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Dose-Response Relationship of CD8+ Tumor Infiltrating Lymphocytes and Survival Time in High-Grade Serous Ovarian Cancer

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Abstract

Importance—Cytotoxic CD8+ T lymphocytes (TILs) participate in immune control of ovarian cancer; however, little is known about prognostic patterns of CD8+ TILs by histotype and in relation to other clinical factors.

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Objective—To define the prognostic role of $CD8^+$ TILs in epithelial ovarian cancer.

Design—Prospective survival cohort.

Setting—Multi-center observational.

Participants—Over 5,500 patients, including 3,196 high-grade serous ovarian carcinomas (HGSOCs), followed prospectively for over 24,650 person-years.

Exposure(s)—Following immunohistochemistry, $CD8^+$ TILs were identified within the epithelial components of tumor islets. Patients were grouped based on the estimated number of $CD8^+$ TILs per high-powered field: negative (none), low (1–2), moderate (3–19), and high (≥20). $CD8^+$ TILs in a subset of patients were also assessed in a quantitative, uncategorized manner, and the functional form of associations with survival was assessed using penalized B-splines.

Main Outcome Measure(s)—Overall survival time.

Results—Among the five major invasive histotypes, HGSOCs showed the most infiltration. $CD8^+$ TILs in HGSOCs were significantly associated with longer overall survival; median survival was 2.8 years for patients with no $CD8^+$ TILs and 3.0 years, 3.8 years, and 5.1 years for patients with low, moderate, or high levels of $CD8^+$ TILs, respectively (p-trend=4.2×10^{-16}). A survival benefit was also observed among women with endometrioid and mucinous carcinomas, but not the other histotypes. Among HGSOCs, $CD8^+$ TILs were favorable regardless of extent of residual disease following cytoreduction, known standard treatment, and germline *BRCA1* pathogenic mutation, but were not prognostic for *BRCA2* mutation carriers. Evaluation of uncategorized $CD8^+$ TIL counts showed a near linear functional form.

Conclusions and Relevance—This study demonstrates the histotype-specific nature of immune infiltration and provides definitive evidence for a dose-response relationship between $CD8^+$ TILs and HGSOC survival. That the extent of infiltration is prognostic, not merely its presence or absence, suggests that understanding factors which drive infiltration will be key to unravelling outcome heterogeneity in this cancer.

INTRODUCTION

Epithelial ovarian cancer (OC) is the most lethal gynecologic cancer and is responsible for approximately 14,000 deaths annually in the United States.[1] While initial remission is often achieved, most patients relapse and die from their disease. Immune checkpoint inhibitors have demonstrated clinical activity in a small subset of OC patients.[2,3] Understanding the endogenous immune response to OC—including the frequency of $CD8^+$ tumor infiltrating lymphocytes (TILs) and their impact on prognosis—has biological and clinical relevance.

Earlier studies demonstrated that OC prognosis is associated with TILs at the time of primary cytoreductive surgery.[4–8] $CD8^+$ T cells are stimulated by peptides from degraded proteins bound to human leukocyte antigen class I molecules.[9] This can trigger $CD8^+$ T cells to kill tumor cells and secrete proinflammatory cytokines. While the presence of $CD8^+$ TILs within the epithelial component of OCs has been associated with favorable prognosis,[2,6–8,10–12] most prior analyses used simple dichotomous classification of $CD8^+$ TILs and neglected to specify the inclusion/exclusion of stromal tissue. Prior analyses have

been inadequately powered to evaluate histotype-specific survival associations. This is critical, as the invasive histotypes (high-grade serous, HGSOC, the most common and most lethal;[13] endometrioid, ENOC;[14] clear cell, CCOC;[14,15] mucinous, MOC;[16] and low-grade serous, LGSOC[17–20]) represent distinct biological processes, with distinct proposed cells of origin, clinical courses, and responses to chemotherapy.[21–23]

We conducted a large-scale assessment of intra-epithelial $CD8^+$ TILs in over 5,000 prospectively followed OC patients. Our goals were to clarify the associations and evaluate the functional form of $CD8^+$ TILs with overall survival in HGSOCs, and to explore association of $CD8^+$ TIL levels with overall survival in other histotypes.

METHODS

Study Design and Participants

We assembled a prospective cohort of 7,377 women with a primary diagnosis of epithelial ovarian, peritoneal, or fallopian tube cancer. Patients were followed from enrollment in an IRB-approved protocol until death from any cause (eTable 1).[24–26] Tumors were obtained at initial debulking surgery, formalin-fixed, paraffin embedded, and arrayed on tissue microarrays (TMAs). Clinical covariates and vital status underwent standardized quality control measures. We excluded 288 patients due to loss to follow-up, 11 with missing age at diagnosis, 65 with non-epithelial disease, and 1,436 due to inadequate quality or amount of arrayed tumor tissue, resulting in a final sample size of 5,577, including 5,078 women with tumors of the five major invasive histotypes (HGSOC, ENOC, CCOC, MOC, and LGSOC) (eTable 2). Median time from diagnosis to enrollment was zero days (interquartile range, 0 – 63); however, 38% of patients were enrolled > one month from diagnosis. As some HGSOC may be mistaken as ENOC,[27] we utilized WT1 and TP53 immunohistochemical staining from 17 studies to re-classify 82 ENOC cases as HGSOC; overall survival of these reclassified cases was consistent with HGSOC (eFigure 1).

Immunohistochemistry and Scoring

For most patients (84%), staining was performed at the Mayo Clinic using the Leica Bond RX stainer (Leica, Buffalo, IL); however, for patients enrolled at the SEA and MAY1 study sites (8% and 9%, respectively), previously stained slides were used. Immunohistochemical methods are provided in Online-Only Text. Scoring was conducted at the University of Calgary; each core was screened for a hotspot of $CD8^+$ TILs using a Nikon eclipse 80i microscope at 200 × magnification. Within each hotspot, one high power field at 400 × magnification with a 0.55 mm field diameter was evaluated, ensuring comparable area despite different core sizes across studies. Only $CD8^+$ TILs within the epithelial component of the tumor (tumor islets) were considered, disregarding $CD8^+$ cells in the stroma or abutting tumor cells (as seen, for example, in eFigure 2 classified as negative). A four-point ordinal score was defined *a priori* based on $CD8^+$ TIL counts per high-powered field: negative (none), low (1–2 TILs), moderate (3–19 TILs), and high (≥20 TILs), similar to the validated method of Zhang and colleagues,[4] except that we decreased the low to moderate cut-off from 6 TILs to 3 TILs. We did this to increase ease and consistency of scoring, as the ≥3 TIL cut-off is routinely used in colorectal carcinoma reporting to assess Lynch

syndrome.[28] Multiple cores from 156 cases were evaluated blindly by two gynecologic pathologists (WC, MK), and a weighted kappa statistic was estimated. Differences in interpretation were discussed at a multi-headed microscope, and WC scored 24% and MK 76% of the remaining cohort. TMAs included an average of 2.4 cores per patient; for cases with more than one scored core, the maximum score was used, consistent with the scoring of hotspot regions.

Analysis

Chi-square tests compared $CD8^+$ TIL categories across clinical factors. Kaplan-Meier curves visually compared survival across categories. Cox proportional hazards regression estimated hazard ratios (HRs) and 95% confidence intervals (CIs). Primary analyses were based on tests for trend, modelling the ordered $CD8^+$ TIL categories as a one degree-of-freedom linear term. Regression models included age at diagnosis (continuous), stage (I/II, III/IV, unknown), and study site as covariates; we also ran sensitivity analyses adjusting for extent of residual disease and post-surgical treatment. Separate analyses were conducted by histotype and among histopathological groupings (e.g., combining LGSOC with their suspected precursor, serous borderline tumors), and by relevant clinical factors. This report meets REMARK reporting recommendations for tumor marker prognostic studies;[29] additional statistical methods are provided in Online-Only Text.

$CD8^+$ TIL Cutpoint Analysis

Because categorical $CD8^+$ TIL cutpoints may artificially restrict variability in the data and can be somewhat arbitrary, MK rescored all cores from a subset of 2,175 patients (1,449 with HGSOC), recording $CD8^+$ TIL count as a numeric marker. Each core was given a value between 0 and 20+, using a threshold of 20 for counts that exceeded that number. As before, the maximum score was used for cases with more than one scored core. Among HGSOC cases, we compared survival distributions of those with rescored levels to those without using Cox proportional hazards regression. Among HGSOC cases with rescored $CD8^+$ TIL levels, we ran five additional sets of Cox regression analyses. We first categorized the levels using our original thresholds (0, 1–2, 3–19 and 20+ $CD8^+$ TILs) to confirm that our original results using all HGSOC cases did not differ from the subset who were rescored. Second, we categorized the levels using the thresholds of Zhang and colleagues[4] to determine the robustness of our original results to these cutpoints. Third, we assessed the functional form of the association between $CD8^+$ TIL levels and survival using penalized B-splines.[30] Fourth, we fit the numerically-valued $CD8^+$ TIL levels as a one-degree-of-freedom linear term. Finally, we carried out a formal cutpoint analysis similar to that described by Budczies and colleagues.[31] Briefly, this approach examines all possible contiguous dichotomizations of TIL levels (i.e., 0 vs. 1+, 0–1 vs. 2+, 0–2 vs. 3+, etc.) using Cox proportional hazards regression to identify the threshold which best discriminates survivors from non-survivors based on evidence of association.

RESULTS

Distribution of CD8+ TILs by Histotype

Epithelial $CD8^+$ TILs were assessed using a four-tiered scoring system (inter-observer agreement 81.8%; weighted kappa 0.846, 95% CI 0.804–0.888). We observed intratumoral heterogeneity in $CD8^+$ TILs across cores per patient (intraclass correlation coefficient = 0.56, 95% CI 0.54–0.57). $CD8^+$ TILs varied across the major invasive OC histotypes (HGSOC, ENOC, CCOC, MOC, and LGSOC, chi-square $p=2.8 \times 10^{-103}$; eFigure 3). Most HGSOC cases (83%) had evidence of $CD8^+$ TILs, with a lower proportion seen in LGSOC and ENOC cases (73%, 72% respectively) and CCOC and MOC cases (52%, 51%, respectively). Most borderline tumors showed evidence of $CD8^+$ TILs (serous 84%, mucinous 70%; eTable 3).

Associations of CD8+ TILs with Overall Survival by Histotype

We observed a striking association for longer survival time with increasing levels of $CD8^+$ TILs in HGSOC cases (p-trend adjusted for study, age, and stage = 4.2×10^{-16}; Table; Figure). Median survival was 2.8 years for women negative for $CD8^+$ TILs, and 3.0 years, 3.8 years, and 5.1 years for low, moderate, or high levels. At the extremes, women with high levels of $CD8^+$ TILs (≥20 per field) had a 43% reduced risk of death compared to women with no evidence of $CD8^+$ TILs (HR 0.57, 95% CI 0.49–0.65; Table). Associations were similar after adjustment for residual disease (eTable 4).

Increasing levels of $CD8^+$ TILs were also associated with longer survival time among women with ENOC (p-trend=0.0084; Table; Figure). This association was also apparent in separate analyses of grade 1 ENOC and grades 2 and 3 ENOC, although these were limited in sample size (eTable 5). While there was a statistically significant dose-response similar to HGSOC, it is noteworthy that ENOCs with moderate levels (3–19 per field) showed the greatest improvement in survival time compared to women with ENOC and no detectable $CD8^+$ TILs (HR 0.50, 95% CI 0.34–0.74).

A similar association was observed for women with MOC (p=0.037; Table; Figure), although, as the histotype with the lowest overall levels of $CD8^+$ TILs, only 13 women (4%) had high TIL levels. Kaplan-Meier plots indicate a dose-response relationship, at least for negative to moderate levels (Figure). In contrast, CCOCs and LGSOCs showed no apparent association between $CD8^+$ TILs and survival time (Table, eFigure 4). Because LGSOC is the rarest of the invasive histotypes, the null association in this group should be interpreted with caution. As some prior studies combined LGSOC and HGSOC, we also analyzed invasive serous cases as a group, including those with missing grade. We found that the striking HGSOC results were attenuated (eTable 5), suggesting that the relevance of $CD8^+$ TILs among serous cases may be limited to HGSOC and confirming that immunohistochemistry-aided histotype classification is a critical first step to improving the classification of OC cases.[27,32] No other patterns were observed in analyses of histopathological groups (eTable 5).

Among the five major invasive histotypes, time to disease progression was known for 52% of cases (N=2,681). Progression-free survival results were remarkably similar to overall survival results (eTable 6).

Associations of CD8+ TILs with Clinical Features in HGSOC

The extent of residual disease following primary cytoreductive surgery was available for 2,173 HGSOC cases. Our results showed a greater proportion of tumors without macroscopic residual disease had high $CD8^+$ TIL levels than those with macroscopic disease (26% *v* 20%; p=0.0064; eFigure 3). Increasing $CD8^+$ TILs were associated with improved survival in a dose-response manner in both surgical outcome groups, indicating that immune response improves prognosis regardless of the remaining residual disease after surgery (eTable 7).

Our study included 133 *BRCA1* and 66 *BRCA2* mutation carriers and 844 tested non-carriers. The extent of $CD8^+$ TILs differed by mutation status (p=0.024), as 29% of *BRCA1* mutation carriers had high TIL counts, yet only 18% of non-carriers and 15% *BRCA2* mutation carriers did (eFigure 3). The survival benefit associated with $CD8^+$ TILs was also found to differ by mutation status (p-interaction=0.0055). Increased $CD8^+$ TILs were associated with favorable survival among cases without mutations ($p=5.1 \times 10^{-7}$) and among cases with a *BRCA1* mutation (p=0.0025, eTable 7). Among *BRCA2* mutation carriers, there was no evidence of association between $CD8^+$ TILs and survival (p=0.62).

Treatment details were documented for 501 HGSOC cases who received standard first line chemotherapy, including 295 who received the standard dose (carboplatin AUC 5 or 6 and paclitaxel 135 mg/m^2 or 175 mg/m^2). Association with $CD8^+$ TIL level and overall survival was also observed within this group (p trend=0.003, eTable 7).

Among HGSOC cases, $CD8^+$ TIL level was associated with earlier stage ($p=4.3 \times 10^{-4}$) and younger age at diagnosis ($p=1.6 \times 10^{-4}$). In stratified analyses $CD8^+$ TIL level was consistently prognostic in stage and age subgroups (eTable 5). We also observed that cases born more recently showed higher levels (n=2,734, p=0.001); we adjusted all analyses additionally for year of birth, and results were similar. $CD8^+$ TIL level was not associated with year of diagnosis (p=0.71), self-reported racial group (p=0.74), or pre- or post-treatment CA125 (p=0.42 and 0.89; respectively).

Analysis of CD8+ TIL Cutpoints in HGSOC

Of the 3,196 HGSOC cases, 1,449 (45%) were rescored using a numeric count. There were no differences in survival between women who were rescored and those who were not (p=0.12; kappas comparing original values to rescored values 0.91 (95% CI 0.89–0.92). eTable 8 displays associations of categorized $CD8^+$ TIL levels and survival in women with rescored tumors. After adjustment for age and stage, strong dose-response associations were observed using both the original threshold values (0, 1–2, 3–19 and 20+ $CD8^+$ TILs) and those used by Zhang and colleagues (0, 1–5, 6–19, 20+ $CD8^+$ TILs) ($p<10^{-5}$ for each). As before, associations were slightly attenuated but remained significant after adjustment for extent of residual disease and post-surgical treatment ($p<10^{-4}$ for each).

Assessment of the functional form of the association between numeric $CD8^+$ TIL levels and survival using penalized B-splines, after adjustment for age and stage, is shown in eFigure 5. We observed a strong negative association with survival, indicating that increasing $CD8^+$ TIL levels are progressively protective across this spectrum of values. The results of fitting $CD8^+$ TIL levels as a one-degree-of-freedom linear term are also shown in eFigure 5 and track very closely to those using penalized B-splines, indicating that the association between $CD8^+$ TIL levels and survival in women with HGSOC is virtually log-linear in nature.

Results of a formal cutpoint analysis examining all possible sets of contiguous dichotomizations of TIL levels can be found in eTable 9. The best discrimination of survivors from non-survivors occurred when comparing those with 0–13 TILs to those with 14 or more (HR 0.75, 95% CI 0.65–0.86, $p=1.5 \times 10^{-5}$). However, each of the 19 dichotomizations yielded highly significant results (all $p<=1.1 \times 10^{-3}$), with HRs consistently ranging from 0.75 to 0.83, again indicating that greater TIL levels are protective across the entire spectrum of values examined.

DISCUSSION

Our study is by far the largest report on intra-epithelial $CD8^+$ TILs in OC to date and shows a robust dose-dependent increase in survival for increasing TIL levels in women with HGSOC. Analyses on a subset of individuals using numeric TIL counts confirmed a progressively protective, nearly log-linear survival effect as $CD8^+$ TILs counts increased from 0 to 20 or more per high-powered field, suggesting that the quantity of $CD8^+$ TILs, not merely their presence, is informative and that the most immune-rich HGSOCs are the most likely to have improved clinical outcome. This effect was not modified or confounded by the extent of residual disease after cytoreductive surgery. As there are fewer than a handful of other validated prognostic biomarkers for HGSOC, e.g., *BRCA1* and *BRCA2* status[33] and PR expression,[26] these results may provide increased prognostic prediction.

This is the first $CD8^+$ TIL study in histotypes other than HGSOC; we revealed a significant reduction in risk of death for patients diagnosed with ENOC and MOC. In ENOC, cases with moderate $CD8^+$ TIL levels had the most favorable survival, with no additional benefit observed beyond this threshold. As prior reports suggest that ENOCs with high $CD8^+$ TIL are more commonly mismatch repair deficient,[34] we speculate that, similar to endometrial cancers,[35,36] ENOC with high $CD8^+$ TIL levels may be associated with an intermediate outcome due to the association with mismatch repair deficiency. No survival associations were seen in CCOC.

Other investigations have noted higher response rates to immune checkpoint blockade among patients with a higher burden of neoantigens,[37,38] suggesting that increased neoantigens increases likelihood that T lymphocytes recognize tumor as foreign and mount an immune response. It has also been demonstrated that *BRCA1*-mutated HGSOC tumors have a higher average neoantigen number than non-mutated tumors.[39] Here, HGSOC patients with germline *BRCA1* mutations demonstrated higher $CD8^+$ TILs than patients with *BRCA2* mutations or those tested mutation negative. While neoantigen load may explain

higher CD8+ TILs in *BRCA1*-mutated tumors, and their association with better outcome, it does not explain the better outcome of *BRCA*2-mutated tumors.[40,41]

Given its robust prognostic ability, relative ease of testing, and low inter-observer variability (percent agreement=81.8%, weighted kappa=0.846), quantitation of CD8+ TILs should be considered for clinical evaluation as suggested for other cancers.[42–44] Unfortunately, as expected, we found intratumoral heterogeneity in CD8+ TILs across cores per patient (intraclass correlation coefficient = 0.56, 95% CI 0.54–0.57). To account for this, we utilize the maximum score, which is akin to the hotspot assessment of proliferation in other cancers, and is more feasible for surgical specimens with many tumor-containing slides. We also propose that, similar to the breast cancer community, a practical and robust scoring system should be developed.[43] Additional issues requiring large-scale study which were not evaluated here include: utility of image analysis; evaluation of stromal CD8+ TILs; consistency across multiple tumor sites per patient; impact of neoadjuvant chemotherapy;[45–47] relationships between CD8+ TIL levels, HGSOC molecular subtypes,[48–50] common genetic variation,[51] and epidemiologic risk factors;[52] and evaluation of other lymphocyte subsets, such as CD4+ TILs, CD20+ TILs (B cells), tertiary lymphoid structures, and plasma cells.[10,12–14,53] Clinically, it will be important to test whether CD8+ TILs predict response to certain therapies including standard chemo- and immune therapy, as, for example, CD8+ TILs predict chemo-response in subtypes of breast cancer.[54] It will also be critical to study whether the immune response of CD8+ TILs can be activated by checkpoint blockade.

In summary, these large-scale analyses show that CD8+ TILs vary by histotype with HGSOC tumors having the highest levels and a strong association with survival, regardless of extent of residual disease or first line chemotherapy treatment. Penalized B-splines revealed that this association was nearly log-linear in nature, indicating that progressively greater TIL counts yield progressively better prognoses for HGSOC tumors. We showed for the first time that CD8+ TILs in HGSOC cases with germline *BRCA2* mutations may not associate with survival. Finally, we find that ENOC and MOC tumors show trends associating CD8+ TILs with survival time and that CCOC do not. A clinically applicable scoring system for CD8+ TILs should be developed in order to incorporate into clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

TIL	tumor infiltrating lymphocytes
HGSOC	high-grade serous ovarian carcinoma
OC	ovarian cancer
PD-1	programmed death 1
ENOC	endometrioid ovarian cancer
CCOC	clear cell ovarian cancer
MOC	mucinous ovarian cancer
LGSOC	low-grade serous ovarian cancer
TMA	tissue microarray

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KEY POINTS

Question

To what extent are $CD8^+$ tumor infiltrating lymphocytes (TILs) prognostic in epithelial ovarian cancer?

Findings

Analysis of over 24,000 person-years of follow-up on over 5,500 cases shows improved survival with increasing $CD8^+$ TIL counts in high-grade serous, endometrioid, and mucinous ovarian cancers (p-trends 4.2×10^{-16}; 0.0084, and 0.037, respectively). Among high-grade serous ovarian cancers, this nearly log-linear relationship was present regardless of extent of residual disease following cytoreduction, receipt of standard treatment, and germline *BRCA1* mutation.

Meaning

$CD8^+$ TILs are a key prognostic factor in certain ovarian cancer histotypes and warrant additional study in the context of immunotherapy.

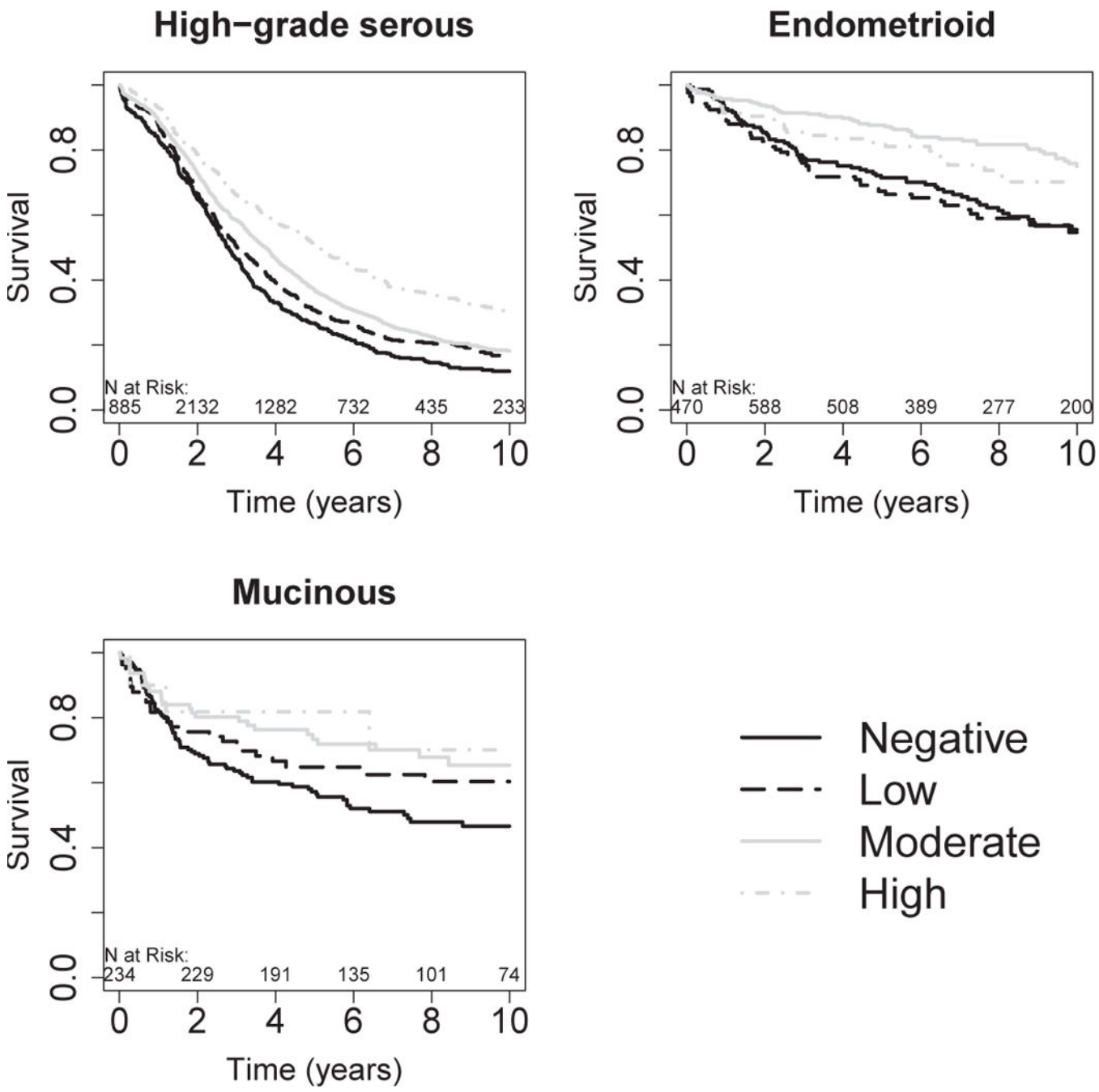

Figure. Kaplan-Meier Overall Survival Plots by CD8$^+$ Tumor-Infiltrating Lymphocyte (TIL) Levels for the High-Grade Serous, Endometrioid, and Mucinous Ovarian Cancer

Negative, no CD8$^+$ TILs; low, 1–2 CD8$^+$ TILs; moderate, 3–19 CD8$^+$ TILs; high, 20 or more CD8$^+$ TILs per high-powered field. The numbers just above the x-axis represent the number of women at risk in two year time intervals. Number at risk on date of diagnosis may be smaller than number at risk later due to left truncation of follow-up resulting from delayed study enrollment.

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Table

Multivariate-adjusted association of $CD8^+$ tumor-infiltrating lymphocytes (TIL) and overall survival among cases with the five most common invasive epithelial ovarian cancer histotype (N=5,078)

Histotype	$CD8^+$ TILs	N	Person-Years	% events	HR (95% CI)	P value trend	P value 3 d.f.
High-grade serous	Negative	546	1,709.75	76.2%	ref	4.2×10^{-16}	7.2×10^{-15}
	Low	546	1,908.39	72.3%	0.86 (0.75–0.99)		
	Moderate	1,394	5,264.82	69.0%	0.77 (0.69–0.87)		
	High	710	3,110.87	56.5%	0.57 (0.49–0.65)		
Endometrioid	Negative	206	1,118.53	33.5%	ref	0.0084	0.006
	Low	130	675.44	34.6%	0.80 (0.54–1.18)		
	Moderate	283	1,844.53	18.0%	0.50 (0.34–0.74)		
	High	110	657.59	22.7%	0.76 (0.47–1.23)		
Clear cell	Negative	309	1,640.28	41.1%	ref	0.50	0.52
	Low	141	658.25	45.4%	1.16 (0.84–1.60)		
	Moderate	118	618.79	41.5%	0.88 (0.62–1.24)		
	High	80	412.44	40.0%	0.92 (0.61–1.39)		
Mucinous	Negative	168	750.75	44.0%	ref	0.037	0.16
	Low	77	375.26	31.2%	0.91 (0.55–1.51)		
	Moderate	85	470.62	27.1%	0.56 (0.34–0.93)		
	High	13	72.35	23.1%	0.79 (0.23–2.68)		
Low-grade serous	Negative	43	198.06	48.8%	ref	0.91	0.996
	Low	44	184.89	65.9%	0.94 (0.50–1.74)		
	Moderate	63	272.13	47.6%	0.98 (0.52–1.83)		
	High	12	49.53	41.7%	0.92 (0.33–2.59)		

Adjusted for study, age (continuous), and stage (I/II, III/IV, unknown); levels based on counts of TIL per high-powered field: negative, none; low, 1–2; moderate, 3–19; high, 20+; HR, hazard ratio; CI, confidence interval; P value trend, from a one degree-of-freedom trend test; P value 3 d.f., from an unordered three degree-of-freedom test.