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Tumour infiltrating lymphocytes and survival after adjuvant chemotherapy in patients with gastric cancer: post-hoc analysis of the CLASSIC trial

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ABSTRACT

Background

Only a subset of gastric cancer (GC) patients with stage II-III benefits from chemotherapy after surgery. Tumour infiltrating lymphocytes per area (TIL density) has been suggested as a potential predictive biomarker of chemotherapy benefit.

Methods

We quantified TIL density in digital images of haematoxylin-eosin (HE) stained tissue using deep learning in 307 GC patients of the Yonsei Cancer Center (YCC) (193 surgery+adjuvant chemotherapy [S+C], 114 surgery alone [S]) and 629 CLASSIC trial GC patients (325 S+C and 304 S). The relationship between TIL density, disease-free survival (DFS) and clinicopathological variables was analysed.

Results

YCC S patients and CLASSIC S patients with high TIL density had longer DFS than S patients with low TIL density (*P*=0.007 and *P*=0.013, respectively). Furthermore, CLASSIC patients with low TIL density had longer DFS if treated with S+C compared to S (*P*=0.003). No significant relationship of TIL density with other clinicopathological variables was found.

Conclusion

This is the first study to suggest TIL density automatically quantified in routine HE stained tissue sections as a novel, clinically useful biomarker to identify stage II-III GC patients deriving benefit from adjuvant chemotherapy. Validation of our results in a prospective study is warranted.

INTRODUCTION

Despite a decline in gastric cancer (GC) incidence, it remains the fifth most common cancer worldwide, with one million new cases and over 769,000 deaths in 2020.[1] Disease stage, patient performance status and patient preferences are currently used to determine patient treatment.[2] In Asia, the standard of care for patients with TNM stage II-III GC is D2 gastrectomy followed by adjuvant chemotherapy, based on results from the ACTS-GC trial[3] and the CLASSIC trial.[4] Benefit from adjuvant chemotherapy remains modest with a 9-11% improved 5-year overall survival.[4,3] Intensifying treatment by increasing the number of drugs or chemotherapy cycles did not improve survival in GC patients with resectable disease.[5-8] It has therefore been suggested that only a subset of GC patients benefits from adjuvant chemotherapy after surgery, irrespective of treatment intensity or modality.

A number of studies identified molecular GC subtypes.[9,10] A previous study in the CLASSIC trial patients identified 4 genes able to predict prognosis and benefit from adjuvant chemotherapy potentially complementing pTNM staging in deciding eligibility for adjuvant chemotherapy.[11] However, none of the published GC molecular classifiers is currently used in clinical routine to determine individual patient's treatment with the exception of HER2 and PD-L1 status in patients with metastatic GC.[10,12,13,2] Thus, there remains an urgent clinical need to identify biomarker that can predict which GC patient likely benefits from adjuvant chemotherapy and which are easy to measure reproducibly in routinely available patient material at relatively low costs.

There has been a growing interest in the role of the tumour microenvironment in cancer progression and response to therapy.[14] Tumour infiltrating lymphocytes (TILs) are a major cellular component of the tumour microenvironment reflecting the host's status of anti-tumour immunity. Although there is currently no consensus on how TILs should be evaluated in GC and investigators have used different methodological approaches in the past, there seems to be a general agreement that a high number of TILs is associated with improved survival in patients treated by surgery (for recent meta-analysis see [15]). Evaluation of TILs in routine haematoxylin-eosin (HE) stained tissue sections would be very convenient, not require any additional material or special staining and be less costly compared to immunohistochemistry or RNA based assays. The feasibility and clinical value of quantifying TILs in HE stained tissue sections has been

demonstrated in a number of different cancers including breast,[16] lung,[17] urothelial,[18] and colorectal cancer.[19] Previous studies in GC quantifying TILs in HE stained tissue sections with different methods suggest a relationship between high TIL density (e.g. number of TILs/mm^2 tissue area) and lower pT (depth of invasion), lower number of lymph node metastases, absence of lymphovascular or perineural invasion as well as improved survival in GC patients treated with surgery.[20-22] However, there has been no study in patients with resectable stage II-III GC to date investigating the relationship between TIL density in HE stained tissue sections and potential patient benefit from adjuvant chemotherapy.

We hypothesised that (a) only GC patients with low TIL density have a survival benefit from adjuvant chemotherapy and (b) postoperative survival of GC patients with high TIL density cannot be further improved by adjuvant chemotherapy.

The aim of this study was to establish whether TIL density has clinical utility as predictive and/or prognostic biomarker in GC patients with locally advanced resectable disease. In order to investigate this question, we used a deep learning-based image analysis approach to quantify TIL density in gastrectomy specimens from two patient cohorts: (1) a well characterised local series of GC patients treated at the Yonsei Cancer Center, Seoul, Republic of Korea and (2) a larger cohort of GC patients recruited into the CLASSIC trial. The relationship between TIL density, clinicopathological variables and 5-year disease-free survival comparing patients treated with surgery alone versus those treated with adjuvant chemotherapy was analysed.

MATERIAL AND METHODS

Yonsei Cancer Center (YCC) patients

One representative HE stained tumour section from gastrectomy specimens from 307 patients with resectable, stage II-III GC treated with either surgery alone (*N* = 114) or surgery followed by fluorouracil-based adjuvant chemotherapy (*N* = 193) treated at the Yonsei Cancer Center (YCC), Seoul, Republic of Korea,[11] was selected and scanned at 40x magnification (Leica Aperio AT scanner). Two virtual 3 mm diameter cores (circles) were sampled from areas with highest tumour density (tumour hotspots) avoiding lymphoid aggregates (supplement figure S1). The study was approved by the local institutional review board, which waived the need for patient informed consent for this retrospective study.

As the YCC cohort was a historical single centre cohort e.g., not randomised for adjuvant therapy, there was an unequal TNM stage distribution with different treatment strategy per stage and we decided to also study a patient cohort randomised for adjuvant therapy, the CLASSIC trial.

CLASSIC trial patients

The CLASSIC trial (NCT00411229) was a randomised, open-label, multicentre, phase 3 study comparing D2 gastrectomy followed by adjuvant capecitabine and oxaliplatin chemotherapy with surgery alone in 1035 stage II-III GC patients demonstrating better survival in the adjuvant chemotherapy arm.[4] Tissue microarrays (TMAs) were constructed previously sampling two 3 mm diameter cores from areas with the highest tumour density (tumour hotspots) avoiding lymphoid aggregates from 629 resected primary tumour (304 patients treated with surgery alone [S], 325 patients treated with surgery followed by adjuvant chemotherapy [S+C]).[23] Four µm sections were stained with HE using a standard laboratory protocol and scanned at 40x magnification (Leica Aperio AT scanner, University of Leeds, UK). There was no significant difference in clinicopathological characteristics or survival when comparing the subset of patients included in the current study to the whole CLASSIC trial population as previously reported.[23] This study was approved by the local institutional review board, which waived the need for patient informed consent for this retrospective study.

Image analysis pipeline to establish TILs/mm^2

In CLASSIC, individual TMA cores were manually outlined to obtain the exact area in mm^2 and linked to a core identifier. In YCC, two 3 mm diameter circles were manually placed onto the slide using HeteroGenius-MIM image analysis software (HeteroGenius Ltd., Leeds, UK) (supplement figure S1). Digital slides and annotations from both cohorts were uploaded to HeteroGenius-MIM image analysis software. The number of tumour infiltrating lymphocytes (TILs) per mm^2 tumour area (TIL density) was obtained using the same multistep process in both cohorts.

For lymphocyte detection we used the Cell Analysis module in HeteroGenius-MIM. This implements a UNET [24] based cell detector that learns to detect, segment, and classify cells by type in whole slide images. We trained this detector by manually labelling approximately 50,000 cells as either lymphocyte, tumour cell, plasma cell, endothelial cell, normal epithelial cell, fibroblast, smooth muscle cell or other in 397 subimages (200µm x 200µm) from 118 HE stained tissue sections from oesophagogastric cancer resections or biopsies from a range of institutions from Europe, Japan, and Korea. The model was trained for 58,000 epochs with a learning rate of 1e-7. Cells labelled as lymphocytes within annotated regions were used for subsequent analysis.

Manual quality control of the TILs detection was performed at different steps in the process by a senior pathologist (HIG) on 10% randomly selected cores blinded for clinicopathological variables, all cores with TIL density values greater or smaller than 2 standard deviations of the mean, and cases where the TIL density measurement varied substantially between cores from the same patient (<50% or >200% TIL density of the other core).

Furthermore, all cores were reviewed by two senior gastrointestinal pathologists (HIG, MCK) to (1) identify cores which contained only tumour epithelium e.g. no non-neoplastic gastric epithelium and (2) to determine the histological tumour phenotype according to World Health Organisation (WHO) classification.[25]

For an example of image analysis-based TILs detection in HE stained gastric cancer, see figure 1.

Tumour infiltrating lymphocyte density (TIL density) was calculated by dividing the total number of lymphocytes detected within an annotated region of interest (ROI) by the size of the area of the ROI.

We did not distinguish between TILs located in the stroma and TILs located within the tumour epithelium. This choice was made based on results from a previous study which identified total TILs per tumour area as the best index for TILs evaluation in GC.[22] Our methodology differs from the one used by the International Immuno-Oncology Biomarker Working Group on Breast Cancer [26] and by Zhang et al[22], as these studies estimated the percentage of the stroma area 'covered' with TILs.

TIL counts of all ROIs available per patient were added and divided by the sum of the area size of all ROIs (mm^2) to calculate TIL density per patient. In CLASSIC, TMA cores with a total tissue area of less than 1 mm^2 and cores containing any amount of non-neoplastic gastric epithelium were excluded from analyses (see supplement figure S2).

Statistical analyses

In the YCC cohort, the relationship between TIL density and clinicopathological variables (age, sex and pTNM stage) was analysed using the Kruskal-Wallis test. In the YCC cohort, the disease stage was reported using AJCC TNM 6th edition.[27] As details on pT and pN categories were not available, we were unable to convert to TNM 7th edition for the YCC cohort.

In the CLASSIC trial cohort, the relationship between TIL density and clinicopathological variables (age, sex, pT category [depth of invasion], pN category [lymph node status], pTNM stage, core-based WHO histological tumour type, EBV status, and microsatellite instability [MSI] status) was analysed using the Kruskal-Wallis test. In the CLASSIC trial, the disease stage was originally reported using UICC TNM 6th edition.[28] For our study, we converted the pT and pN category to UICC TNM 7th edition.[29]

As recurrent disease has a significant impact on quality of life, prevention of recurrence by individualising adjuvant treatment based on biomarker selection is of particular clinical importance. We therefore focussed our analyses on disease-free survival (DFS) as the primary endpoint rather than overall survival. DFS was defined as the time from the date of surgery or

randomisation (as appropriate) to the date of the first event (recurrent disease or death) or date of last follow up. Analyses were performed using the Kaplan-Meier method, log-rank tests and Cox proportional hazards models.[30] A TIL density classifier (high versus low) was established based on the median TIL density of each cohort. In each cohort, treatment interaction was analysed.
The multivariable Cox proportional hazards model was adjusted for factors significant in univariate analysis. *P* values of less than 0.05 were considered significant. Statistical analyses were performed using SPSS, version 27 (IBM Corporation, Somers, NY, USA).

RESULTS

Yonsei Cancer Center gastric cancer patients

After quality control, values for tumour infiltrating lymphocyte counts per mm^2 (TIL density) were available from 302 GC patients (111 S, 191 S+C, see supplement figure S2A). The median TIL density of all 302 YCC patients was 1290 TILs/mm^2 (range: 59 – 11,162 TILs/mm^2). There was no significant difference in TIL density between treatment arms in the YCC cohort (S patients: median [range] TIL density 1164 TILs/mm^2 [59 – 9461 TILs/mm^2], S+C patients: median [range] TIL density 1335 TILs/mm^2 [77 – 11,162 TILs/mm^2], *P* = 0.099).

The analysis of the relationship between TIL density and clinicopathological variables (age, sex, and pTNM stage) using data from all YCC patients showed no relationship between TIL density, age, sex, or pTNM stage (table 1). The median follow-up time for 5-year DFS was 3.5 years for S patients (range: 0.08 – 5.33 years) and S+C patients (range: 0.17 – 5.00 years). The disease stage distribution in the YCC cohort was significantly different between treatment (S patients: 62.2% (*N* = 69) stage II, 37.8% (*N* = 42) stage III; S+C patients: 36.6% (*N* = 70) stage II, 63.4% (*N* = 121) stage III; P < 0.001).

TIL density and survival

There was no difference in DFS between YCC patients with low or high TIL density GC related to treatment (figure 2A and 2B).

We explored the relationship between TIL density and DFS in YCC patients per treatment. YCC S patients and YCC S+C with high TIL density had significantly longer DFS compared to YCC S patients and YCC S+C patients with low TIL density, respectively (S: HR 2.496, 95%CI 1.288-4.837, *P* = 0.007; S+C: HR 1.792, 95%CI 1.134-2.833, *P* = 0.012; figure 2C and 2D). TIL density remained significant in multivariable analysis including pTNM stage and treatment in the model (HR 1.999, 95%CI 1.377-2.902, *P* < 0.001; supplement table S1). TIL density treatment interaction *P*-value was 0.584.

CLASSIC trial patients

After quality control, values for tumour infiltrating lymphocyte counts per mm^2 (TIL density) were available from 549 GC patients (267 S patients, 282 S+C patients, see supplement figure S2B). The median TIL density of all 549 CLASSIC trial GC patients was 1360 TILs/mm^2 (range: 93 – 7989 TILs/mm^2). There was no significant difference in TIL density between treatment arms in the CLASSIC cohort (S patients: median [range] TIL density 1363 TILs/mm^2 [93 – 6060 TILs/mm^2], S+C patients: median [range] TIL density 1359 TILs/mm^2 [157 – 7989 TILs/mm^2], *P* = 0.602).

The relationship between TIL density and clinicopathological variables (pT, pN, pTNM stage, age, sex, histological tumour type, microsatellite instability [MSI] status, and Epstein-Barr Virus [EBV] status) was analysed in the whole group of patients irrespective of treatment allocation. CLASSIC trial patients with EBV positive GC had more frequently high TIL density (>1360 TILs/mm^2) compared to CLASSIC trial patients with EBV negative GC (*P* < 0.001). TIL density was not related to age, sex, pT, pN, pTNM stage, WHO histological tumour type, or MSI status. For details see table 2. The median follow-up time for 5-year DFS was 52 months (range: 1 month – 60 months) and 60 months (range: 1 month – 60 months) for S patients and S+C patients, respectively. The disease stage distribution in the CLASSIC trial cohort with available TIL density data was more balanced between treatment arms compared to the YCC cohort (S patient: 37.8% [*N* = 101] stage I/II, 62.2% [*N* = 166] stage III; S+C patients: 29.4% [*N* = 83] stage I/II, 70.6% [*N* = 199] stage III, *P* = 0.037). Originally, using TNM 6^{th} ed, all CLASSIC trial patients had either stage II or stage III disease. With the reclassification according to TNM 7^{th} ed, one of the CLASSIC trial patients had to be classified as stage I.

TIL density and survival

CLASSIC trial patients with low TIL density GC (*N* = 273, 49.73% of the study population) treated with S+C had longer DFS compared to CLASSIC trial patients with low TIL density GC treated with S (HR 1.760, 95%CI 1.213-2.553, *P* = 0.003; figure 3A).

CLASSIC trial S+C patients with high TIL density GC (*N* = 276, 50.27%) had similar DFS to S patients with high TIL density GC (HR 1.248, 95%CI 0.830-1.876, *P* = 0.287; figure 3B). TIL density treatment interaction *P*-value was 0.099.

We explored the relationship between TIL density and DFS per treatment arm. CLASSIC trial S patients with high TIL density GC had significantly longer DFS compared to CLASSIC trial S patients with low TIL density GC (HR 1.591, 95%CI 1.104-2.292, *P* = 0.013; figure 3C). There was no significant relationship between TIL density and DFS within CLASSIC trial S+C patients (HR 1.128, 95%CI 0.746-1.706, *P* = 0.567; figure 3D). TIL density remained significant in multivariable analysis when including pTNM stage and treatment in the model (HR 1.345, 95%CI 1.023-1.768, *P* = 0.034; table 3).

DISCUSSION

Asian patients with stage II-III gastric cancer (GC) are routinely treated with adjuvant chemotherapy.[4,3] However, prognosis prediction and risk stratification for an individual GC patient remains challenging and there is currently no biomarker in routine clinical use that can predict benefit from adjuvant chemotherapy. We aimed to investigate the potential clinical utility of measuring tumour infiltrating lymphocytes per area mm^2 (TIL density) in areas with high tumour density using routine haematoxylin-eosin (HE) stained GC tissue sections from patients who had been treated at the Yonsei Cancer Center, Seoul, Republic of Korea (YCC, non-randomised cohort, treated by surgery alone or surgery followed by adjuvant chemotherapy) and from patients randomised to either surgery alone or surgery followed by adjuvant chemotherapy within the CLASSIC trial.[11,4] This is the first study to suggest that in a trial setting adjuvant

chemotherapy can significantly improve the relatively poor survival of patients with low TIL density GC but cannot improve the survival of GC patients with high TIL density.

As expected from the current literature, high TIL density was related to better disease-free survival (DFS) in the YCC cohort and the CLASSIC trial cohort. However, only in the CLASSIC trial cohort, patients with low TIL density GC treated with adjuvant chemotherapy had a significantly longer DFS compared to patients with low TIL density GC treated by surgery alone. The discrepant results for GC patients with low TIL density between the YCC cohort and the CLASSIC trial cohort could be related to the nature of the YCC cohort which included fewer patients, was not randomised for treatment, originated from a single hospital comparing historical cases, had more imbalances with respect of patients per treatment (37% S patients in YCC versus 49% in CLASSIC) and stage subgroups (for example: 62% S patients in stage II in YCC versus 38% in CLASSIC) and less well defined adjuvant chemotherapy treatment regimens.

Our HE based TIL density findings in the CLASSIC trial patients support previous results from the same cohort investigating RNA expression levels.[11] CLASSIC trial patients with low RNA expression of granzyme B and WARS (surrogate marker of low levels of immune cells, previous study) or low TIL density (current study) benefitted most from adjuvant chemotherapy.

Whereas our data seem to suggest that GC patients with high TIL density derive no additional benefit from chemotherapy after D2 gastrectomy, studies in breast cancer seem to suggest the opposite (for an overview of breast cancer studies, see table 1 in [26]). However, in contrast to the current GC study where there was a surgery alone control group enabling the distinction between prognostic and predictive effect of TIL density, breast cancer studies did usually not include a surgery alone control patient group limiting interpretability of findings. There is a single breast cancer study (*N* = 190) which compared a surgery alone patient group (mastectomy) versus a patient group treated by lumpectomy followed by radiotherapy, suggesting that adjuvant radiotherapy improves the disease-free survival and overall survival in a subset of HER2 positive and triple negative breast cancers patients with elevated TILs.[31] As this is a relatively small single-

centre study limited to a special molecular subgroup of breast cancer with different treatment modalities, our findings are difficult to compare with this study. The prognostic value of high TILs measured in HE stained tissue sections was also shown in a large lung cancer study. However, the authors did not compare survival by treatment (surgery alone versus adjuvant chemotherapy) and TILs level, and concluded there was no predictive value of TILs level based on treatment interaction analyses alone.[17] Whilst in the lung cancer study, TIL density was manually estimated in resection specimens, we used automated image analysis to quantify TILs in areas with high density of tumour. These different methodologies might potentially explain the differences in the results between the studies.

Although there is currently no consensus in GC on cut offs to define high versus low level of TILs, nor on how and where within the tumour one should TILs be scored, most previous GC studies concluded that 'high TILs' are predictive for a good prognosis often independent of TNM stage,[32,33] which is supported by the findings in the CLASSIC trial patients and the YCC patient cohort. As expected, our study showed a significantly higher TIL density in EBV-associated GC patients.[34,35]

Our study has some limitations. This is a retrospective *post hoc* analysis from a local hospital non-randomised gastric cancer patient cohort (YCC) and a subset of randomised patients from the CLASSIC trial where resection material was available for research. However, we confirmed that this subset of CLASSIC trial GC patients is representative of the whole CLASSIC trial population with respect to clinical characteristics and estimated 5-year DFS rates. In both cohorts, the investigated tissue was sampled from areas with the highest tumour content on visual inspection irrespective of the tumour location within the wall, which could have introduced bias. As treatment of Asian GC patients has changed to surgery followed by adjuvant chemotherapy after the publication of the ACTS-GC and CLASSIC trials, immediate validation of our results in a similar GC patient cohort with a surgery alone control arm was not possible. Studies into the predictive value of TILs in pre-treatment biopsies from patients receiving neoadjuvant chemotherapy might be informative in this context and support clinical decisions in the adjuvant setting based on TIL

density. By investigating TILs in the CLASSIC trial and YCC patient cohorts, the patient selection was restricted to Asian patients with stage II-III resectable GC. We have previously shown that quantity and phenotype of TILs varied between GCs from Asian and non-Asian patients.[36] Validation of our results in non-Asian GC patients to establish the generalisability across different ethnicities is needed. Some authors investigated the impact of TILs in solid tumours, including subtyping immune cells by immunohistochemistry.[37] We considered that immunohistochemical staining will have different pre-analytical, analytical and post-analytical challenges compared to the assessment of TILs in routine HE stained tissue sections. The International Immuno-Oncology Biomarker Working Group recommends using HE stained tissue slides[26] with a good reproducibility.[38] Considering the current literature and in particular our aim to develop a predictive biomarker that is relatively simple, reproducible, has shown clinical utility in previous studies in other cancer types, is cost-effective and would be easy to implement into the routine practice, we opted for TIL quantification on HE stained tissue sections. In the CLASSIC trial cohort and the YCC cohort, surgery alone treated GC patients with HE based high TIL density had a better survival as expected from studies in other tumour types. This confirmed that the prognostic value of TILs can be determined reproducibly based on HE stained sections in GC as shown for other tumour entities previously.

In summary, this is the first study in patients with locally advanced resectable gastric cancer (GC) to measure the density of tumour infiltrating lymphocytes (TIL density) in Haematoxylin-Eosin (HE) stained tissue sections using either virtual cores or actual TMA cores from gastrectomy specimens from patients treated at Yonsei Cancer Center, Seoul, Republic of Korea and patients recruited into the Korean CLASSIC trial. Low TIL density proved to be an independent predictive biomarker for benefit from adjuvant chemotherapy in GC patients from the CLASSIC trial. The use of 3 mm diameter tissue cores in the current study suggests that TIL density can also be measured in very small tumours or endoscopic biopsies. Our study confirmed that patients with high levels of TIL density have an excellent prognosis after being treated with surgery alone. As a relatively large number of GC seem to have relatively low TIL density, further studies are warranted to

better understand the interaction between cancer cells, host immune system and chemotherapy to personalise GC treatment in the near future.

ADDITIONAL INFORMATION

Authors' contribution

HIG and J-HC conceived and designed the study. HIG, J-HC and RL supervised the study. Y-WK, M-CK, HK and J-HC collected the specimens, constructed the TMAs and provided the database. M-CK, LCH and HIG performed the pathological review. JL, VV, YJ, GEF, LHC and DM set up the image analysis pipeline. HIG and GEF performed the quality control. DHWL, ND, SJ, LHC and VM carried out the statistical analysis. DHWL, NS, HIG, RL, LCH and AFI wrote and revised the manuscript. Y-WK and J-HC provided significant input for the manuscript. LHC, VV, YJ did the computation analysis. All authors had full access to the study data, discussed and reviewed the manuscript, and approved the manuscript for publication.

Ethics declarations

The study was study was conducted in compliance with the Declaration of Helsinki and was approved by the Institutional Review Boards of all participating institutions. The local institutional review boards, which waived the need for patient informed consent for this retrospective study.

Conflict of interest

Derek R. Magee is director of HeteroGenius Limited. Rupert Langer received consulting fees from Astellas, Janssen, Roche, MSD not related to the current study. Heike I. Grabsch received consulting fees from AstraZeneca and BMS not related to the current study.

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Data availability

The data sets analysed during the current study are available from the corresponding author on reasonable request.

REFERENCES

1 Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A. et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin* **71**, 209-249 (2021).

2 Smyth, E. C., Verheij, M., Allum, W., Cunningham, D., Cervantes, A., Arnold, D. et al. Gastric cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann. Oncol.* **27**, v38-v49 (2016).

3 Sasako, M., Sakuramoto, S., Katai, H., Kinoshita, T., Furukawa, H., Yamaguchi, T. et al. Five-year outcomes of a randomized phase III trial comparing adjuvant chemotherapy with S-1 versus surgery alone in stage II or III gastric cancer. *J. Clin. Oncol.* **29**, 4387-4393 (2011).

4 Noh, S. H., Park, S. R., Yang, H. K., Chung, H. C., Chung, I. J., Kim, S. W. et al. Adjuvant capecitabine plus oxaliplatin for gastric cancer after D2 gastrectomy (CLASSIC): 5-year follow-up of an open-label, randomised phase 3 trial. *Lancet Oncol* **15**, 1389-1396 (2014).

5 Kang, Y. K., Yook, J. H., Park, Y. K., Lee, J. S., Kim, Y. W., Kim, J. Y. et al. PRODIGY: A Phase III Study of Neoadjuvant Docetaxel, Oxaliplatin, and S-1 Plus Surgery and Adjuvant S-1 Versus Surgery and Adjuvant S-1 for Resectable Advanced Gastric Cancer. *J Clin Oncol* **39**, 2903-2913 (2021).

6 Tsuburaya, A., Yoshida, K., Kobayashi, M., Yoshino, S., Takahashi, M., Takiguchi, N. et al. Sequential paclitaxel followed by tegafur and uracil (UFT) or S-1 versus UFT or S-1 monotherapy as adjuvant chemotherapy for T4a/b gastric cancer (SAMIT): a phase 3 factorial randomised controlled trial. *Lancet Oncol* **15**, 886-893 (2014).

7 Yu, J., Gao, Y., Chen, L., Wu, D., Shen, Q., Zhao, Z. et al. Effect of S-1 Plus Oxaliplatin Compared With Fluorouracil, Leucovorin Plus Oxaliplatin as Perioperative Chemotherapy for Locally Advanced, Resectable Gastric Cancer: A Randomized Clinical Trial. *JAMA Netw Open* **5**, e220426 (2022).

8 Zhang, X., Liang, H., Li, Z., Xue, Y., Wang, Y., Zhou, Z. et al. Perioperative or postoperative adjuvant oxaliplatin with S-1 versus adjuvant oxaliplatin with capecitabine in patients with locally advanced gastric or gastro-oesophageal junction adenocarcinoma undergoing D2 gastrectomy (RESOLVE): an open-label, superiority and non-inferiority, phase 3 randomised controlled trial. *Lancet Oncol* **22**, 1081-1092 (2021).

9 Cancer Genome Atlas Research Network. Comprehensive molecular characterization of gastric adenocarcinoma. *Nature* **513**, 202-209 (2014).

10 Ho, S. W. T. & Tan, P. Dissection of gastric cancer heterogeneity for precision oncology. *Cancer Sci* **110**, 3405-3414 (2019).

11 Cheong, J. H., Yang, H. K., Kim, H., Kim, W. H., Kim, Y. W., Kook, M. C. et al. Predictive test for chemotherapy response in resectable gastric cancer: a multi-cohort, retrospective analysis. *Lancet Oncol* **19**, 629-638 (2018).

12 Japanese Gastric Cancer, A. Japanese gastric cancer treatment guidelines 2018 (5th edition). *Gastric Cancer* **24**, 1-21 (2021).

13 Muro, K., Chung, H. C., Shankaran, V., Geva, R., Catenacci, D., Gupta, S. et al. Pembrolizumab for patients with PD-L1-positive advanced gastric cancer (KEYNOTE-012): a multicentre, open-label, phase 1b trial. *Lancet Oncol* **17**, 717-726 (2016).

14 Salvatore, V., Teti, G., Focaroli, S., Mazzotti, M. C., Mazzotti, A. & Falconi, M. The tumor microenvironment promotes cancer progression and cell migration. *Oncotarget* **8**, 9608-9616 (2017).
15 Tian, C., Jing, H., Wang, C., Wang, W., Cui, Y., Chen, J. et al. Prognostic role of tumour-infiltrating lymphocytes assessed by H&E-stained section in gastric cancer: a systematic review and meta-analysis. *BMJ Open* **11**, e044163 (2021).
16 Denkert, C., von Minckwitz, G., Brase, J. C., Sinn, B. V., Gade, S., Kronenwett, R. et al. Tumor-Infiltrating Lymphocytes and Response to Neoadjuvant Chemotherapy With or Without Carboplatin in Human Epidermal Growth Factor Receptor 2-Positive and Triple-Negative Primary Breast Cancers. *J Clin Oncol* **33**, 983-991 (2015).
17 Brambilla, E., Le Teuff, G., Marguet, S., Lantuejoul, S., Dunant, A., Graziano, S. et al. Prognostic Effect of Tumor Lymphocytic Infiltration in Resectable Non-Small-Cell Lung Cancer. *J Clin Oncol* **34**, 1223-1230 (2016).
18 Huang, H. S., Su, H. Y., Li, P. H., Chiang, P. H., Huang, C. H., Chen, C. H. et al. Prognostic impact of tumor infiltrating lymphocytes on patients with metastatic urothelial carcinoma receiving platinum based chemotherapy. *Sci Rep* **8**, 7485 (2018).
19 Shibutani, M., Maeda, K., Nagahara, H., Fukuoka, T., Iseki, Y., Matsutani, S. et al. Tumor-infiltrating Lymphocytes Predict the Chemotherapeutic Outcomes in Patients with Stage IV Colorectal Cancer. *In Vivo* **32**, 151-158 (2018).
20 Ahn, B., Chae, Y. S., Kim, C. H., Lee, Y., Lee, J. H. & Kim, J. Y. Tumor microenvironmental factors have prognostic significances in advanced gastric cancer. *APMIS* **126**, 814-821 (2018).
21 Kim, J. Y., Kim, C. H., Lee, Y., Lee, J. H. & Chae, Y. S. Tumour infiltrating lymphocytes are predictors of lymph node metastasis in early gastric cancers. *Pathology* **49**, 589-595 (2017).
22 Zhang, D., He, W., Wu, C., Tan, Y., He, Y., Xu, B. et al. Scoring System for Tumor-Infiltrating Lymphocytes and Its Prognostic Value for Gastric Cancer. *Front Immunol* **10**, 71 (2019).
23 Choi, Y. Y., Kim, H., Shin, S. J., Kim, H. Y., Lee, J., Yang, H. K. et al. Microsatellite instability and programmed cell death-ligand 1 expression in stage II/III gastric cancer: Post hoc analysis of the CLASSIC randomized controlled study. *Ann Surg* 10.1097/SLA.0000000000002803 (2018).
24 Falk, T., Mai, D., Bensch, R., Cicek, O., Abdulkadir, A., Marrakchi, Y. et al. Author Correction: U-Net: deep learning for cell counting, detection, and morphometry. *Nat Methods* **16**, 351 (2019).
25 Board, W. C. o. T. E. *Digestive system tumours*. 5th ed. edn, Vol. vol. 1 (International Agency for Research on Cancer: Lyon (France), 2019).
26 Salgado, R., Denkert, C., Demaria, S., Sirtaine, N., Klauschen, F., Pruneri, G. et al. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. *Ann Oncol* **26**, 259-271 (2015).
27 Cancer, A. J. C. o. *AJCC Cancer Staging Manual*. https://doi.org/10.1007/978-1-4757-3656-4 (Springer New York, NY: New York, NY, 2002).
28 *International Union Against Cancer. TNM Classification of Malignant Tumours*. 6th edn, (Wiley-Liss: New York, 2002).

29 Sobin, L. H., Gospodarowicz, M. & Wittekind, C. *International Union against Cancer. TNM classification of malignant tumours* 7th edition edn, (2009).
30 Royston, P. & Sauerbrei, W. Two techniques for investigating interactions between treatment and continuous covariates in clinical trials. *Stata J* **9**, 230-251 (2009).
31 Mouabbi, J. A., Chand, M., Asghar, I. A., Sakhi, R., Ockner, D., Dul, C. L. et al. Lumpectomy followed by radiation improves survival in HER2 positive and triple-negative breast cancer with high tumor-infiltrating lymphocytes compared to mastectomy alone. *Cancer Med* **10**, 4790-4795 (2021).
32 Lee, J. S., Won, H. S., Sun, S., Hong, J. H. & Ko, Y. H. Prognostic role of tumor-infiltrating lymphocytes in gastric cancer: A systematic review and meta-analysis. *Medicine (Baltimore).* **97**, e11769 (2018).
33 Yu, P. C., Long, D., Liao, C. C. & Zhang, S. Association between density of tumor-infiltrating lymphocytes and prognoses of patients with gastric cancer. *Medicine (Baltimore).* **97** (2018).
34 Cheng, N., Li, P., Cheng, H., Zhao, X., Dong, M., Zhang, Y. et al. Prognostic Value of Tumor-Infiltrating Lymphocytes and Tertiary Lymphoid Structures in Epstein-Barr Virus-Associated and -Negative Gastric Carcinoma. *Front Immunol* **12**, 692859 (2021).
35 Kang, B. W., Seo, A. N., Yoon, S., Bae, H. I., Jeon, S. W., Kwon, O. K. et al. Prognostic value of tumor-infiltrating lymphocytes in Epstein-Barr virus-associated gastric cancer. *Ann Oncol* **27**, 494-501 (2016).
36 Lin, S. J., Gagnon-Bartsch, J. A., Tan, I. B., Earle, S., Ruff, L., Pettinger, K. et al. Signatures of tumour immunity distinguish Asian and non-Asian gastric adenocarcinomas. *Gut* **64**, 1721-1731 (2015).
37 Hendry, S., Salgado, R., Gevaert, T., Russell, P. A., John, T., Thapa, B. et al. Assessing Tumor-Infiltrating Lymphocytes in Solid Tumors: A Practical Review for Pathologists and Proposal for a Standardized Method from the International Immuno-Oncology Biomarkers Working Group: Part 2: TILs in Melanoma, Gastrointestinal Tract Carcinomas, Non-Small Cell Lung Carcinoma and Mesothelioma, Endometrial and Ovarian Carcinomas, Squamous Cell Carcinoma of the Head and Neck, Genitourinary Carcinomas, and Primary Brain Tumors. *Adv Anat Pathol* **24**, 311-335 (2017).
38 Kojima, Y. A., Wang, X., Sun, H., Compton, F., Covinsky, M. & Zhang, S. Reproducible evaluation of tumor-infiltrating lymphocytes (TILs) using the recommendations of International TILs Working Group 2014. *Ann Diagn Pathol* **35**, 77-79 (2018).

LEGENDS OF FIGURES AND TABLES

Figure 1. Example of image analysis-based tumour infiltrating lymphocyte detection in haematoxylin-eosin (HE) stained gastric cancer tissue

A. Original HE stained tissue.
B. HE stained tissue with segmentation mask (green circles) around the lymphocytes.

Figure 2. Disease-free survival (DFS) and tumour infiltrating lymphocyte (TIL) density in the Yonsei Cancer Center cohort

A. Patients with low TIL density (≤ 1290 TILs/mm^2) gastric cancer have similar survival when treated by surgery or surgery followed by adjuvant chemotherapy (HR 1.038, 95%CI: 0.662-1.628, *P* = 0.870).

B. Patients with high TIL density (> 1290 TILs/mm^2) gastric cancer have similar survival when treated by surgery or surgery followed by adjuvant chemotherapy (HR 0.775, 95%CI: 0.398-1.510, *P* = 0.454).

C. Patients treated with surgery alone with high TIL density gastric cancer have significantly longer DFS compared to patients treated with surgery alone with low TIL density (HR 2.496, 95%CI: 1.288-4.837, *P* = 0.007).

D. Patients treated with surgery plus adjuvant chemotherapy with high TIL density have significantly longer DFS compared to patients treated with surgery plus adjuvant chemotherapy with low TIL density (HR 1.792, 95%CI: 1.134-2.833, *P* = 0.012).

Figure 3. Disease-free survival (DFS) and tumour infiltrating lymphocyte (TIL) density in the CLASSIC trial cohort

A. Patients with low TIL density (≤ 1360 TILs/mm^2) gastric cancer have a significant longer DFS when treated by surgery followed by adjuvant chemotherapy (HR 1.760, 95%CI: 1.213-2.553, *P* = 0.003).

B. Patients with high TIL density (> 1360 TILs/mm^2) gastric cancer have similar survival when treated by surgery alone or surgery followed by adjuvant chemotherapy (HR 1.248, 95%CI: 0.830-1.876, *P* = 0.287).

C. Patients treated with surgery alone with high TIL density gastric cancer have significantly longer DFS compared to patients treated with surgery alone with low TIL density gastric cancer (HR 1.591, 95%CI: 1.104-2.292, *P* = 0.013).

D. Patients treated with surgery followed by adjuvant chemotherapy have similar survival irrespective of TIL density high or low gastric cancer (HR 1.128, 95%CI: 0.746-1.706, *P* = 0.567).

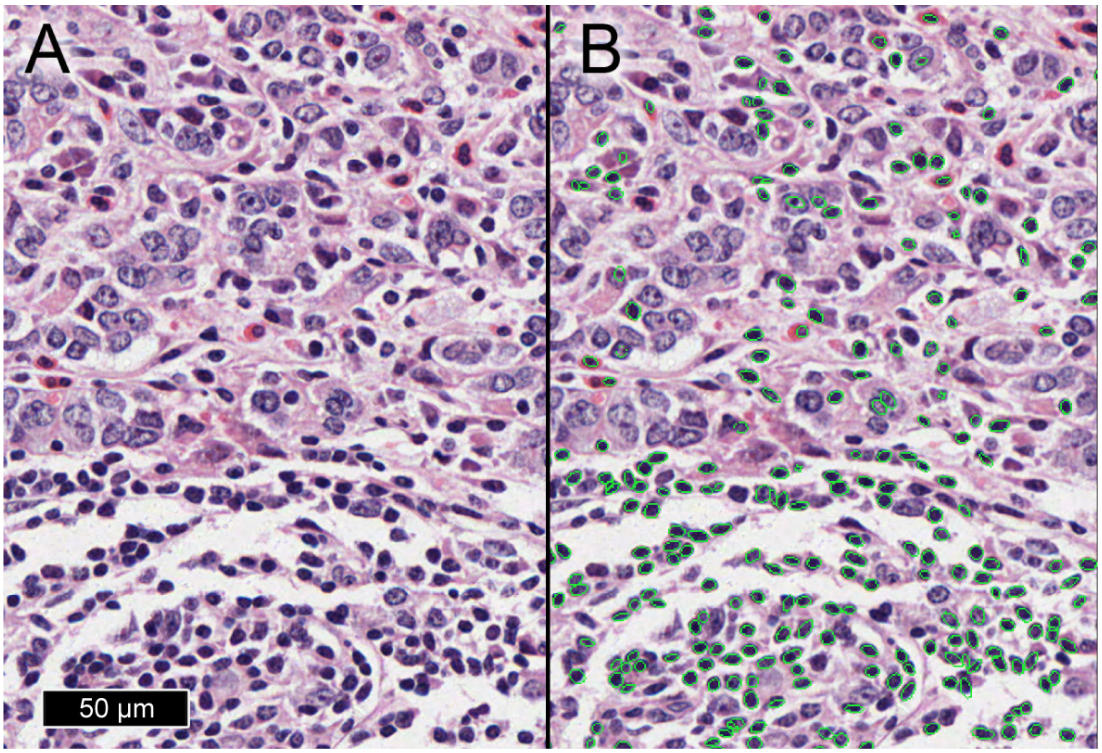
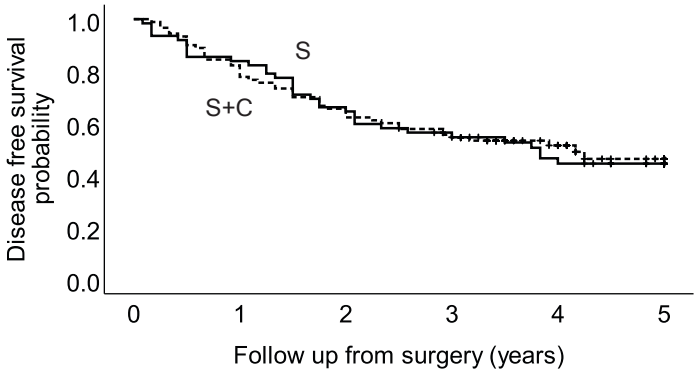


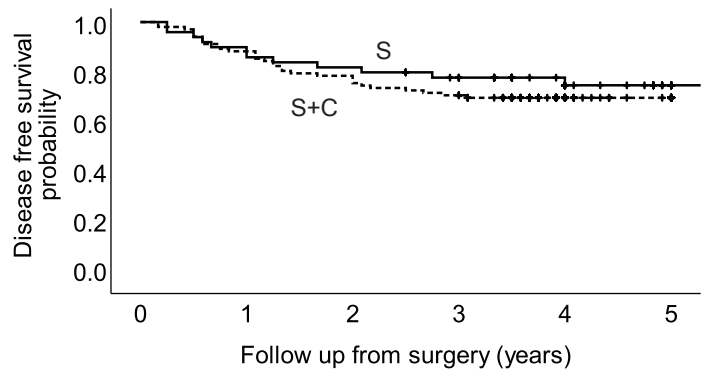
Figure 1. Example of image analysis-based tumour infiltrating lymphocyte (TIL) detection in haematoxylin-eosin (HE) stained gastric cancer tissue

A **Low TIL density**



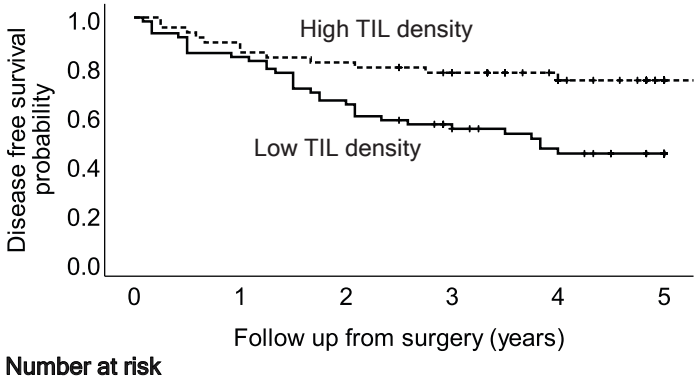
Number at risk						
S	62	52	40	29	22	13
S+C	90	70	56	49	24	10

B **High TIL density**



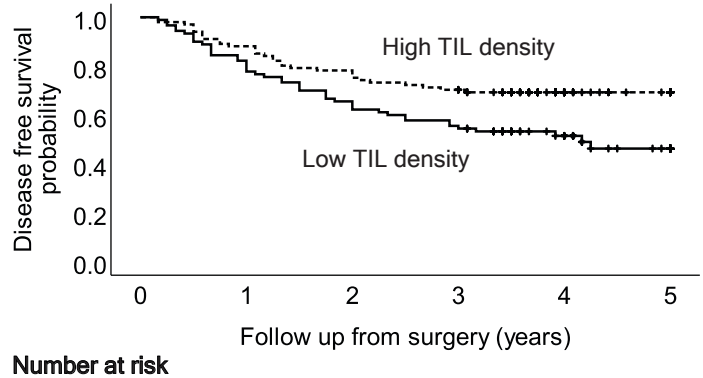
Number at risk						
S	49	42	40	33	19	9
S+C	101	89	76	69	23	11

C **Surgery alone**



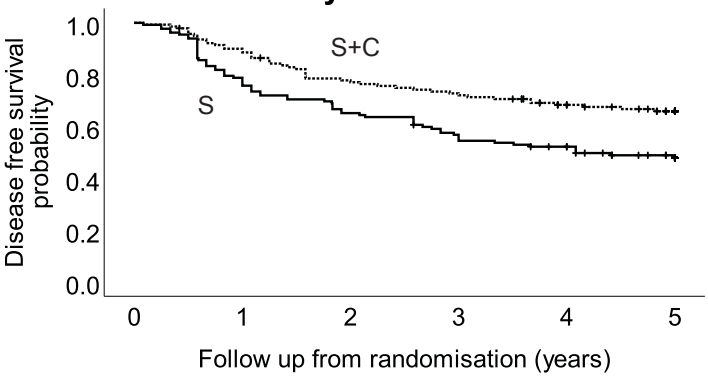
Number at risk						
Low TIL density	62	52	40	29	22	13
High TIL density	49	42	40	33	19	9

D **Surgery + adjuvant chemotherapy**



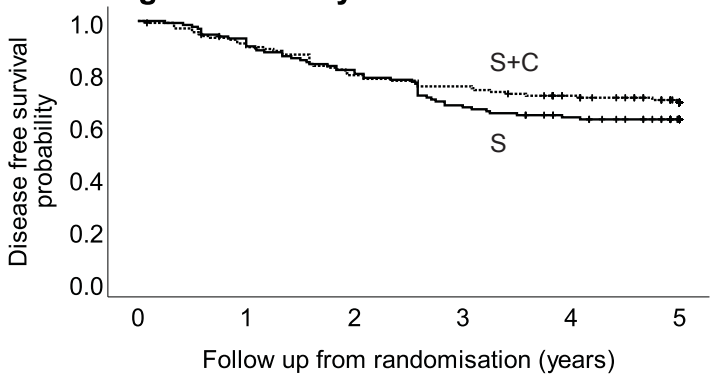
Number at risk						
Low TIL density	90	70	56	49	24	10
High TIL density	101	89	76	69	23	11

A **Low TIL density**



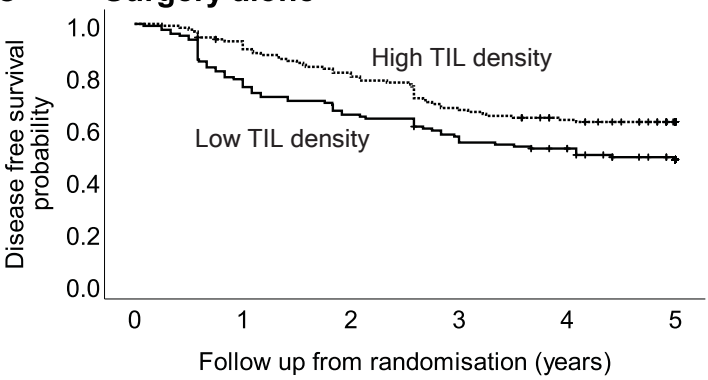
Number at risk						
S	132	104	86	74	66	51
S+C	141	126	108	101	87	72

B **High TIL density**



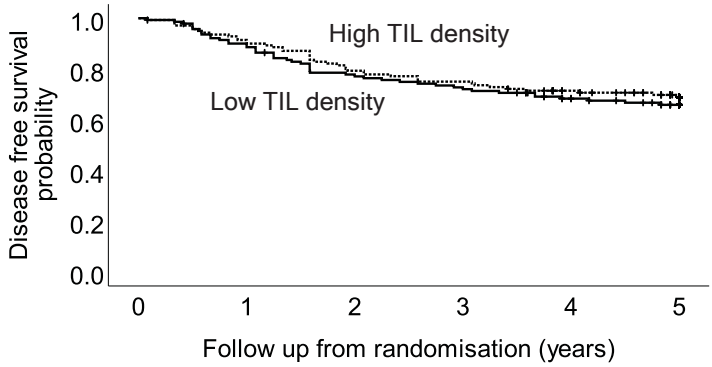
Number at risk						
S	135	124	108	90	79	61
S+C	141	128	111	105	92	72

C **Surgery alone**



Number at risk						
Low TIL density	132	104	86	74	66	51
High TIL density	135	124	108	90	79	61

D **Surgery + adjuvant chemotherapy**



Number at risk						
Low TIL density	141	126	108	101	87	72
High TIL density	141	128	111	105	92	72

Table 1. Relationship between tumour infiltrating lymphocyte (TIL) density and clinicopathological variables Yonsei Cancer Center cohort

		TIL density				
	Patients	≤ 1290 TILs/mm^2		> 1290 TILs/mm^2		
	n	n	%	n	%	P value
Age						
<65 years	183	89	48.6	94	51.4	0.465
≥65 years	119	63	52.9	56	47.1	
Sex						
Male	215	110	51.2	105	48.8	0.650
Female	87	42	48.3	45	51.7	
TNM stage[#]						
II	139	70	50.4	69	49.6	0.993
III	163	82	50.3	81	49.7	

[#] American Joint Committee on Cancer 6th edition

Table 2. Relationship between tumour infiltrating lymphocyte (TIL) density and clinicopathological variables CLASSIC trial

		TIL density				
	Patients	≤ 1360 TILs/mm^2		> 1360 TILs/mm^2		
	n	n	%	n	%	P value
Age						
<65 years	388	188	48.5	200	51.5	0.355
≥65 years	161	85	52.8	76	47.2	
Sex						
Male	395	201	50.9	194	49.1	0.385
Female	154	72	46.8	82	53.2	
pT (depth of invasion)						
T1/T2	101	48	47·5	53	52·5	0.625
T3/T4	448	225	50·2	223	49·8	
pN (lymph node status)						
N0	44	21	47.7	23	52.3	0.347
N1	160	75	46.9	85	53.1	
N2	168	85	50.6	83	49.4	
N3	177	92	52.0	85	48.0	
TNM stage[#]						
I / II	184	89	48.4	95	51.6	0.652
III	365	184	50.4	181	49.6	
WHO classification (2010)						
Tubular well differentiated	33	18	54.5	15	45.5	0.126
Tubular moderately differentiated	124	64	51.6	60	48.4	
Tubular poorly differentiated	202	83	41.1	119	58.9	
Poorly cohesive	131	67	51.1	64	48.9	
Papillary	15	10	66.7	5	33.3	
Mucinous	24	23	95.8	1	4.2	
Mixed	20	8	40.0	12	60.0	
MSI Status[@]						
MSS/MSI-L	484	235	48.6	249	51·4	0.622
MSI-H	34	18	52.9	16	47·1	
EBV Status[^]						
Negative	505	266	52.7	239	47.3	**<0.001**
Positive	43	6	14.0	37	86.0	

[#] Union for International Cancer Control 7th edition

[@] Microsatellite Instability (MSI); MSI-low (MSI-L); MSI-high (MSI-H); Microsatellite Stable (MSS)

[^] Epstein-Barr virus

Table 3. Multivariable analysis CLASSIC trial

	Hazard Ratio	95% confidence interval	P value
TNM stage#			
Stage I/II versus Stage III	0.490	0.353 – 0.678	**<0.001**
Treatment			
Surgery alone versus Surgery + adjuvant chemotherapy	1.579	1.199 – 2.078	**0.001**
TIL density*			
Low (≤ 1360 TILs/mm^2) versus High (> 1360 TILs/mm^2)	1.345	1.023 – 1.768	**0.034**

Union for International Cancer Control 7th edition

* Tumour infiltrating lymphocyte (TIL) density