.013 | 2.337 (1.436–3.803) | 0.001 |
| Histotype (type I vs II) | 3.061 (1.705–5.495) | <0.001 | 3.200 (1.801–5.687) | <0.001 |
| Stage (IIIA/IIIB vs IIIC/IV) | – | – | – | – |
| Grade (G1/G2 vs G3) | – | – | – | – |
| Ascites (positive vs negative) | – | – | – | – |
| Chemotherapy response (sensitive vs resistant) | 2.281 (1.334–3.901) | 0.003 | 1.755 (1.036–2.973) | 0.036 |
| Model B: immune cells | | | | |
| Islet CD4+ TILs (positive vs negative) | 2.114 (1.299–3.442) | 0.003 | – | – |
| Stroma CD4+ TILs (positive vs negative) | – | – | – | – |
| Islet CD8+ TILs (positive vs negative) | – | – | – | – |
| Stroma CD8+ TILs (positive vs negative) | – | – | – | – |
| CD68+ TAMs (positive vs negative) | 3.148 (1.883–5.263) | <0.001 | 3.138 (1.879–5.242) | <0.001 |
| Model C: adjusted for significant variables from models A and B | | | | |
| CA125 (≤667 vs >667 U/mL) | 2.003 (1.244–3.226) | 0.004 | 1.920 (1.164–3.165) | 0.011 |
| Histotype (type I vs II) | 3.026 (1.711–5.351) | <0.001 | 3.340 (1.935–5.765) | <0.001 |
| Chemotherapy response (sensitive vs resistant) | 2.181 (1.295–3.671) | 0.003 | – | – |
| Islet CD4+ TILs (positive vs negative) | 2.315 (1.387–3.864) | 0.001 | – | – |
| CD68+ TAMs (positive vs negative) | – | – | 1.989 (1.155–3.423) | 0.013 |

PFS: progression-free survival; OS: overall survival; HR: hazard ratio; CI: confidence interval; CA125: carbohydrate antigen 125; TILs: tumor-infiltrating lymphocytes; TAM: tumor-associated macrophages.

with better survival (Figure 2a–d, i, j). However, poorer PFS ($p < 0.001$) and OS ($p < 0.001$) were observed in the lower serum CA125 level subgroup with a high density of CD68+ TAMs and type I histotype cases with a high density of CD68+ TAMs in omental tumors. Similar results were found in the groups of patients with chemotherapy sensitivity who had increased omental metastatic CD68+ TAM infiltration (Figure 5). Another screened independent immune predictor, intra-islet CD4+ TILs, showed weaker interactions with clinical predictors. Poorer PFS ($p = 0.028$) but not poorer OS was observed in the lower serum CA125 level groups with CD4+ TIL infiltration in omental metastatic tumors, and similar results (poorer PFS, $p = 0.002$; but not poorer OS) were observed in the sensitive-to-chemotherapy groups with CD4+ TIL infiltration in omental metastatic tumors (Figure 6). These results indicated that patients with advanced EOC and better clinical outcome indicators (lower CA125, type I histotype, and sensitivity to chemotherapy) could be further distinguished by CD68+ TAM and intra-islet CD4+ TIL infiltration in omental metastatic tumors. Moreover, favorable survival was observed in the low CD68+ TAM infiltration group with resistance to chemotherapy (better PFS, $p = 0.041$; better OS, $p = 0.036$) and low CD4+ TIL infiltration group with higher CA125 levels (better PFS, $p < 0.001$; better OS, $p = 0.023$), type II histotype (better PFS, $p < 0.001$; but not better OS), and resistance to chemotherapy (better PFS, $p = 0.019$; but not better OS) (Supplement Figure 2 and 3).

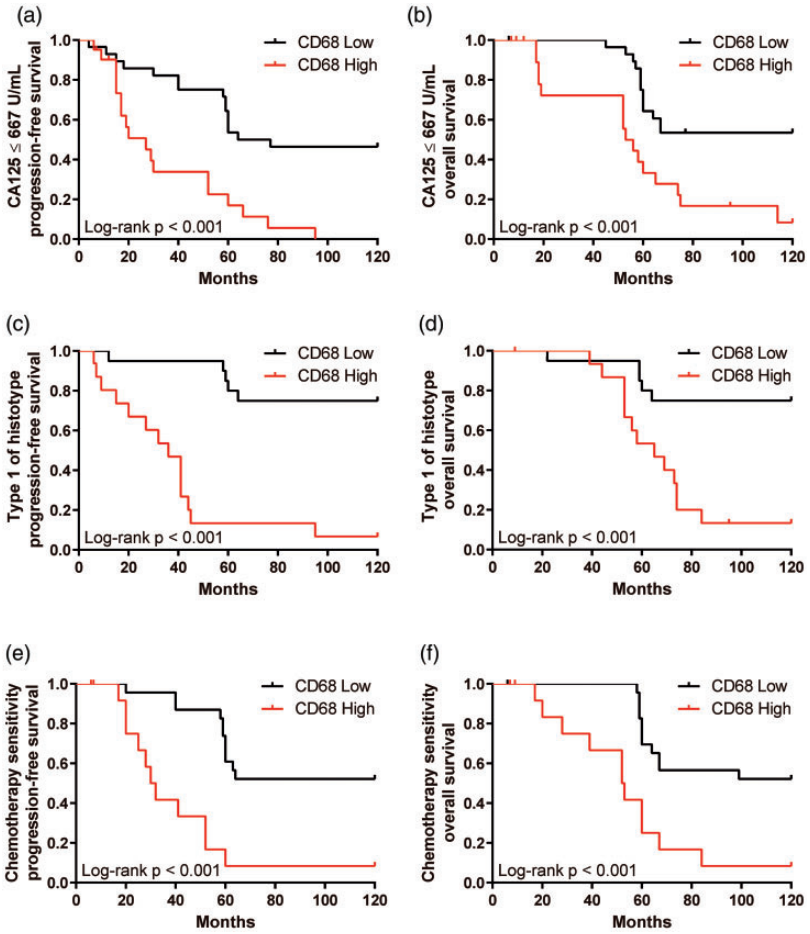


**Figure 5.** Kaplan–Meier survival analyses of the relationship between CD68+ TAMs and favorable clinical predictors. (a) Comparison of PFS by CD68+TAMs (low vs high) in the lower CA125 group. (b) Comparison of OS by CD68+TAMs in the lower CA125 group. (c) Comparison of PFS by CD68+TAMs in the type I histotype group. (d) Comparison of OS by CD68+TAMs in the type I histotype group. (e) Comparison of PFS by CD68+TAMs in the chemotherapy sensitivity group. (F) Comparison of OS by CD68+TAMs in the chemotherapy sensitivity group.
TAM: tumor-associated macrophage; OS: overall survival; PFS: progression-free survival; CA125: cancer antigen 125.

## Discussion

The TME and immunotherapy of advanced EOC have been topics of high interest over the past decade. Many studies have shown that TILs are associated with better outcomes in ovarian cancer[17,18] and that TAMs are mainly involved in ovarian cancer metastasis.[19,20] A small number of studies reported that neoadjuvant chemotherapy improved survival outcomes by promoting TIL accumulation in ovarian tumors.[21] A recently published review article mentioned an improved understanding of the key factors involved in the ovarian TME of not only primary tumors but also ascites and metastases.[17] Thus, the TME of

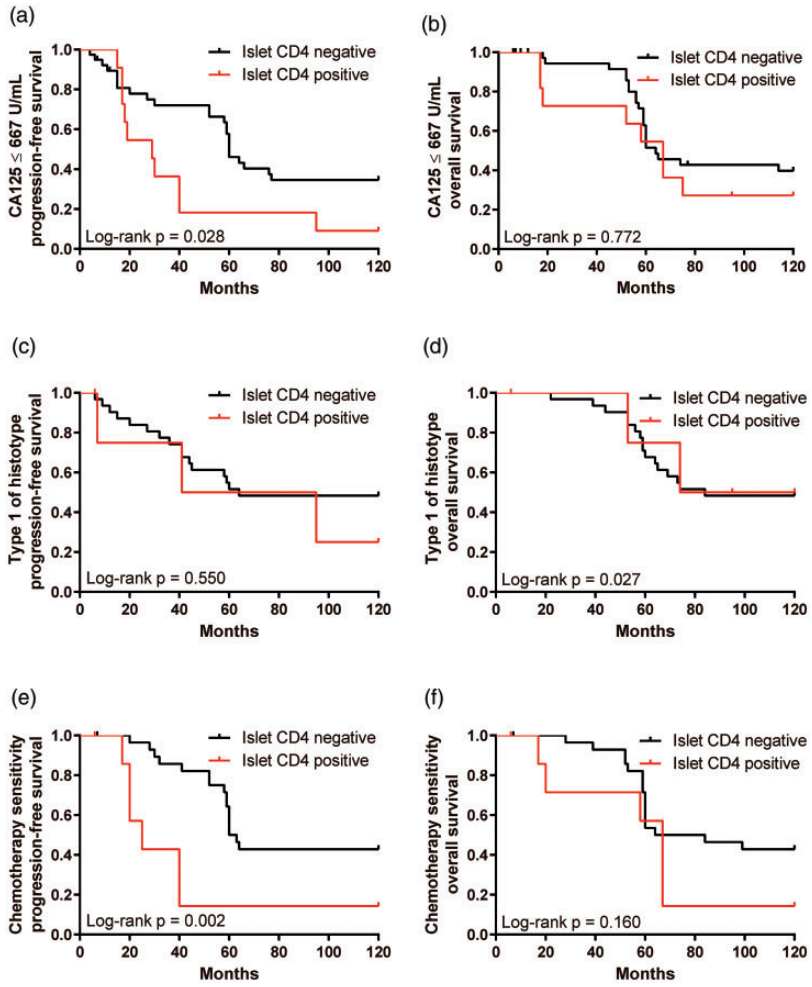


**Figure 6.** Kaplan–Meier survival analyses of the relationship between CD4 expression in cancer islets and favorable clinical predictors. (a) Comparison of PFS by CD4 expression in TILs (negative vs positive) in the lower CA125 group. (b) Comparison of OS by CD4 expression in TILs in the lower CA125 group. (c) Comparison of PFS by CD4 expression in TILs in the type I histotype group. (d) Comparison of OS by CD4 expression in TILs in the type I histotype group. (e) Comparison of PFS by CD4 expression in TILs in the chemotherapy sensitivity group. (f) Comparison of OS by CD4 expression in TILs in the chemotherapy sensitivity group.
TIL: tumor-infiltrating lymphocyte; OS: overall survival; PFS: progression-free survival; CA125: cancer antigen 125.

the omental fatty tissue in which ovarian cancer cells preferentially implant cannot be overlooked. However, in previous studies, there has been an increased focus on milky spots and the unique immune structure of the omentum in terms of the mechanisms underlying ovarian cancer metastasis.[22–24] Interestingly, no evidence has verified the consistency of immune cell-relevant survival outcomes between the primary and metastatic sites. To our knowledge, the current study is the first to

analyze the prognostic role of TILs and TAMs in omental specimens and determine essential clinical parameters in patients with advanced EOC. Our investigation led to several important findings.

This study showed that multiple clinical factors, including higher serum CA125 levels, the type II histotype, stage IIIC or IV disease, grade 3 disease, and chemotherapy resistance, were associated with worse PFS and OS in advanced EOC. These observations are consistent with those of a previous study showing that various clinical factors are associated with short-term survival.[25] We found that the EOC histotype, which is a new classification proposed approximately 15 years ago,[26] was an essential predictor of outcomes, with an impact that almost exceeded that of all other risk factors. Low-grade serous and endometrioid carcinoma, mucinous carcinoma, and clear cell carcinoma are classified as type I EOC, which harbors mutations in *KRAS*, *BRAF*, *PIK3CA*, *ERBB2*, *CTNNB1*, and *PTEN* with microsatellite instability. Type II EOC includes high-grade serous and endometrioid carcinoma, which are mainly characterized by mutations in p53.[27,28] This group of tumors contains more pathological and genetic features, which may contribute to its unfavorable outcomes.

The response to chemotherapy is an independent prognostic factor for patients with advanced EOC. Correlative studies have indicated that chemoresistance is linked to a shorter PFS and OS.[29] Identifying accurate predictors of the response to chemotherapy may enable individualized regimens for these patients and prolong their survival times. Notably, another study reported that chemotherapy resistance was more likely to occur in patients with type I EOC.[15]

It has been reported that CD68+ TAMs are related to advanced ovarian cancer but have no association with PFS or OS.[10,11] However, our study showed that CD68+ TAMs located in omental metastases from EOC samples are an independent and strong prognostic predictor of the survival outcomes of patients with advanced EOC. CD68+ TAM infiltration indicates different clinical outcomes according to high or low density and further groups other survival outcomes in subgroups of clinical prognostic indicators, such as CA125 and stage. Thus, the assessment of omental TAM infiltration is important for patients with advanced EOC to plan individualized follow-up strategies.

Only intra-islet CD4+ TILs were identified to be associated with worse PFS in the study population through multivariate Cox models, and intra-islet CD4+ TILs had a significant correlation with shorter PFS in patients with lower serum CA125 levels who were sensitive to chemotherapy (Figure 6). CD8+ TILs were not identified as a key prognostic factor in our study. Similarly, a previous study reported that CD4+, CD8+, and Foxp3+ TILs were not associated with improved PFS or OS in advanced EOC.[21] However, accumulating reports have confirmed that increased intra-islet CD8+ TILs are linked to better PFS and OS.[18,28] As evident in our data, intra-islet CD8+ TILs were moderately related to CD68+ TAMs (Figure 3), and their influence may be less significant, ranking behind TAMs in the statistical model. We assume that our findings differ from those of previous studies that examined specimens from primary (ovarian) sites. The metastatic (omental) site may have an immune TME that is distinct from that of the primary site. Only one study investigated whether increased B-cells in omental tissue were associated with poorer survival[30], and their results showed a similar trend as ours. As previously asked by Fraggetta et al.,[31] whether the immune cell predictors found by Zhang et al.[32] differ

between primary and metastatic lesions remains unknown.

This study has several limitations. Although we analyzed them separately, the small sample size limits the precise comparison of all pathological subtypes (serous, endometrioid, mucinous, clear cell, and undifferentiated carcinoma) (Figure 4). We examined only omental specimens but not paired primary ovarian tumors. However, numerous relevant papers based on the immune TME of the primary site have been published. Additionally, we were unable to distinguish between the two main phenotypes (M1/M2) of TAMs. Decreased M2 TAMs and a higher M1/M2 ratio were reported to be associated with a better prognosis of ovarian cancer.[10,11] Finally, our study enrolled a relatively small number of patients (n = 100), and some results should be further confirmed by increasing our sample size.

## Conclusion

Our work is the first to review the influence of TILs in omental metastases of EOC on clinical outcomes. Elevated CD68+ TAMs and intra-islet CD4+ TILs in omental metastases of advanced EOC are independent prognostic factors associated with worse PFS and OS. In addition, increased densities of the above immune cells resulted in worse survival of patients with lower serum CA125 levels, chemotherapy sensitivity, or the type I histotype. However, type I EOC and lower serum CA125 are favorable survival predictors. These findings are inconsistent with previous research showing no associations between CD68+ TAMs and survival outcomes in ovarian cancer. In our work, we determined that the immune TMEs of ovarian cancer and omental metastases may be different. Further investigation with a larger sample size is needed.

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### Supplemental material

Supplemental material for this article is available online.

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