Cytotoxic and immunosuppressive inflammatory cells predict regression and prognosis following neoadjuvant radio chemotherapy of esophageal adenocarcinoma

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CYTOTOXIC AND IMMUNOSUPPRESSIVE INFLAMMATORY CELLS PREDICT REGRESSION AND PROGNOSIS FOLLOWING NEOADJUVANT RADIOCHEMOTHERAPY OF OESOPHAGEAL ADENOCARCINOMA

DISSERTATION

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Dedication

To my family

The present work was performed in fulfilment of the requirements for obtaining the PhD degree within the Clinical Evidence-Based Medicine postgraduate program at USSM.

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List of Abbreviations

APC	Antigen presenting cells
CPS	Combined positive score
CTL	Cytotoxic T-lymphocytes
DFS	Disease free survival
FoxP3	Forkhead box P3
HR	Hazard ratio
MHC	Major histocompatibility complex
nCT	Neoadjuvant chemotherapy
NED	No evidence of disease
nRCT	Neoadjuvant radiochemotherapy
OAC	Oesophageal adenocarcinoma
OC	Oesophageal cancer
OS	Overall survival
OSCC	Oesophageal squamous cell cancer
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death ligand-1
RCT	Radiochemotherapy
RD	Risk difference
RR	Relative risk
TMA	Tissue microarray
TAM	Tumour associated macrophages
TIC	Tumour infiltrating cells
TIL	Tumour infiltrating lymphocytes
Treg	Regulatory T-cells
TRG	Tumour regression grade
USSM	University of Split School of Medicine

Table 1. Definitions and categories

Siewert AEG type (3)	Classification of adenocarcinomas of the oesophagogastric junction according to the centre of the tumour in relation to the oesophagogastric junction: I = -5 to -1 cm II = -1 to +2 cm III = +2 to +5 cm
Mandard TRG (4)	Grade of histological tumour regression after neoadjuvant RCT of oesophageal carcinoma: 1 = complete regression 2 = rare residual cancer 3 = increased number of residual cells, predominantly fibrosis 4 = residual cancer outgrowing fibrosis 5 = no regressive changes
PD-L1/PD-1 expression	(Number of positive cells)/(total number of cells), estimated 0 = <1% 1 = ≥1% to <10% 2 = ≥10% to <50% 3 = ≥50% Positive Score = ≥1%
OS, DFS and NED (5)	Survival endpoints in studies subsuming different events: *OS: Overall survival;* time to death, irrespective of cause. *DFS: Disease free survival;* time to any event, irrespective of cause. All events are included, except of loss to follow-up. *NED: No evidence of disease* (synonymous TTR = time to recurrence); time to any event related to the same cancer (recurrence and death). Deaths from other cancers, non-cancer-related deaths, treatment-related deaths, and loss to follow-up are censored observations. *Events:* locoregional recurrence, distant metastases, second primary same cancer, second primary other cancer, death from same cancer, death from other cancer, non-cancer-related death, treatment-related death, loss to follow-up
Ivor-Lewis-procedure (6)	Radical oesophagectomy by laparatomy and right-sided thoracotomy followed by an immediate intrathoracic gastrooesophageal anastomosis.

AEG adenocarcinoma of the esophagogastric junction, *TRG* tumour regression grade, *RCT* radiochemotherapy, *OS* overall survival, *DFS* disease free survival, *NED* no evidence of disease

Introduction

In the top ten of worldwide cancer incidences, oesophageal cancer (OC) ranks in the lower third. In 2018, it was the ninth most common cancer worldwide with an age standardised incidence of about 5.5 for both sexes. On the other hand, it was the sixth leading death of cancer due to a rapid progression and a low five-year survival rate of about 20%. More than 80% of cases and deaths occur in developing countries, especially in the so called "Asian oesophageal cancer belt" (beginning from the East of Turkey to the East Asian countries), and in Eastern and Southern Africa with an age standardised incidence of up to 22 in men, in some regions up to 100 (7–9). In developing countries, oesophageal squamous cell cancer (OSCC) is the vast predominant histological subtype, whereas in the Western industrialised countries 40–50% of OC are oesophageal adenocarcinomas (OAC). Here, the incidence of OAC increased enormously in the last five decades (probably reaching a plateau in the last 15 years), and in some countries it exceeds that of OSCC (10, 11). For 2012, the worldwide incidence of OAC was estimated around 52,000 cases (12), and the five-year mortality in Sweden from 2010 to 2013 was about 85% (13).

The increase of OAC in Western industrialised countries may be due to changes in lifestyle and a steady rise of reflux disease (14), since gastro-oesophageal reflux disease, Barrett's oesophagus and obesity are considered to be risk factors for OAC, but not for OSCC, whereas tobacco use increases the risk of both entities, and alcohol consumption is associated with an increased risk of OSCC, but not of OAC (10).

In limited disease (cT1–T2 N0 M0), resection is the treatment of choice, in very early stages (in OAC T1a without other risk criteria) by endoscopic therapy, otherwise by surgery. In locally advanced disease (cT3–T4 or cN1-3 M0), surgery alone is not recommended, since a complete tumour resection cannot be achieved in about 30% (T3) to 50% (T4) of cases, and even after complete tumour resection, long-term survival rarely exceeds 20% (15). At this stage of disease, both neoadjuvant chemotherapy (nCT) and neoadjuvant radiochemotherapy (nRCT) are able to reduce overall mortality significantly (16–19). To the present, the optimal multimodality treatment for oesophageal adenocarcinoma remains undetermined, but nRCT seems to be superior to nCT in local tumour control, and in patients treated without radiotherapy, survival and recurrence depend significantly on the extent of lymph node harvest (20, 21). Irrespectively of these considerations, it seems widely accepted that nRCT in locally advanced stages can decrease overall mortality by about 25% (18, 19).

In the Dutch CROSS trial (18), however, 19% of the patients had minor or no response to radiochemotherapy (RCT). Since tumour regression grade (TRG) is an important prognostic factor (22), this subgroup of patients will probably have no prognostic benefit, and hence experience mainly toxicity by neoadjuvant therapy. On the other hand, it is still not clear whether patients with response to RCT will benefit from subsequent surgery (23, 24). Therefore, it is of paramount clinical interest to identify biomarkers in pre-RCT biopsies which can predict the response to RCT and may influence the prognosis of the patients.

Apart from preliminary data on possibly predictive markers including p53, SOX2 (25), ERCC1, DPYD, ERBB2 (26) and stromal-derived interleukin 6 (27), specific interest has recently arisen from the investigation of the complex tumour microenvironment. It is well established that fibroblasts, endothelial cells, blood vessels, lymph vessels, and cells of the immune system are in intensive contact with tumour cells and influence development of cancer in a great extent. Especially, adaptive immune cell infiltration was shown to have a superior prognostic value, leading to an ongoing process of constituting a TNM-I (TNM-Immune) tumour classification based on an Immunoscore stratification (28–30).

Immune cell infiltration of tumour and peritumour tissue has to be considered as a complex network of interactions, where tumour infiltrating lymphocytes (TIL), such as CD8+ and FoxP3+ TIL (31–36), and tumour associated macrophages (TAM), such as CD68+ and CD163+ TAM (37, 38), have central relevance for modifying the tumour microenvironment.

CD8+ cytotoxic T-lymphocytes (CTL) are regarded as key players in anticancer surveillance. They are able to recognize cellular alterations presented by major histocompatibility complex (MHC) class I, and subsequently to mediate cytotoxicity. To maintain immunological self-tolerance, they are controlled on multiple levels, primarily in the thymus. Some self-reactive CTL, however, escape the negative selection in the thymus and have to be controlled by peripheral tolerance mechanisms, mainly by the activity of regulatory T-cells (Treg). Treg derive from natural and activated CD4+ T-cells and express the protein “forkhead box P3” (FoxP3), which acts as a master regulator of transcription and is critically important for the differentiation of Treg. On the other hand, presence of self-reactive CTL is necessary to combat cancer development, as tumour cells mainly express endogenous antigens and may not produce any molecules that can activate dendritic cells. So, the right balance of activation and control of CTL is extremely important in the surveillance of autoimmunity and tumour genesis (39, 40). In this context, it has to be taken into account that with chronic antigen exposure, effector

T-cells can lose their functional activity, becoming progressively exhausted (recoverable), accompanied by low expression of programmed cell death protein 1 (PD-1), or even hyper-exhausted (unrecoverable), along with high levels of PD-1 (41–43). Furthermore, it has to been kept in mind that FoxP3+ T-cells are composed of heterogeneous subpopulations. Besides suppressive Treg (naïve and effector Treg), there exist activated non-Treg FoxP3+ T-cells without suppression function. Most cancers are infiltrated predominantly by effector Treg. Not surprisingly, in those cases decreased ratios of CD8+ to FoxP3+ cells or high frequency of FoxP3+ cells were shown to correlate with poor prognosis, especially in patients with breast, gastric and ovarian cancer, and in patients with solid tumours in the cervix, kidney, breast, and melanomas, respectively. In contrast, in Hodgkin lymphoma or colorectal cancer, some studies indicated a better prognosis in patients with high tumour infiltration of FoxP3+ cells, whereas others showed the contrary. These contradictory results may be due to the fact that heterogeneity of FoxP3+ subpopulations was omitted to be taken into consideration (44).

Given the dilemma to interpret this extraordinary complexity, a key could be to look at the final result of the diverse differentiations and interactions, and to focus on the functional activity of TIL in the prevailing tumour environment. The idea is that spatial distribution of CD8+ CTL and FoxP3+ cells may reflect their functional interactions, since Treg suppress immune response by cytokines and cell-to-cell contact (45), both needing a certain proximity. Thus, in an environment where cell-to-cell distances of CD8+ CTL and FoxP3+ cells are significantly shorter than random distances, functional interactions may be assumed (*Figure 1*). Recently, the Erlangen Radiotherapy study group presented comprehensive data for gastric, rectal and anal cancer that the analysis of cell-to-cell distances may offer a tool to predict outcome, supporting the hypothesis that short cell-to-cell distances may identify functionally active, interacting infiltrating inflammatory cells in different tumour compartments (46–48).

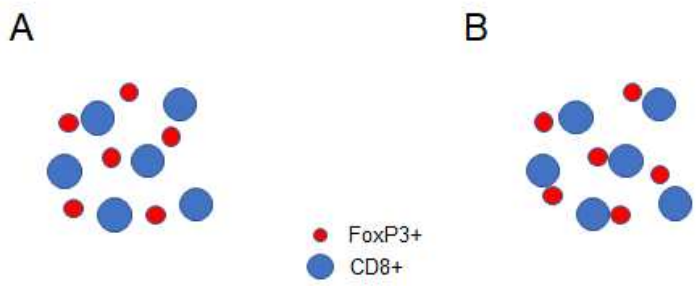


Figure 1. Distribution of CD8+ and FoxP3+ cells.

A Random distribution
B Short distances, possibly indicating functional activity of tumour infiltrating lymphocytes

Macrophages constitute a dominant fraction of tumour infiltrating cells (TIC). They are recruited by tumour derived signals and – depending on the tumour microenvironment – subsequently polarized toward an M1 or M2 phenotype, within most solid malignancies to an M2 phenotype. M2 macrophages, in turn, foster a microenvironment that supports tumour evolution by promoting tumour angiogenesis, providing growth factors, facilitating invasion and metastasis, and protecting developing tumours from adaptive immunosurveillance. However, macrophages polarized to an M1 phenotype may show potent antitumour properties. They are able to eliminate tumour cells, to inhibit tumour-induced angiogenesis, and to deplete tumour-associated stromal fibrosis. Moreover, depending on their phenotype, macrophages regulate T-cell activity. They are able to suppress T-cell activation and even induce T-cell exhaustion, and, on the other hand, sustain T-cell activation. CD68 is a general marker for macrophages, most subgroups of M2 macrophages are CD163 positive (49, 50).

PD-1 is an inhibitory receptor on T-cells which was initially considered as a regulator of cell death, but it is now recognized that its main function is to act as an immune checkpoint receptor to maintain immune tolerance. Activation of the T-cell antigen receptor and cytokine receptors induce PD-1 expression, and up-regulation of PD-1 is necessary for the termination of the immune response. PD-L1 and PD-L2 are the ligands for PD-1. They are expressed on antigen presenting cells (APC), like dendritic cells, and on a wide variety of nonhematopoietic cell types, like vascular endothelial cells. But PD-L1 (to a lesser extent, PDL-2) is also expressed in several cancers. By this mechanism, termed “adaptive immune resistance”, cancer cells protect themselves from attack by the immune system (51, 43). In the last years, treatment with PD-1 and PD-L1 antibodies led to a substantial progress in anticancer therapy of many tumour

entities like melanoma, non-small-cell lung carcinoma, Hodgkin lymphoma, head-neck cancer, squamous cell carcinoma of the oesophagus, pleural mesothelioma, breast, renal, urothelial, hepatocellular, colorectal (MSI-H), endometrial and cervical carcinoma. Several studies also investigated PD-1 and PD-L1 antibody treatment in gastric carcinoma and adenocarcinoma of the oesophagogastric junction (52, 53), and based on KEYNOTE-590 (54) and on CheckMate 649 (55), the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) approved Pembrolizumab and Nivolumab as first-line therapy for this indication under certain conditions in 2021.

Only few publications regarding anticancer surveillance deal with OAC. Data addressing the role of FoxP3+ and CD8+ TIL mainly focussed on surgical approach only (32, 56, 57), or were obtained from posttherapeutic specimens (33). Moreover, results considering their influence on prognosis are conflicting, and data of pretherapeutic immunologic tumour parameters predictive for response to RCT and prognosis of patients are lacking. Regarding PD-L1 and PD-1 expression, there is uncertainty about a reasonable threshold to classify into positive and negative groups with respect to prognosis. Thus, there is urgent need to contribute reliable data in order to understand the role of relevant biomarkers in this context.

Aims and Hypotheses

It was the primary aim of the present study to investigate immunological markers with possible effects on response to RCT and prognosis of patients with locally advanced, non-metastatic OAC. Therefore, we analysed pre-RCT tumour biopsies and – if available – post-RCT tissue samples, focussing on FoxP3+, CD8+, CD68+ and CD163+ tumour infiltrating cells and on the expression of PD-L1 and PD-1. We hypothesize that the prognostic value of immunologic biomarkers may be enhanced by including parameters of possibly functional activity, such as cell ratios and FoxP3+ to CD8+ cell-to-cell distance. A secondary aim was to study possible alterations of the immunological markers induced by RCT.

Patients and Methods

Patient cohort

Patient selection and characteristics are summarized in *Figure 2* and *Table 2*, definitions and categories in *Table 1*.

Between October 2004 and June 2018, 106 patients with locally advanced, non-metastatic OAC and AEG (3) were treated at the Coburg Cancer Centre, Germany. In Coburg, a multidisciplinary team was established in 2007, including defined lead clinicians in surgery, medical and radiation oncology, radiology, pathology, and gastroenterology, according to the guidelines of the Deutsche Krebsgesellschaft (German Cancer Society). Staging procedures included upper endoscopy with multiple biopsies of suspicious lesions, endoscopic ultrasound, computed tomography scans of thorax and abdomen with oral and intravenous contrast enhancement (58).

Eighty-eight of the patients underwent neoadjuvant RCT. The total dose of radiation was 50.4 Gy, 73 patients received taxol and infusional 5-fluorouracil (FU), 15 patients platin and FU. Details of diagnostic and therapeutic procedures are described elsewhere (58).

Pretherapeutic biopsies were available from 76 patients (tumoural compartment: 71 specimens, peritumoural: 57), of whom 58 patients underwent radical oesophagectomy by laparatomy and right-sided thoracotomy followed by an immediate intrathoracic gastrooesophageal anastomosis (Ivor-Lewis-procedure (6)). Eighteen patients were not eligible for surgery or refused the procedure.

Of the 76 included patients, 16 patients were female (21%) and 60 male (79%); mean age at the time of diagnosis was 66.4 years (SD ±10.5, range 44.3-86.5 years). Median follow-up time for all patients was 18 months (IQR 9-43 months), and 54 months (IQR 25-97 months) for surviving patients.

TRG was categorized according to Mandard (1: complete regression; 2: rare residual cancer; 3: increased number of residual cells, predominantly fibrosis; 4: residual cancer outgrowing fibrosis; 5: no regressive changes) (4).

Informed consent was obtained from all living patients, and the study was approved by the Ethics Committee of the University-Hospitals of Erlangen *(No. 133_17B)*.

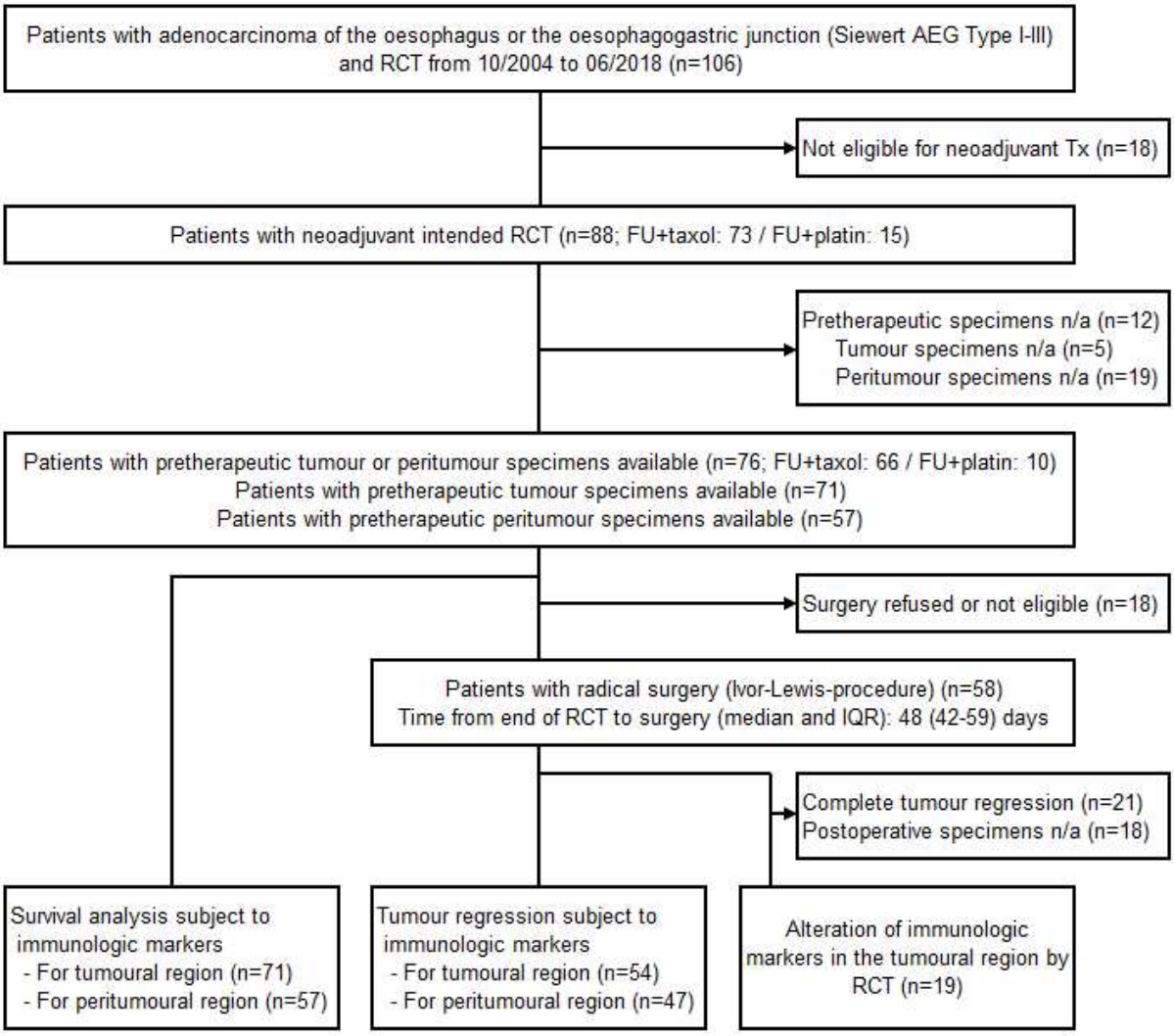


Figure 2. CONSORT diagram of patient selection

AEG adenocarcinoma of the esophagogastric junction, *RCT* radiochemotherapy, *Tx* therapy, *FU* infusional 5-fluorouracil, *n/a* not available

Table 2. Patients' characteristics

	All patients	Patients with surgery	Patients w/o surgery	p-value[1]
Number	76 (100%)	58 (100%)	18 (100%)	
Gender				
Female	16 (21%)	11 (19%)	5 (28%)	
Male	60 (79%)	47 (81%)	13 (72%)	n.s.[2]
Age				
Mean (± SD) - yr	66.4 (±10.5)	64.2 (±9.5)	73.5 (±10.9)	p=0.001[3]
Range - yr	44.3-86.5	44.3-85.4	48.4-86.5	
Staging pre-Tx				
UICC				
I	0 (0%)	0 (0%)	0 (0%)	
II	4 (5%)	3 (5%)	1 (6%)	
III	70 (92%)	53 (91%)	17 (94%)	
IV	2 (3%)	2 (3%)	0 (0%)	n.s.[2]
cTNM				
cT1	1 (1%)	0 (0%)	1 (6%)	
cT2	12 (16%)	10 (17%)	2 (11%)	
cT3	53 (70%)	39 (67%)	14 (78%)	
cT4	10 (13%)	9 (16%)	1 (6%)	n.s.[2]
cN0	12 (16%)	10 (17%)	2 (11%)	
cN+	64 (84%)	48 (83%)	16 (89%)	n.s.[2]
cM0	75 (99%)	57 (98%)	18 (100%)	
cM1*	1 (1%)	1 (2%)	0 (0%)	n.s.[2]
Grading pre-Tx (biopsy)				
pG1 & pG2	35 (46%)	28 (48%)	7 (39%)	
pG3	41 (54%)	30 (52%)	11 (61%)	n.s.[2]
Localization (3)				
OAC w/o AEG	7 (9%)	4 (7%)	3 (17%)	
AEG Siewert I	40 (53%)	32 (55%)	8 (44%)	
AEG Siewert II	26 (34%)	19 (33%)	7 (39%)	
AEG Siewert III	3 (4%)	3 (5%)	0 (0%)	n.s.[2]
Follow-up **				
All patients				
Median (IQR) - months	18 (9-43)	22 (8-62)	16 (13-21)	n.s.[4]
Patients alive, number =	24 (32%)	20 (34%)	4 (22%)	n.s.[2]
Median (IQR) - months	54 (25-97)	68 (34-111)	15 (14-21)	p=0.013[4]
Time from diagnosis to RCT				
Median (IQR) - days	38 (28-49)	37 (28-52)	39 (29-47)	n.s.[4]
Time from diagnosis to surgery				
Median (IQR) - days		126 (115-140)		
Resection quality				
R0		51 (88%)		
R1		6 (10%)		
R2		1 (2%)		
TRG (Mandard)				
1		20 (34%)		
2		22 (38%)		
3		5 (9%)		
4		9 (16%)		
5		2 (3%)		

Postoperative mortality		
All patients (age 44.3-85.4 yr)	12/58 (21%)	
Age < 63.1 yr	1/24 (4%)	p=0.010[2]
Age ≥ 63.1 yr	11/34 (32%)	

RCT radiochemotherapy, *w/o* without, *n.s.* not significant, *SD* standard deviation, *yr* years, *IQR* interquartile range, *Tx* therapy, *UICC* International Union against Cancer, *OAC* oesophageal adenocarcinoma, *AEG* adenocarcinoma of the esophagogastric junction, *TRG* tumour regression grade

* One patient with limited hepatic metastasis firstly underwent liver resection and secondly radical oesophagectomy.

** Last verification: 2019/04/01

Postoperative mortality death caused by a clearly postoperative complication, like bleeding, fistula or insufficiency of the anastomosis, within 20 weeks after surgery; age threshold obtained by ROC analysis

[1] Except "Postoperative mortality", p-value for the difference between patients with and without surgery

[2] Fisher's exact test, [3] Student's t-test, [4] Mann-Whitney-U test

Immunohistochemistry and evaluation of TIC

Tissue microarrays (TMA) with a core diameter of 2 mm were constructed from pretherapeutic biopsies and, if available, from resection specimens according to the original HE and immunohistologically stained slides. Tissue sections were de-paraffinised and one of neighbouring histological sections was HE stained, others were double stained using antibodies against CD8/FoxP3 (Dako/Abcam, *Figure 3A*), CD68/CD163 (Dako/Leica, *Figure 3B*), and PD-1/PD-L1 (Cell Marque/Abcam, *Figure 3C*), respectively. For detection, alkaline phosphatase detection kit (POLAP-100, Zytomed Systems GmbH, Berlin, Germany) with Fast red and Fast blue as chromogens (Sigma-Aldrich, Deisenhofen, Germany) were used according to the manufacturer's instructions. CD68/CD163 double positive cells were considered as M2 macrophages, further referred to as CD163+, remaining CD68+/CD163- cells as M1 macrophages, further referred to as CD68+. We are aware that this is an approximate graduation of macrophages (59). Stained slides were scanned at a magnification of 1:400 (Zeiss, Imager Z2, Göttingen, Germany; Metapher software MetaSystems, Altlussheim, Germany) and transferred to PC. TIC were counted using image analysis software (Biomas Software, Version 3.0; MSAB, Erlangen, Germany). Tumoural and peritumoural compartments were marked separately according to the neighbouring HE stained section (*Figure 4*), their sizes were calculated automatically, TIC were identified semiautomatically (*Figure 3D-F*). The compartments were analysed if their size exceeded a minimum of 0.3 mm^2. To determine

thresholds between high and low cell densities and ratios, ROC analysis was used for TRG, and median for survival analysis, respectively.

As described previously (46–48), the positions of the FoxP3+ and CD8+ cells were used to calculate the mean of the shortest cell-to-cell distances. Since distances are influenced by cell density, cell-to-cell distances of randomly distributed cells with an identical density were simulated in order to compare the random results with the measured distances. The simulation was performed with the aid of Visual Basic for Applications software of the spreadsheet program Excel. Random x and y coordinates of the desired quantity of "cells" were generated and the shortest distances between the cells were calculated and averaged. This procedure was repeated 100 times and the mean of shortest distances was taken as the expected value of randomly distributed cells. If the median of the measured shortest cell-to-cell distances was less than 90% of the mean simulated ones, the sample was classified as "short cell-to-cell distance", otherwise as "long cell-to-cell distance". Assuming that interactions between FoxP3+ and CD8+ cells are mediated by direct cell contact and by soluble factors, short cell-to-cell distance was presumed to be functionally interactive.

For PD-1 and PD-L1 categorisation we used a modified score referred to the CPS which is defined as the proportion of the number of positive PD-L1 cells related to the number of tumour cells. Because tumour cells are absent in the peritumoural area, we instead evaluated the percentages of positive cells related to the total number of cells for a better comparison. Category 0 to 3 were defined as an estimated percentage of <1%, ≥1% to <10%, ≥10% to <50%, and ≥50%, respectively. According to survival analysis, a percentage of <1% was considered as negative, of ≥1% as positive (*Figure 8*). At least in tumoural area, our modified score of PD-L1 should be in good approximation to the widely used CPS of PD-L1.

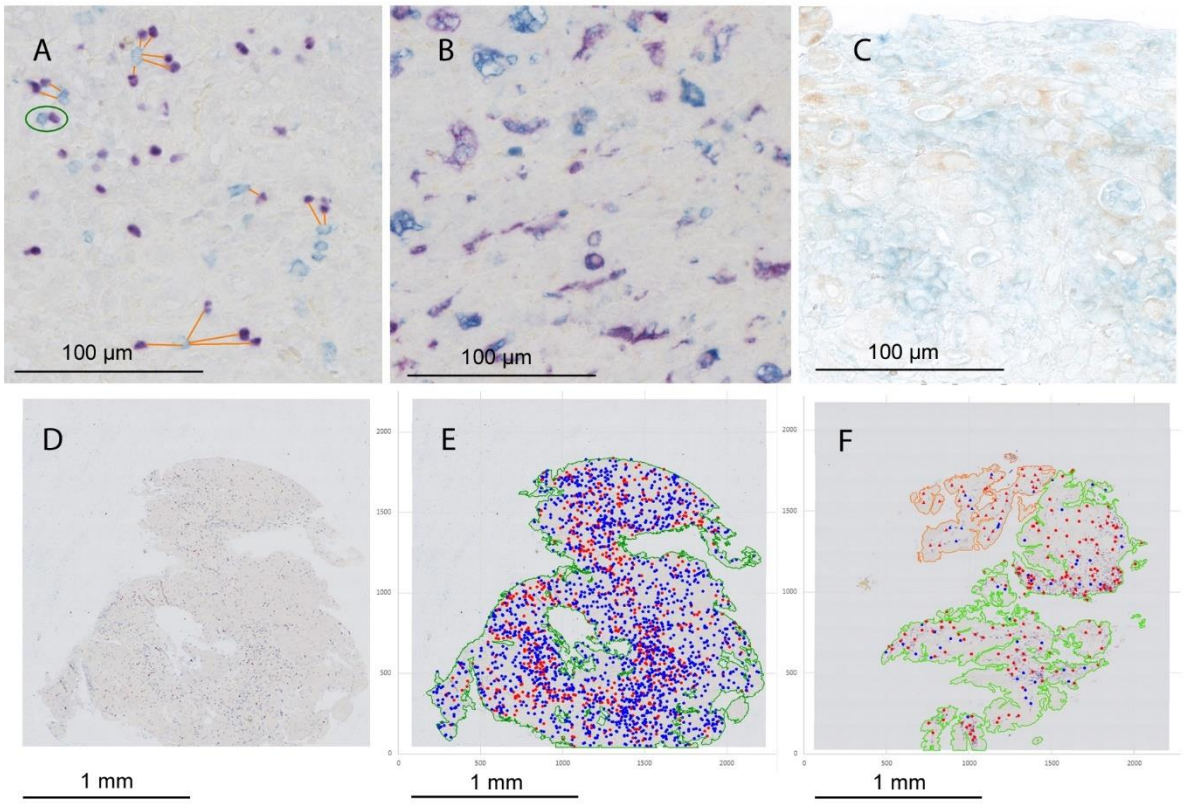


Figure 3. Evaluation of FoxP3+, CD8+, CD68+ and CD163+ tumour infiltrating cells, and PD-1 and PD-L1 expression in adenocarcinoma of the oesophagus and the oesophagogastric junction

A: Double staining of FoxP3+ (violet) and CD8+ (blue) tumour infiltrating lymphocytes (400x original magnification). Orange lines: shortest FoxP3+ to CD8+ cell-to-cell distance for some of the cells. Green ellipse: direct cell contact. **B:** Double staining of CD68+ (blue) and CD163+ (violet) tumour associated macrophages. **C:** Double staining of PD-L1 (blue) and PD-1 (brown) expression. **D:** Tissue microarray of a FoxP3+/CD8+ sample. **E:** Corresponding evaluation by image processing (red markers: positions of FoxP3+ cells, blue markers: positions of CD8+ cells). **F:** Evaluation of a CD68+/CD163+ sample; green lines: surroundings of tumoural compartment; orange lines: surroundings of peritumoural compartment; red markers: CD163+ cells; blue markers: CD68+ cells; circles in tumoural compartment and triangles in peritumoural compartment.

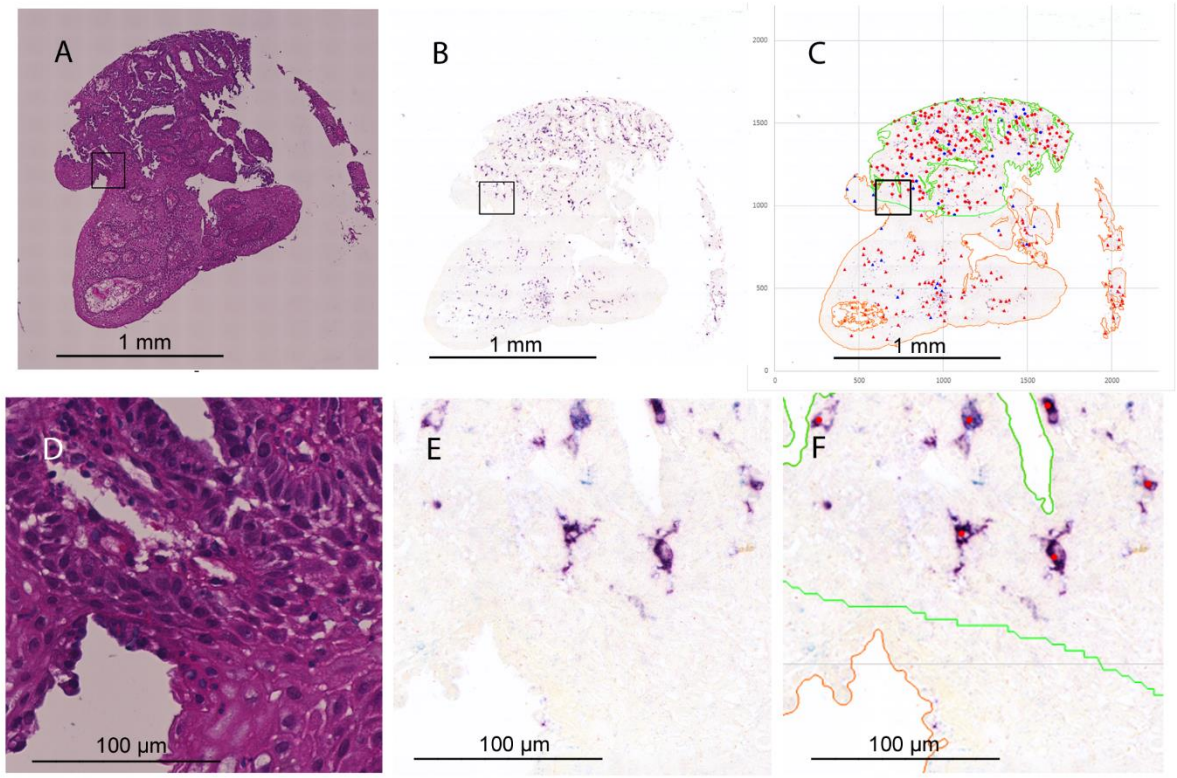


Figure 4. Identification of compartments and tumour infiltrating cells

A: HE stained section of a tissue microarray (TMA). **B:** Neighbouring section of (A), double stained for CD68+ (blue) and CD163+ (violet) tumour associated macrophages (TAM). **C:** Specifying tumour and non-tumour area of (B) according to (A) and semiautomatic identification of TAM. Green lines: surroundings of tumoural compartment; orange lines: surroundings of peritumoural compartment; red markers: CD163+ cells; blue markers: CD68+ cells; circles in tumoural compartment and triangles in peritumoural compartment.
D–F: Details of marked areas (black rectangles) in (A)–(C).

Statistical analysis

Statistical analysis was performed using MedCalc Statistical Software version 20.014 (MedCalc Software bvba, Ostend, Belgium; 2021) and PAST Paleontological Statistics version 3.25 (Oslo, Norway; 2019) (60). Subgroups of patients were compared by Student's t-test, Fisher's exact test, Wilcoxon test and Mann-Whitney-U test. Influence of immunologic markers on TRG was estimated by risk analysis, Pearson's chi-squared test and ROC analysis. Correlation of PD-L1 and PD-1 expression were calculated by Spearman's rank-order correlation. Overall survival (OS), disease free survival (DFS) and no evidence of disease (NED) were analysed by Kaplan-Meier method. NED is defined by time to any event related to the same cancer (recurrence and death) (5). Logrank test and Cox regression (possible

confounders: age, surgery, cN, TRG, resection quality) were used to compare survival between subgroups of patients. A p-value less than 0.05 was considered significant.

Results

Surgery analysis

After RCT, 76% of the patients underwent surgery, 24% did not (*Table 2*). Patients with surgery following RCT were distinctly younger than those without (mean 64.2±9.5 years and 73.5±10.9 years, respectively, p=0.001), and the median follow-up time of patients being alive at the end of the study was longer in the surgery group (68 (IQR 34-111) months and 15 (IQR 14-21) months, respectively, p=0.013). All other parameters tested (gender, staging, grading, localization, overall follow-up time, time from diagnosis to RCT) did not differ significantly.

Eighty-eight percent of the patients could be resected without residual tumour (R0). Postoperative mortality was 21% and clearly higher among patients ≥63.1 years compared to younger ones (32% vs. 4%, p=0.010). All deaths caused by any postoperative complication within 20 weeks were defined as postoperative mortality.

Tumour regression

Eighty-one percent of the patients experienced major response to RCT (Mandard regression score 1-3 vs. 4&5, *Table 2*). Among the clinical parameters, only clinically positive nodal disease (cN+) was associated with an unfavourable TRG (p=0.001). Age, depth of tumour infiltration and histological grading of pretherapeutic biopsies had no influence on TRG in risk analysis (*Figure 5*, *Table 3*).

As for the pretherapeutic immunologic parameters, low intratumoural FoxP3+/CD8+ ratio (p=0.020), short intratumoural FoxP3+ to CD8+ cell-to-cell distance (p=0.106), high CD163+/CD68+ ratio (p=0.070 intratumoural, p=0.045 peritumoural) and high intratumoural TAM density (RR p=0.108; RD p=0.023) were associated with a poor TRG of Mandard 4&5. Albeit intratumoural CD8+ densities were higher in complete responders (Mandard 1; median 163 cells/mm^2, 95% CI 60–203) than in non-complete responders (Mandard 2–5; median 110 cells/mm^2, 95% CI 64–138), the difference was not significant (p=0.223, Mann-Whitney-U test). A favourable TRG was found in patients with a positive score of PD-L1 expression in the peritumoural area (RR p=0.036; RD p=0.023). PD-L1 expression in the tumoural area and PD-1 expression in both areas had no significant influence on TRG.

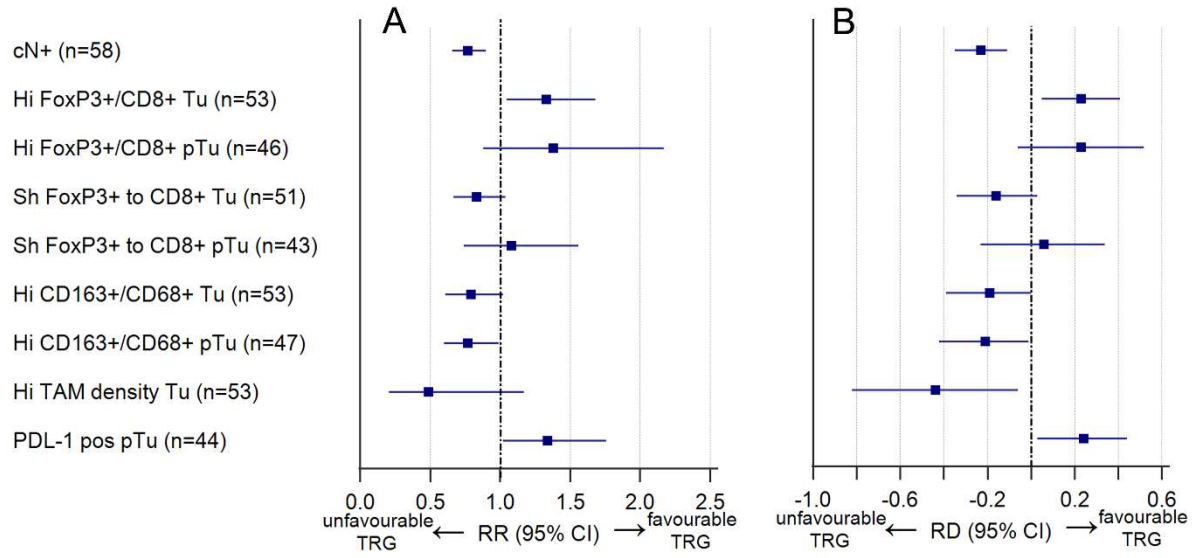


Figure 5. Pretherapeutic variables with possible impact on favourable tumour regression after RCT (risk analysis of prognostic factors).
Forest plots of RR (risk ratio) (**A**) and RD (risk difference) (**B**). Details in *Table 3*.

RCT radiochemotherapy, *TRG* tumour regression grade, *RR* risk ratio, *RD* risk difference, *CI* confidence interval, *Hi* high, *Tu* in tumoural area, *pTu* in peritumoural area, *Sh* short cell-to-cell distance, *TAM* tumour associated macrophages (CD68+ plus CD163+), *pos* positive expression (≥1%)

Favourable TRG: Mandard 1–3 vs. Mandard 4&5
cN+: clinically positive lymph nodes pre-RCT
High ratio (density): The ratio of the cell densities (the density, respectively) in this case is equal to or higher than the best fitting threshold (obtained by ROC-analysis) of all cases.
Short cell-to-cell distance: The median of measured shortest distances is less than 90% of the mean simulated random ones in this case.
Cell-to-cell distance analysis was omitted, if the count or the density of the markers was less than 2 or less than 2/mm², respectively.

Results of risk analysis, Pearson's chi-squared test and two tailed z-test

Survival analysis

Five-year survival with regard to OS, DFS and NED of the whole cohort were 30%, 24% and 42%, respectively (*Figure 6*). Survival analysis comparing patients with and without surgery revealed no significant difference, neither in univariate nor in multivariate analysis adjusted for age and cN status (OS: p=0.314, DFS: p=0.505, NED: p=0.208; *Table 4, Table 5, Table 6*).

Independent predictors for poor survival among the clinical parameters were "resection quality" adjusted for TRG (R1&2 vs. R0: HR 2.88 [95% CI 1.19-6.98], p=0.020) and TRG adjusted for "resection quality" (Mandard 4&5 vs. 1-3: HR 2.27 [1.06-4.85], p=0.034). Pre-RCT cN+ was linked to a higher mortality compared to cN0 adjusted for surgery (p=0.101). Pretherapeutic cT

staging and histological grading (pG) were not associated with significantly different survival rates.

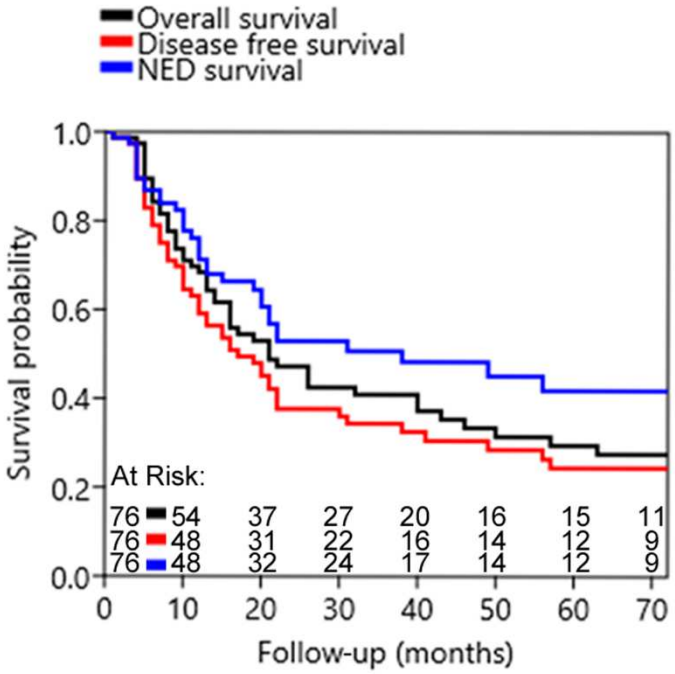


Figure 6. Kaplan-Meier survival curves of the whole cohort

NED No evidence of disease

Considering pretherapeutic immunologic cell types, overall survival was favourable with high amounts of intratumoural (p=0.125) and low amounts of peritumoural CD8+ lymphocytes (p=0.017; *Figure 7A&B, Figure 18A&B, Figure 19A&B*). Intratumoural FoxP3+ density was not associated with survival (p=0.838; *Figure 7C, Figure 18C, Figure 19C*). A low intratumoural FoxP3+/CD8+ ratio tended to be favourable (p=0.186; *Figure 7D, Figure 18D, Figure 19D*). Similar results were seen for long intratumoural FoxP3+ to CD8+ distances (p=0.144); the difference was significant excluding cases within lower and upper quintile of CD8+ density (p=0.036; *Figure 7E&F, Figure 18E&F, Figure 19E&F*), or excluding patients who died postoperatively (p=0.030). There was no significant association of intratumoural CD68+ macrophages with survival (p=0.286; *Figure 7G, Figure 18G, Figure 19G*). CD163+ density had no influence on survival. Low intratumoural ratio of TAM/CD8+ was linked to a favourable survival (p=0.062; *Figure 7H, Figure 18H, Figure 19H*). For detailed information see *Table 4, Table 5, Table 6*.

In multivariate analysis peritumoural CD8+ infiltration (p=0.012) and intratumoural FoxP3+ to CD8+ cell-to-cell distances in middle ranged CD8+ density (p=0.050) were significant prognostic factors.

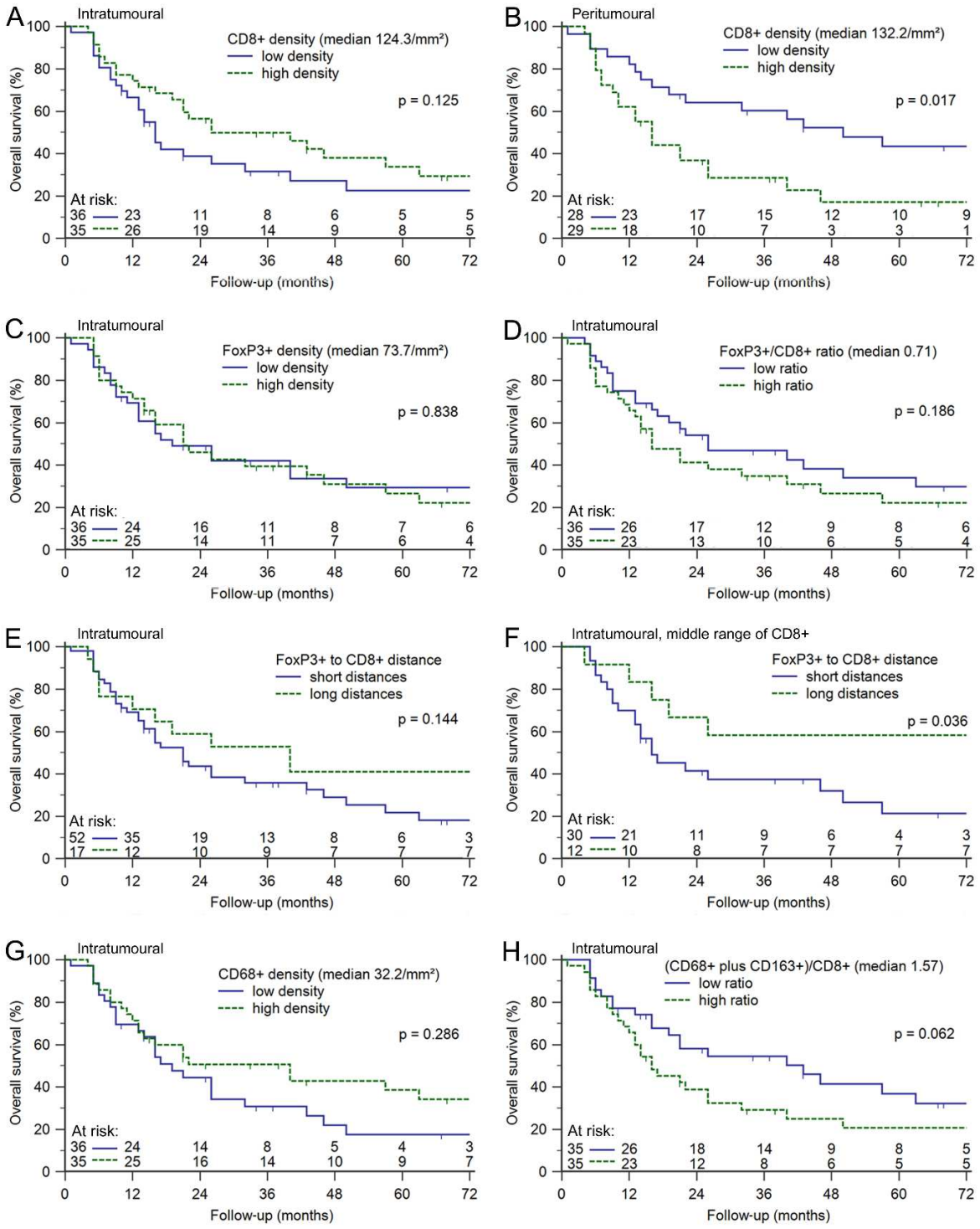


Figure 7. Influence of pretherapeutic immunologic parameters on overall survival

A: Intratumoural CD8+ density, HR 0.63 (95% CI 0.36–1.13). **B:** Peritumoural CD8+ density, HR 2.29 (1.16–4.52). **C:** Intratumoural FoxP3+ density, HR 1.06 (0.60–1.88). **D:** Intratumoural FoxP3+/CD8+ ratio, HR 1.47 (0.83–2.62). **E:** Intratumoural FoxP3+ to CD8+ cell distance, HR 0.62 (0.33–1.18). **F:** Intratumoural FoxP3+ to CD8+ cell distance, lower and upper quintile of underlying CD8+ density excluded (≤53.5/mm^2 and ≥303/mm^2), HR 0.43 (0.19–0.95). **G:** Intratumoural CD68+ density, HR 0.73 (0.41–1.30). **H:** Intratumoural (CD68+ plus CD163+)/CD8+ ratio, HR 1.74 (0.97–3.12).

Results of logrank test

Considering survival analysis of different classes of PD-1/PD-L1 expression, as shown in *Figure 8*, it seemed reasonable to set the threshold of positive expression at 1%.

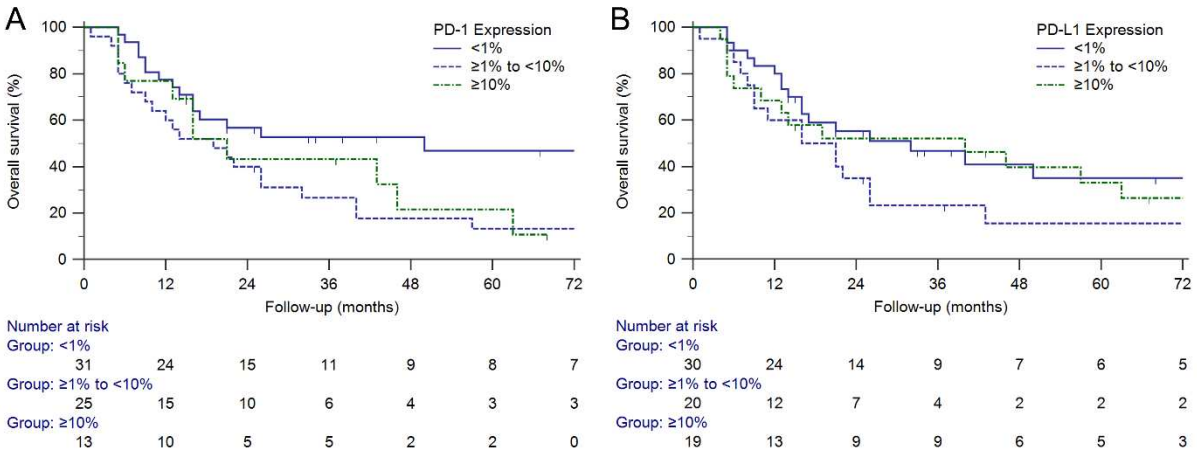


Figure 8. Dependence of overall survival on PD-1 and PD-L1 expression in tumoural area

A: Scores of PD-1 expression. **B:** Scores of PD-L1 expression.

Expression of PD-1 and PD-L1 was estimated as the number of positive cells divided by the number of all cells in the area

In tumoural area, negative PD-1 expression was associated with a significant better prognosis (p=0.028), whereas PD-L1 expression had no significant influence on outcome (p=0.212). Taking into account the density of CD8+ TIL, best prognosis was seen in the group with high CD8+ density and negative PD-1 expression, worst prognosis in the group with low CD8+ density and positive PD-1 expression (p=0.007). Similar effects were seen when combining CD8+ density and PD-L1 expression (p=0.028) (*Figure 9*, *Figure 20*, *Figure 23*).

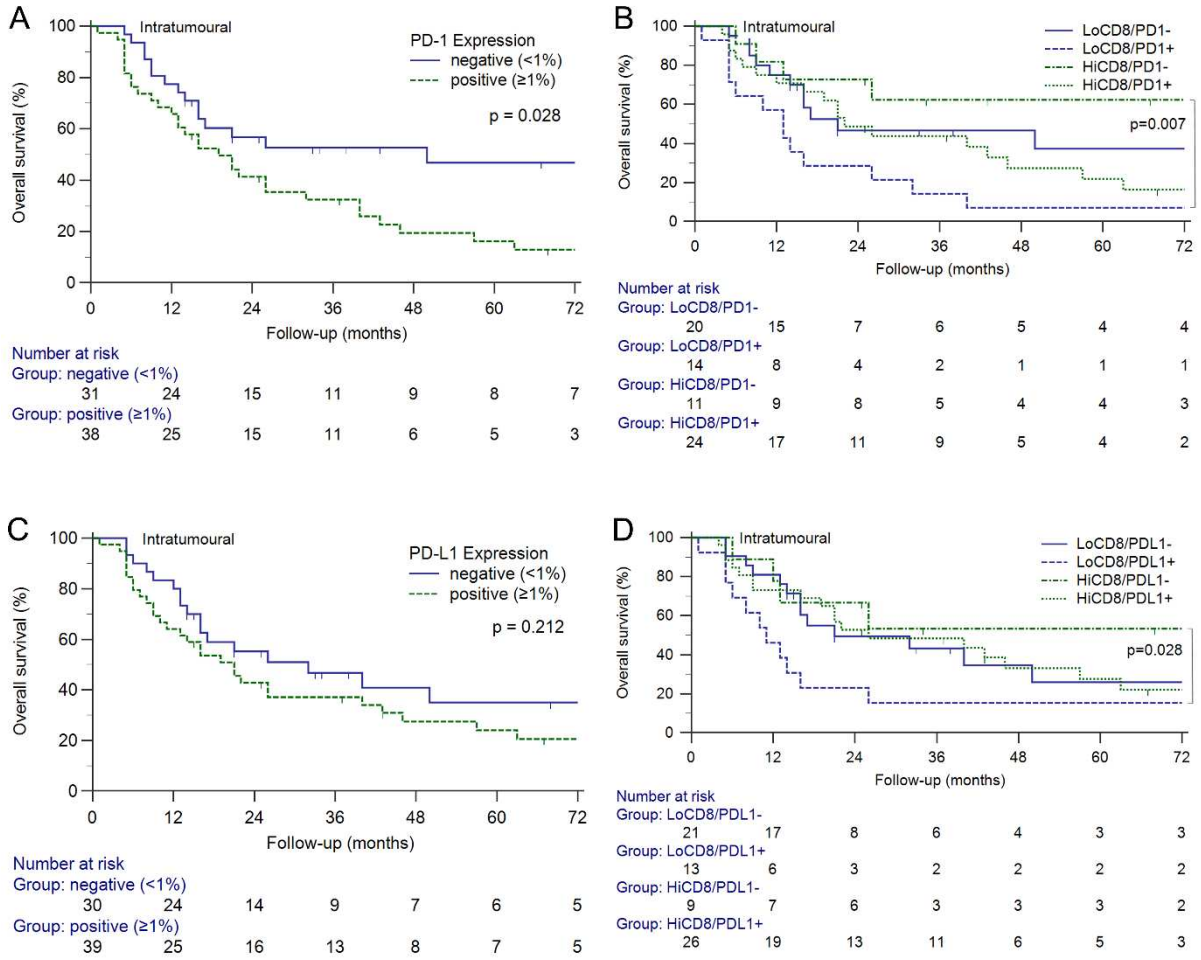


Figure 9. Influence of PD-1 and PD-L1 expression in tumoural area on overall survival

A: PD-1 expression, HR 0.52 (95% CI 0.29–0.93). **B:** PD-1 expression combined with CD8+ density, LoCD8/PD1+ compared to HiCD8/PD1-: HR 0.25 (0.09–0.69). **C:** PD-L1 expression, HR 0.69 (95% CI 0.39–1.23). **D:** PD-L1 expression combined with CD8+ density, LoCD8/PDL1+ compared to HiCD8/PDL1-: HR 0.32 (0.12–0.89).

Results of logrank test

HiCD8/LoCD8 high/low CD8+ density (median 124.3/mm^2), *PD1-/PD1+* negative/positive PD-1 expression, *PDL1-/PDL1+* negative/positive PD-L1 expression (threshold 1%)

In peritumoural area, again negative PD-1 expression was linked to a significant favourable survival (p=0.047), and PD-L1 expression had no distinct influence (p=0.343). Regarding CD8+ density, a negative PD-1 or PD-L1 expression in a low CD8+ density environment was associated with a significant better prognosis than a positive PD-1 or PD-L1 expression in a high CD8+ density environment (p=0.010 and p=0.031, respectively) (*Figure 10*, *Figure 21*, *Figure 24*).

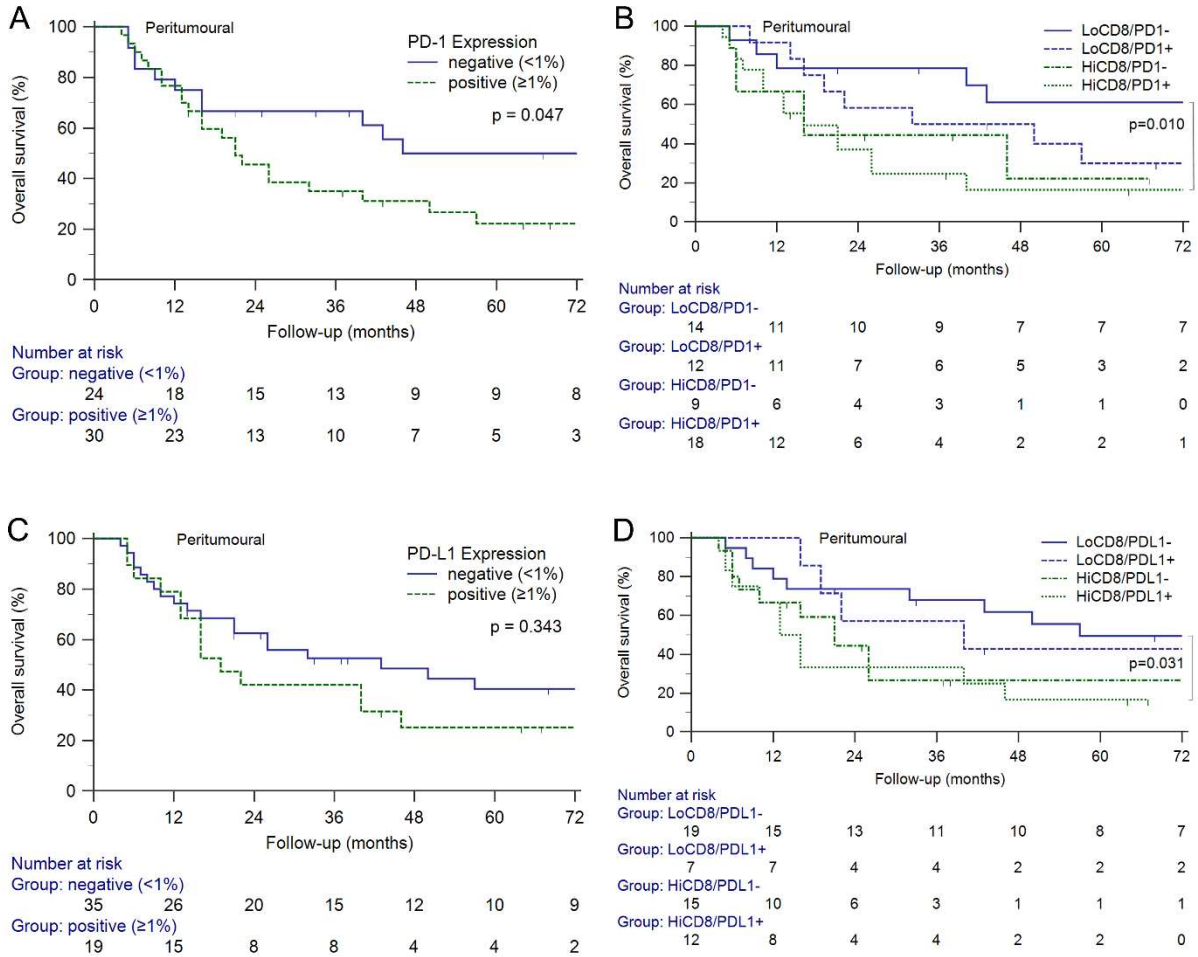


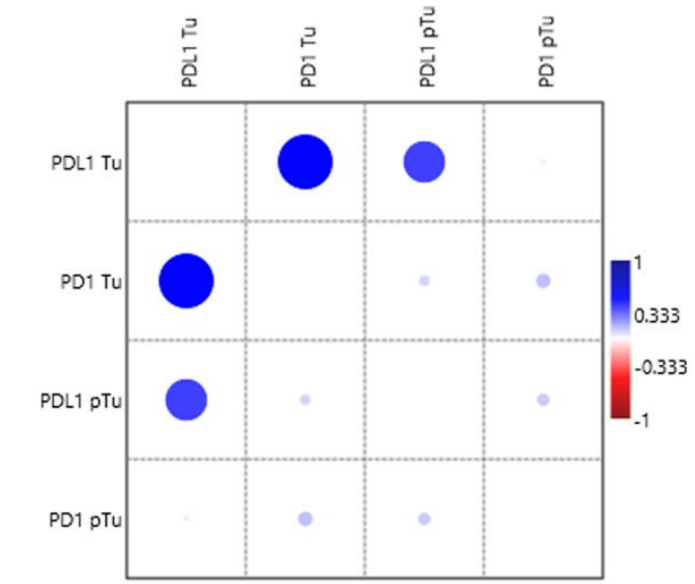
Figure 10. Influence of PD-1 and PD-L1 expression in peritumoural area on overall survival

A: PD-1 expression, HR 0.50 (95% CI 0.25–0.99). **B:** PD-1 expression combined with CD8+ density, HiCD8/PD1+ compared to LoCD8/PD1-: HR 0.29 (0.11–0.74). **C:** PD-L1 expression, HR 0.71 (95% CI 0.34–1.45). **D:** PD-L1 expression combined with CD8+ density, HiCD8/PDL1+ compared to LoCD8/PDL1-: HR 0.33 (0.12–0.90).

Results of logrank test

HiCD8/LoCD8 high/low CD8+ density (median 132.2/mm^2), *PD1-/PD1+* negative/positive PD-1 expression, *PDL1-/PDL1+* negative/positive PD-L1 expression (threshold 1%)

There was a significant correlation between PD-1 and PD-L1 expression in tumoural area (r=0.50, p=0.001) and between PD-L1 expression in tumoural and peritumoural area (r=0.37, p=0.008) (*Figure 11*).



r\p	PDL1 Tu	PD1 Tu	PDL1 pTu	PD1 pTu
PDL1 Tu		<0.001	0.008	0.809
PD1 Tu	0.496		0.568	0.408
PDL1 pTu	0.373	0.083		0.480
PD1 pTu	-0.035	0.120	0.098	

Figure 11. Correlation of PD-L1 and PD-1 expression in tumoural and peritumoural area

Grade of correlation is represented by different colours according to the colour bar on the right, grade of significance by the diameter of the circles.

PD-L1 and PD-1 expressions were scored as 0 = <1%, 1 = ≥1% to <10%, 2 = ≥10% to <50%, 3 = ≥50% number of positive cells compared to the number of all cells in the area

Tu tumoural area, *pTu* peritumoural area, *r* Spearman's Rho, *p* p-value

Results of Spearman's rank-order correlation

Regarding a combination of positive PD-1 and PD-L1 expression, overall survival was significantly worse than in those groups with any of the parameters being negative (*Figure 12*, *Figure 22*, *Figure 25*). Significance of combined testing was more pronounced than significance of testing each parameter alone. In multivariate analysis adjusted for both parameters, PD-1 expression contributed predominantly to prognosis (p=0.034), whereas the influence of PD-L1 expression was not significant.

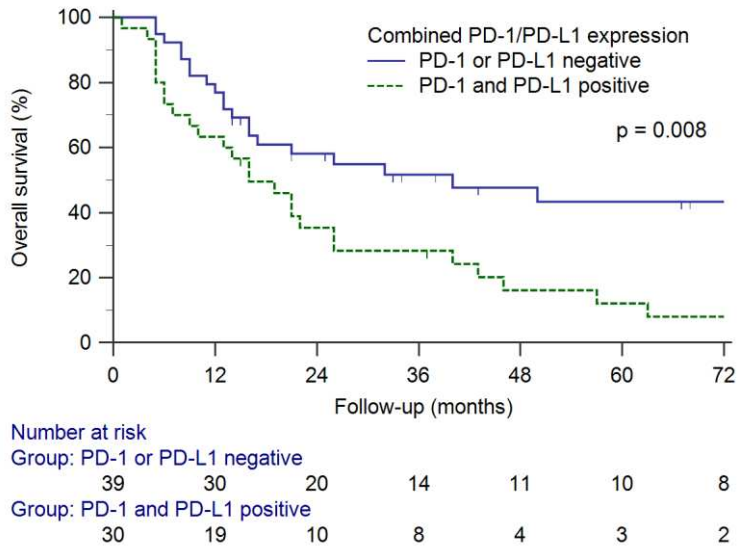


Figure 12. Influence of combined PD-1 and PD-L1 expression in tumoural area on overall survival

PD-1 or PD-L1 negative compared to PD-1 and PD-L1 positive: HR 0.44 (0.24–0.81).
Results of logrank test, threshold of positive expression ≥1%

Alteration of immunologic markers

We found no significant correlation between histological grade and pre-RCT TIC density, only intratumoural CD8+ showed a trend to lower density in the G3 group compared to G1&G2 (p=0.086, Student's t-test).

Prior to RCT in peritumoural compartment fewer FoxP3+ and CD163+ cells were present compared to tumoural compartment. Considering post-RCT TIC infiltration, only samples without complete tumour regression and with clearly discriminable compartments were evaluated. Albeit the low sample number is limiting statistical power, FoxP3+ and CD163+ cells were significantly depleted by RCT ($p \leq 0.010$) as compared to a slight and insignificant reduction of CD8+ and CD68+ cells (*Figure 13A*). Consequently, FoxP3+/CD8+ and CD163+/CD68+ ratio decreased clearly comparing pre- and post-RCT tumour tissue (*Figure 13B&C*). Median FoxP3+ to CD8+ cell-to-cell distance was lowest in pretherapeutic peritumoural and highest in posttherapeutic peritumoural compartment; the difference between pre- and posttherapeutic distances in tumoural compartment was not significant (*Figure 13D*). Comparing paired samples, results were similar to those of unpaired evaluation (*Figure 13E-*

H). There was no significant relationship between pretherapeutic intratumoural FoxP3+/CD8+ ratios and FoxP3+ to CD8+ cell-to-cell distances (p=0.250, Fisher's exact test).

In tumoural area before RCT, 46% of the samples were PD-1 negative and 43% PD-L1 negative, 36% and 29% had a positive score of ≥1% to <10%, 14% and 19 % had a score of ≥10% to <50%, 4% and 10% had a score of ≥50%, respectively. The distribution in the pre-RCT peritumoural area was not significantly different from that in the tumoural area. RCT had no significant influence on the expression in both areas, but the percentage of samples with positive PD-1 expression in tumoural and peritumoural area tended to be lower after RCT (p=0.141 and p=0.109, respectively, Fisher's exact test) (*Figure 14*).

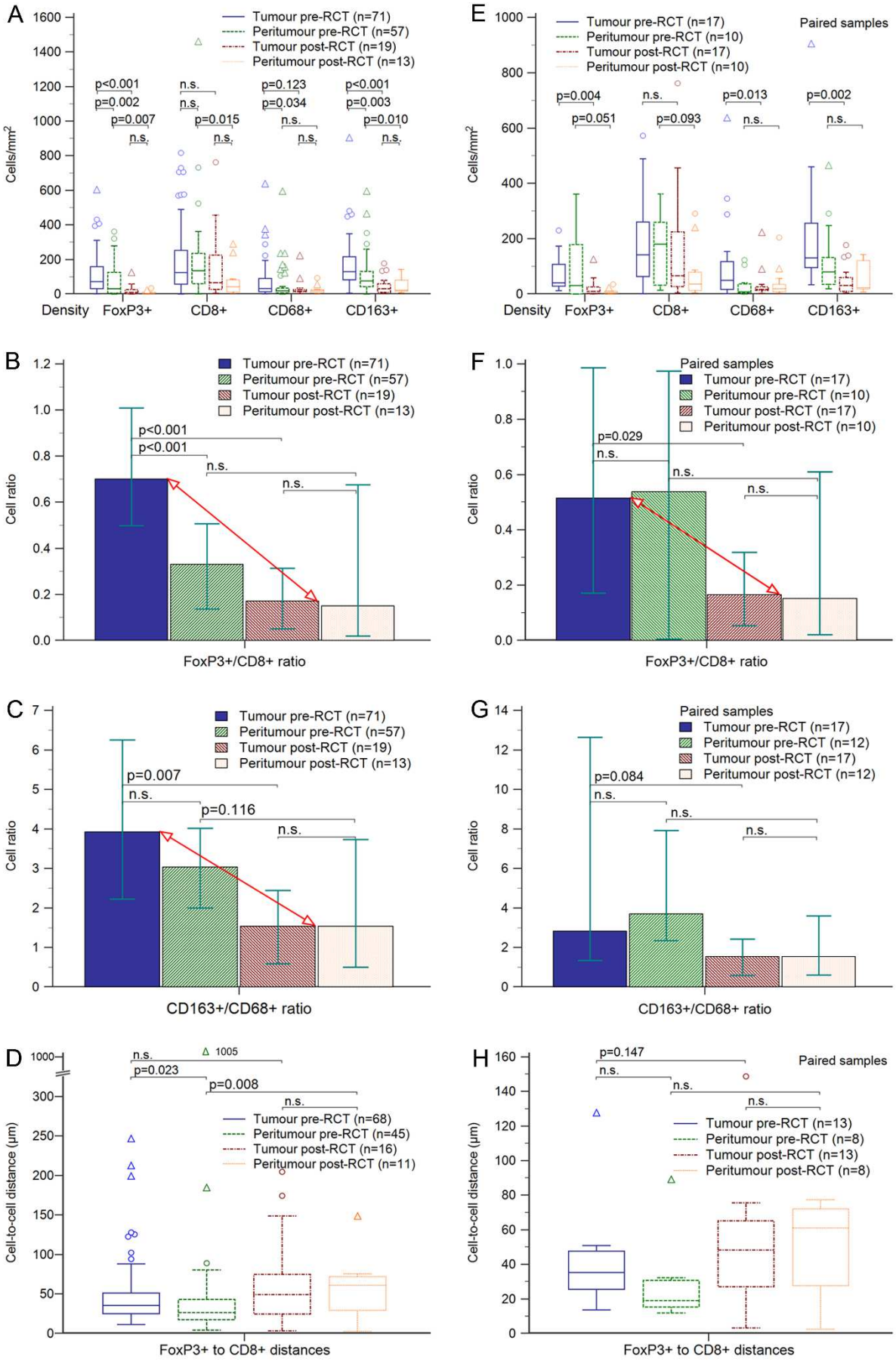

A
Tumour pre-RCT (n=71)
Peritumour pre-RCT (n=57)
Tumour post-RCT (n=19)
Peritumour post-RCT (n=13)
Cells/mm²
p<0.001
p=0.002
p=0.007
n.s.
n.s.
n.s.
p=0.015
n.s.
p=0.123
p=0.034
n.s.
n.s.
p<0.001
p=0.003
p=0.010
n.s.
Density
FoxP3+
CD8+
CD68+
CD163+
E
Tumour pre-RCT (n=17)
Peritumour pre-RCT (n=10)
Tumour post-RCT (n=17)
Peritumour post-RCT (n=10)
Paired samples
Cells/mm²
p=0.004
p=0.051
n.s.
p=0.093
p=0.013
n.s.
p=0.002
n.s.
Density
FoxP3+
CD8+
CD68+
CD163+
B
Tumour pre-RCT (n=71)
Peritumour pre-RCT (n=57)
Tumour post-RCT (n=19)
Peritumour post-RCT (n=13)
Cell ratio
p<0.001
p<0.001
n.s.
n.s.
FoxP3+/CD8+ ratio
F
Paired samples
Tumour pre-RCT (n=17)
Peritumour pre-RCT (n=10)
Tumour post-RCT (n=17)
Peritumour post-RCT (n=10)
Cell ratio
p=0.029
n.s.
n.s.
n.s.
FoxP3+/CD8+ ratio
C
Tumour pre-RCT (n=71)
Peritumour pre-RCT (n=57)
Tumour post-RCT (n=19)
Peritumour post-RCT (n=13)
Cell ratio
p=0.007
n.s.
p=0.116
n.s.
CD163+/CD68+ ratio
G
Paired samples
Tumour pre-RCT (n=17)
Peritumour pre-RCT (n=12)
Tumour post-RCT (n=17)
Peritumour post-RCT (n=12)
Cell ratio
p=0.084
n.s.
n.s.
n.s.
CD163+/CD68+ ratio
D
n.s.
1005
p=0.023
p=0.008
n.s.
Tumour pre-RCT (n=68)
Peritumour pre-RCT (n=45)
Tumour post-RCT (n=16)
Peritumour post-RCT (n=11)
Cell-to-cell distance (µm)
FoxP3+ to CD8+ distances
H
p=0.147
n.s.
n.s.
n.s.
Paired samples
Tumour pre-RCT (n=13)
Peritumour pre-RCT (n=8)
Tumour post-RCT (n=13)
Peritumour post-RCT (n=8)
Cell-to-cell distance (µm)
FoxP3+ to CD8+ distances

Figure 13. TIC densities and FoxP3+ to CD8+ cell distances

A–D: Independent evaluation of all samples. **E–H:** Dependent evaluation of paired samples. **A&E:** Cell density of TIC in different compartments (Box-Whisker). **B&F:** FoxP3+/CD8+ ratio of cell density (median and 95% CI). The double arrows mark the significant reduction of the supposed immunosuppressive status caused by RCT. **C&G:** CD163+/CD68+ ratio of cell density. **D&H:** FoxP3+ to CD8+ cell distances in different compartments. Cell-to-cell distance analysis omitted, if the count or the density of the markers was < 2 or < 2/mm², respectively.

RCT radiochemotherapy, *TIC* tumour infiltrating cells, *CI* confidence interval, *p* two-tailed p-value

Results of Mann-Whitney-U test (A–D) and Wilcoxon test (E–H)

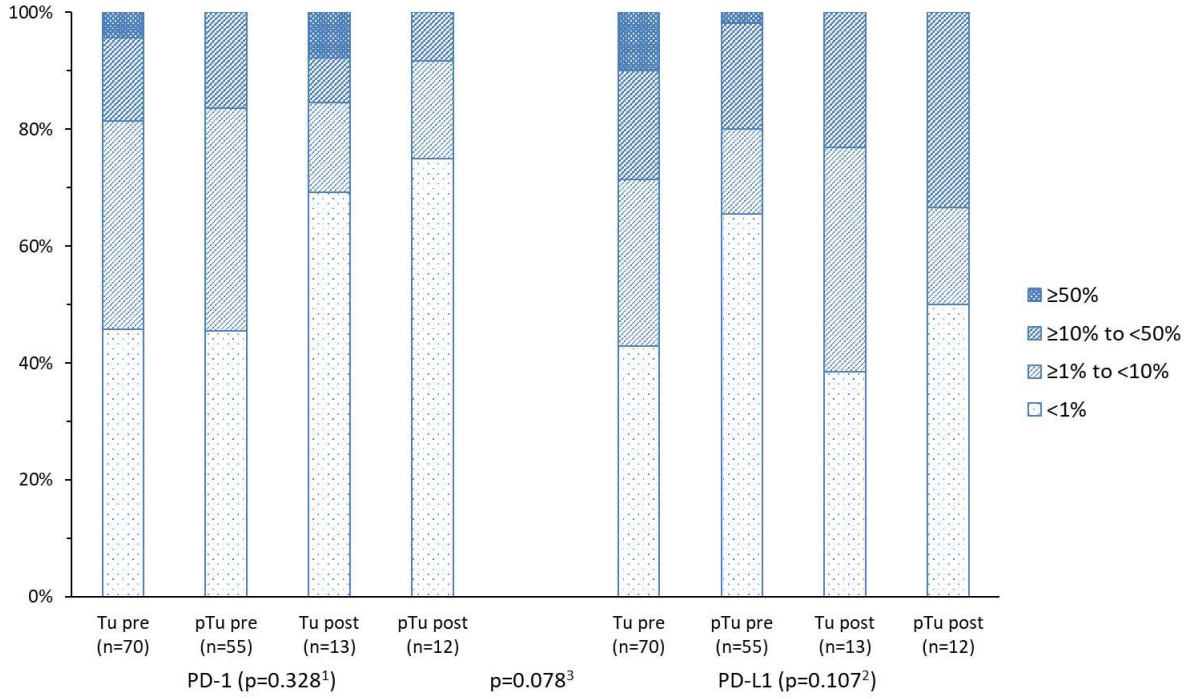


Figure 14. PD-1 and PD-L1 expression in tumoural and peritumoural area, pre- and post-RCT

Expression of PD-1 and PD-L1 was estimated as the number of positive cells divided by the number of all cells in the area.

RCT radiochemotherapy, *Tu* tumoural area, *pTu* peritumoural area, *pre* pre-RCT, *post* post-RCT

Results of Chi-squared test, [1]PD-1 group, [2]PD-L1 group, [3]all groups

Discussion

Trimodality therapy of OAC has been identified as a major advance by recent evidence-based data. To predict a good response following neoadjuvant RCT would be a further step forward, since only patients experiencing a reasonable regression grade of Mandard 1-3 will benefit from this approach (22). To the best of our knowledge no data on predictive immunologic tumour parameters had been identified up to the publication of our results (1). However, a recently published study confirmed substantial parts of our findings (61).

Apart from some clinical parameters, we found significant influence of pretherapeutic immunological biomarkers on TRG and survival. In addition, their prognostic value was enhanced by including parameters of possibly functional activity, such as cell ratios, FoxP3+ to CD8+ cell-to-cell distance, and PD-L1/PD-1 expression.

Thus, our results may also contribute important ideas to the ongoing process of constituting a TNM-I (TNM-Immune) classification using an Immunoscore-based stratification. After all, in Immunoscore regarding CD8+- and CD3+ densities in colorectal cancer, adaptive immune cell infiltration was shown to have a prognostic value superior to the classic tumour invasion criteria, including grade, stage, and metastatic status (28–30).

Influence of pretherapeutic clinical and immunologic parameters on TRG

Among all clinical parameters tested, only lymph node status influenced TRG, cN+ diminished the effect of RCT distinctly. This may be due to the fact that especially poorly immunogenic tumours tend to metastasize early and are known to respond worse to RCT (62, 63). However, current ESMO clinical practice guidelines (15) do not recommend to withhold cN+ patients from neoadjuvant RCT, albeit clear evidence addressing cN status is lacking.

Densities of each evaluated TIC alone had no influence on TRG. In contrast, Noble et al. reported a favourable TRG for higher CD8+ (significant) and FoxP3+ (trend) density in OAC (57). However, patients received chemotherapy alone and minor TRG was defined as Mandard 3-5. Our evaluations instead suggest to consider Mandard 4&5 as minor TRG, in accordance to Thies and Langer (64). Goedgebuure, Harrasser et al. recently compared complete responders (TRG 1) to RCT with non-complete responders (TRG 2–5) and found that complete responders had significantly higher numbers of tumour-infiltrating T-cells (61). We also found higher CD8+ densities in complete responders than in non-complete responders, but the difference was not significant.

In patients with rectal cancer, a high intratumoural FoxP3+/CD8+ ratio, assuming an immunosuppressive state, was linked to an unfavourable TRG following RCT (65), whereas another study found no association (66). On the contrary, in our cohort a high intratumoural FoxP3+/CD8+ ratio predicted a significantly favourable TRG. However, as discussed below, FoxP3+ density was lowered to a great extent by RCT, whereas CD8+ density remained nearly constant. Considering a relatively stable balance between immunologic surveillance and tumour escape mechanisms in the pretherapeutic period, it may be hypothesized that a sudden switch to a pronounced pro-inflammatory microenvironment by RCT catapults immunologic surveillance into a superior position (see also *Figure 17*).

In contrast, short intratumoural FoxP3+ to CD8+ cell-to-cell distances were associated with an unfavourable TRG (trend), though they also should constitute an immunosuppressive status. However, FoxP3+ to CD8+ cell-to-cell distances were not significantly altered by RCT. So, the immunosuppressive status seemed to be preserved and consequently could reduce RCT efficacy.

Accordingly, a high CD163+/CD68+ ratio was associated with worse TRG, too, both for tumoural (strong trend) and peritumoural compartment (significant). Goedgebuure, Harrasser et al. recently reported similar findings, low numbers of CD163+ M2 macrophages being associated with a favourable complete response (61). The effect may be due to the outstanding role of M2 macrophages in wound healing (49). As discussed by Schaue et al., tissue damage following RCT causes M2 macrophages to attempt healing at the price of possible tumour immune escape (63).

High infiltration with overall TAM was also linked to worse TRG, supporting data of Sugimura et al. (37), possibly reflecting the tumour-promoting effect of chronic inflammation (67). Dutta et al. pointed out in this context, that high TAM infiltration is associated with a high tumour proliferative index (Ki67) (38).

In OSCC, Fassan et al. found that PD-L1 expression was significantly higher in patients who experienced complete pathological response following nRCT (68). The authors discuss that a strong immune infiltration within the tumour could be counterbalanced by a high expression of PD-L1 at baseline, but the therapeutic effects could unmask the cancer antigens, allowing a strong immune response and a favourable response on therapy. In contrast, Chen et al. described a significant correlation of positive PD-L1 staining with poor treatment response following radiotherapy of OSCC (69). In our cohort, PD-1 and PD-L1 expression in tumour area had no influence on TRG, only PD-L1 expression in peritumoural area was significantly associated

with a significant better response on RCT. Interpretation of our results remains difficult. Regarding that OAC seems to be mostly immune cell excluded (70), the immunological response to tumour spreading may be better characterized in the peritumoural area. The positive correlation of PD-L1 expression in tumoural and peritumoural area found in our cohort may support our assumption, indicating that peritumoural effects may reflect tumoural effects in a more pronounced manner.

Influence of pretherapeutic immunologic parameters on survival

Since surgery had no significant influence on survival in our cohort, we evaluated pretherapeutic parameters including patients with and without surgery:

- *CD8+ density*

As cytotoxic T-lymphocytes (CTL) are able to kill tumour cells, high CD8+ density should predict a favourable survival. But data in OAC are conflicting. In a study with primary resected OAC, high CD8+ cell density in tumoural (but not peritumoural) compartment predicted a better survival (32). Another study, however, could not find any influence of CD8+ density on survival in patients with OAC (36). A recently published review, including 2121 patients with OC, reported high levels of CD8+ TIL being associated with better OS (34).

In our cohort high intratumoural CD8+ density tended to improve survival, but peritumourally it predicted significantly worse survival. Similar contrasting results were found in rectal carcinoma and gastric cancer of the cardia, albeit in various compartments (48, 71). The present results indicate that an inflammatory status in the peritumoural area of OAC may promote tumour spreading. For a more subtle analysis it would be of interest to investigate the milieu at the border between tumoural and peritumoural compartments. From the tumour "excluded" CTL may possibly constitute an overshooting pro-inflammatory status and cause tumour cells to down-sensitize anticancer immunity, e.g. by inhibitory factors downregulating MHC class I molecule expression (43), see *Figure 15*.

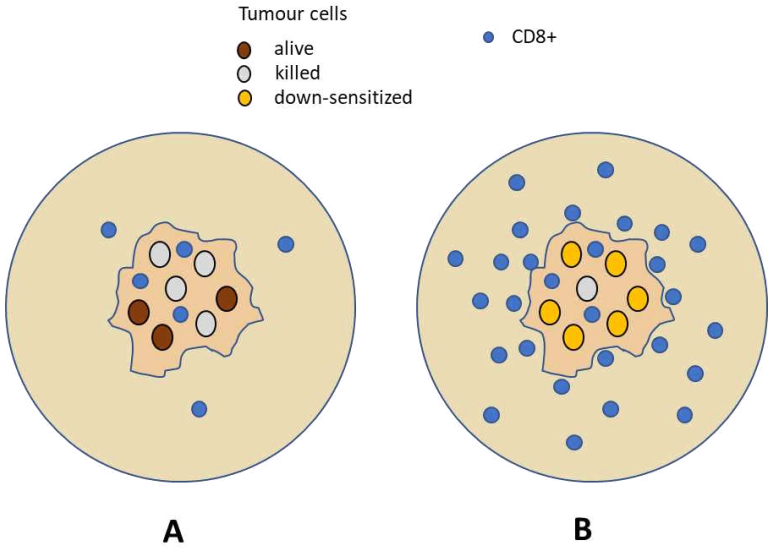


Figure 15. Influence of peritumoural CD8+ density on anticancer-immunity

A: A low number of peritumoural CD8+ cells does not influence substantially the intratumoural microenvironment. Tumour cells keep being vulnerable to attacks of intratumoural CD8+ cells.
B: A high number of peritumoural CD8+ cells causes an overshooting proinflammatory environment and down-sensitizes anticancer immunity of tumour cells. Thus, intratumoural CD8+ cells lose effectiveness.

- *FoxP3+ and CD8+ interactions*

FoxP3+ density had no influence on survival. This is in line with a review of Zheng et al. (34), but it is in contrast with findings in other tumour entities. For example, in gastric cancer of the cardia Haas et al. found a favourable outcome for patients with a high FoxP3+ cell density in tumour stroma (71), whereas in gastric cancer Wang et al. described that a high FoxP3+ cell density was associated with a reduced survival (72), and similar results were seen in ovarian carcinoma (73). Obviously, the role of FoxP3+ Treg seems to vary in a large extent, and therefore it might be crucial to consider functional interactions between FoxP3+ and CD8+ cells.

Indeed, evaluating FoxP3+ to CD8+ cell-to-cell distances in tumoural compartment a short distance was associated with worse survival in our cohort. The difference was clearly pronounced when patients who died postoperatively were excluded, as they do not contribute to studying immunological effects. A similar shift to a more distinct effect was seen excluding cases within lower and upper quintile of CD8+ density. This approach may be justified as an influence of FoxP3+ cells in a very low CD8+ density should be negligible, whereas a high

underlying CD8+ density may cause tumour cells to down-sensitize anticancer immunity (43, 74), see *Figure **16***.

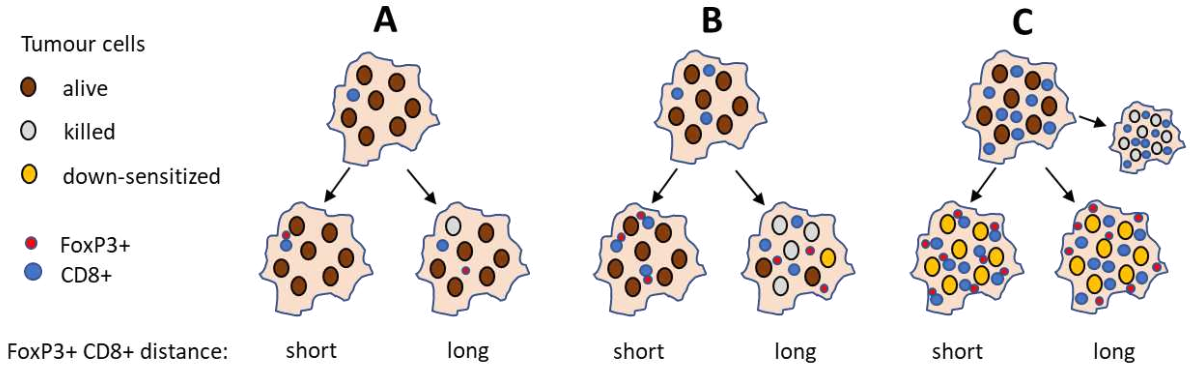


Figure 16. Effects of FoxP3+ to CD8+ distance in different microenvironments according to intratumoural CD8+ density

A: If very few CD8+ cells are present, anticancer surveillance keeps low, nearly independent of any FoxP3+ to CD8+ distance.
B: CD8+ density in a middle range only can attack tumour cells successfully if they are not hampered by the influence of FoxP3+ cells in narrow proximity. In this case, a small part of tumour cells may down-sensitize anticancer immunity, driven by a proinflammatory microenvironment.
C: A very high number of CD8+ cells may lead to an effective tumour control, but due to an overshooting proinflammatory microenvironment, many tumour cells may down-sensitize anticancer immunity. As a consequence, FoxP3+ to CD8+ distance will lose its impact.

These findings support our hypothesis that a short FoxP3+ to CD8+ cell-to-cell distance reflects immunologic activity (46–48), though they should be evaluated, for example, by proximity ligation assays (75). Moreover, it seems difficult to apply this approach for diagnostic purpose.

In most studies, a higher FoxP3+/CD8+ ratio has been related to unfavourable prognosis in different types of cancer, including breast, ovarian, oesophageal squamous cell, tonsillar, gastric and colorectal cancer (except (76)), and osteosarcoma (77–81, 35, 82, 83, 65, 84, 85). In our OAC cohort, a high pre-RCT intratumoural FoxP3+/CD8+ ratio also tended to predict worse survival. At least, high amounts of FoxP3+ Treg seemed to annihilate the positive effects of CD8+ CTL. We do not think that this observation is contradictory to the favourable effects of high FoxP3+/CD8+ ratio on TRG. The latter may describe short-run effects of RCT, whereas influence on survival should be due to a reorganized balance of immunosurveillance and immune escape mechanisms in the long lasting posttherapeutic period (*Figure 17*).

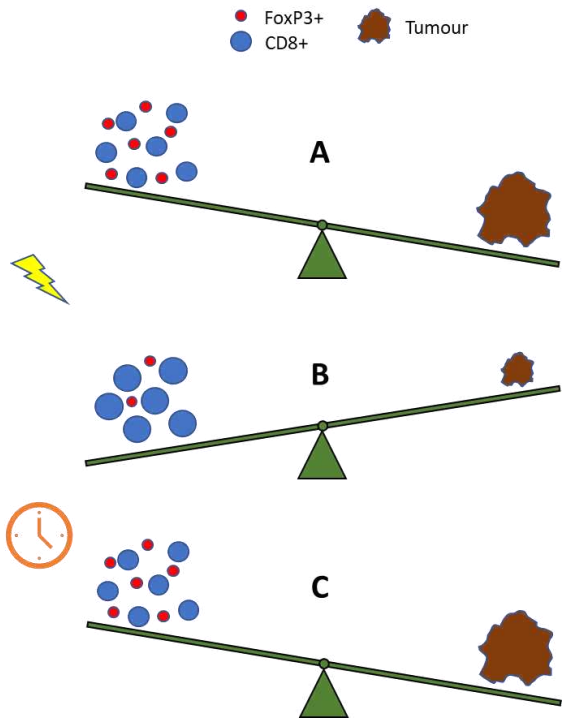


Figure 17. Effects of a high FoxP3/CD8+ ratio on TRG and prognosis.

A: In the pretherapeutic state, a high FoxP3+/CD8+ ratio leads to a low tumour control
B: During radiochemotherapy, CD8+ cells keep constant whereas FoxP3+ cells are reduced in a great extent. No more hampered by FoxP3+ cells, CD8+ cells may increase cancer surveillance, promoting a favourable TRG.
C: In the period after RCT, FoxP3+ cells will recover by the time and interfere with the activity of CD8+ cells, predicting an unfavourable prognosis.

- *Macrophages parameters*

CD163+ density had no significant influence on survival, high intratumoural CD68+ density was associated with a weak trend for better survival. In contrast, Sugimura et al. found high infiltration of CD68+ and CD163+ macrophages in OC being significantly associated with a poor prognosis in patients undergoing neoadjuvant chemotherapy (37). Searching for an explanation for these contrarious results, we explored the ratio of all intratumoural TAM to CD8+ cells. Here, a high ratio was associated with a worse outcome, being more predictive than all parameters alone. In summary, our findings support the hypothesis of Dutta et al., that high macrophage infiltration may promote and high lymphocytic infiltration may prevent tumour progression (38).

- *PD-L1 and PD-1 expression*

Most recently published treatment studies investigating the effect of checkpoint inhibitors in gastric cancer and OAC used the CPS to determine PD-L1 expression (CPS =combined positive score = number of PD-L1 positive tumour cells, lymphocytes, and macrophages divided by the total number of viable tumour cells and multiplied by 100) (53, 86, 55). In our study we evaluated PD-L1 and PD-1 expression simultaneously not only in the tumoural but also in the peritumoural compartment, where tumour cells are absent. For a better comparison of both parameters in both areas we adapted and simplified the CPS and divided the number of cells with positive expression by the total number of all cells. Survival analysis of our cohort revealed that a score of ≥1% was best appropriate to be classified as positive. This threshold may be a bit lower than the CPS of 5 and 10 which was postulated for the recent approval of nivolimumab and pembrolizumab for treatment of gastric cancer and OAC, respectively.

In our cohort, a positive PD-1 expression both in tumoural and in peritumoural area was associated with a significantly worse outcome. In contrast, we could not demonstrate a significant influence of PD-L1 expression on survival, neither in tumoural nor in peritumoural area. PD-1 and PD-L1 expressions were highly correlated in tumoural compartment, and a combined evaluation of PD-1 and PD-L1 expression in this area seemed to increase the grade of influence on prognosis. However, multivariate analysis provided that mainly PD-1 expression was responsible for this effect. Our findings may be somewhat surprising, as in gastric cancer, for example, Gao et al. found a significant unfavourable effect of both PD-1 and PD-L1 expression on prognosis (87), and Chang et al. of PD-L1 expression (88). On the other hand, Wang et al. reported an improved survival of patients with positive tumour PD-L1 expression in gastric cancer (72). There seems to be some evidence of meta-analysis that in patients with digestive system cancer, PD-L1 expression is only a prognostic marker in Asian ethnicity, but not in Non-Asian (89). The same meta-analysis pointed out that the prognostic value in oesophageal cancer may be uncertain. A Swedish study found a prolonged survival for high PD-L1 or PD-1 expression in patients with OAC or gastric cancer; but patients in this study had no neoadjuvant and only 7.5% had adjuvant therapy (90). Results of a Swiss study at least support our findings that high PD-1 expression predicts an unfavourable outcome in OAC (91). As shown for OSCC by Jiang et al., moreover, prediction of PD-L1 expression on survival seems to depend on the tumour stage and lymph node status. In this study, positive tumoural PD-L1 expression was a favourable predictor in UICC stage I-II, but not in III-IV (92).

Däster et al. combined CD8+ and PD-L1/PD-1 evaluation and found that high/high infiltration/expression was associated with significant better survival than low/low infiltration/expression (93). In our cohort we could confirm the impact of CD8+ infiltration on survival, but our results of the combined evaluation of CD8+ infiltration and PD-L1 or PD-1 expression showed that PD-L1 or PD-1 expression was an inversed amplifier of the effect of CD8+ infiltration. That means, that in our cohort a high CD8+ infiltration combined with a low PD-L1 or PD-1 expression predicted a favourable prognosis and vice versa. We think that our results seem to be well plausible in tumoural compartment, as a high immunologic activation should not be hampered by any inhibitory mechanisms, and especially as PD-1 expression is considered to be a sign of T-cell exhaustion or even hyperexhaustion (43). In peritumoural area, we found a similar signature as Däster in tumoural area, but the effect was the opposite: Low/low infiltration/expression was associated with a significant better outcome than high/high infiltration/expression. As previously mentioned, we think that a high expression of PD-L1 and PD-1 is mainly induced by a high immunologic activation of CD8+, and that a high peritumoural immunologic activation may support escape mechanisms of tumour cells.

In our opinion, our results indicate that at least for Non-Asian ethnicities, PD-1 expression could be more meaningful for the prediction of prognosis than PD-L1 expression, and that the expression of PD-L1/PD-1 has to be placed in the context with CD8+ infiltration.

Influence of RCT on TIC density and ratio

As we excluded patients with complete regression from posttherapeutic evaluation and included only samples with clear discrimination of tumoural and peritumoural area, only few post-RCT samples could be evaluated and results have to be interpreted cautiously.

FoxP3+ density in the tumoural compartment was reduced distinctly by RCT, whereas a slight reduction of CD8+ density was not significant. These results are congruent with data of Zingg et al. in OAC (36) and of Mirjolet et al. in rectal cancer (94). Yoneda et al. even found an increase in CD8+-density after RCT in patients with non-small-cell lung cancer (95). In the study of Mirjolet patients with a significant increase of the FoxP3+/CD8+ ratio were more likely to live longer, as patients did in the study of Yoneda with an increase of CD8+ TILs.

Considering TAM, to our knowledge no data are published for OAC. In our cohort CD68+ and CD163+ densities were both reduced by RCT with stronger effects on CD163+. Consequently, FoxP3+/CD8+ and CD163+/CD68+ ratios were significantly reduced by RCT, indicating that

RCT efficacy may partially be mediated by constituting a pronounced pro-inflammatory microenvironment (62, 63).

In our cohort, around half of pre-RCT samples was classified as PD-L1 or PD-1 positive both in tumoural and in peritumoural area. Taking into account different scoring systems, this is approximately in line with published results of OAC and gastric cancer (90, 96, 97). The high proportion of PD-L1 and PD-1 positive samples of peritumoural area in our cohort may reflect the deep involvement of this outside compartment in cancer-related immunologic reactions, and may justify increased interest in further investigation of the peritumoural compartment.

We did not find any significant influence of RCT on PD-L1 or PD-1 expression, at best a weak trend to a reduced PD-1 expression following RCT. In OAC and gastric cancer, Svensson et al. reported no effect of chemotherapy on PD-L1 (98), whereas Yu et al. found increasing PD-L1 and PD-1 expression after chemotherapy of gastric cancer (99). In other tumour entities, an up-regulation of PD-L1 expression was reported after RCT of rectal cancer (100), and following chemotherapy of ovarian cancer (101) and of head-neck cancer (102). It is discussed, that activation of CD8+ CTL, for example by chemotherapy, is accompanied by a shift to a pronounced expression of PD-L1 and PD-1 induced by interferon (IFN)-γ, which is produced by activated CTL themselves, consequently restoring a relatively balanced environment (99, 72). In our cohort, CD8+ infiltration was not altered significantly by RCT, and thus, also the PD-L1 and PD-1 expression could be expected to be unchanged.

Postoperative mortality and impact of surgery on survival

We observed a significant difference in postoperative mortality depending on age. These data may reflect "real world" conditions among rather unselected patients. Therefore, question arises whether especially elderly patients should be treated by RCT alone, mainly after major response to RCT. In a systematic review of Best et al. RCT in OC appeared to be at least equivalent to surgery in people responsive to RCT (23). Similar results were reported in a meta-analysis by Wang et al. (24). Though not stratified for clinical response, in our cohort survival was not significantly different in patients with vs. without surgery in multivariate analysis.

Limitations

Despite the prospective approach studying the influence of pre-RCT taken immunologic markers on TRG and survival, the evaluation of the parameters was made retrospectively, and as the number of patients was relatively small, the analysis of subgroups had reduced statistical

power. Furthermore, the use of tissue microarrays for histological examination may not take into account the heterogeneity of the immune landscape, as described in gastric cancer (103).

Conclusion

We demonstrated substantial influence of various clinical and pretherapeutic immunological parameters on TRG and survival of patients with OAC under "real world" conditions. Our results emphasize the outstanding role of immunologic balance maintained by a complex co-stimulatory and co-inhibitory immunologic network. At a first glance antithetic results may be resolved, if the contributions of the most important players to a certain microenvironment are taken into account. Thus, in the present study simultaneous investigation of CD8+ and FoxP3+ TILs, CD68+ and CD163+ TAMs, and PD-L1 and PD-1 expression could deepen the understanding of immunologic mechanisms responsible for cancer surveillance. Besides forming ratios of corresponding immunologic markers, we verified that evaluating cell-to-cell distance of counteracting FoxP3+ and CD8+ cells was a valuable instrument to investigate functional immunologic interactions, and that the inclusion of peritumoural compartment may enlighten predictive and prognostic mechanisms. Also, PD-L1 and PD-1 expression should preferentially be evaluated in the context of underlying immunological environment, mainly of the CD8+ infiltration grade.

In particular, patients with pretherapeutic cN+, low intratumoural FoxP3+/CD8+ ratio, high peritumoural CD163+/CD68+ ratio, high intratumoural TAM density, and negative peritumoural PD-L1 expression may not expect a reasonable tumour regression following RCT. Regarding prognosis, patients with pretherapeutic high peritumoural CD8+ density, short intratumoural FoxP3+ to CD8+ cell-to-cell distance, and positive intratumoural PD-1 expression seem to be at high risk for disease progression in multivariate analysis. An unfavourable outcome was also seen in a subgroup of patients with low intratumoural CD8+ density combined with positive PD-L1 expression.

We think that our results could help to apply different treatment strategies to patients with OAC in a more targeted manner. But, of course, to validate these results further investigation with larger size datasets is required.

Abstract in Croatian Language

SAŽETAK

Pozadina i svrha: Tumor infiltrišući limfociti (TIL), tumor asocirani makrofagi (TAM) i PD-L1/PD-1 ekspresija igra ključnu ulogu u antikancerskom imunološkom nadzoru. Mi smo proučavali njihov uticaj na odgovor na neoadjuvantnu radiohemoterapiju (RCT) i prognozu kod pacijenata sa adenokarcinomima ezofagusa (OAC).

Materijali i metode: Između 10/2004 i 06/2018, uradjena je pre-RCT biopsija-uzoraka od ukupno 76 pacijenata sa lokalno uznapredovalim, ne metastatskim OAC pripremanih za trimodalnu terapiju. Procenjivali smo intra- i peritumoralnu ekspresiju FoxP3+, CD8+, CD68+, CD163+ i PD-L1/PD-1 da bi utvrdili njihov uticaj na stepen tumorske regresije (TRG) i preživljavanje.

Rezultati: Slaba tumorska regresija je uočena kod cN+ (RR 0.77 [95% CI 0.66-0.90], p=0.001), nizak intratumorski FoxP3+/CD8+ racio (RR 0.75 [0.60-0.96], p=0.020), visok peritumorski CD163+/CD68+ racio (RR 0.77 [0.60-0.99], p=0.045), visoka intratumorska gustina TAM (RD -0.44 [-0.82 to -0.06], p=0.023), i negativna peritumorska PD-L1 ekspresija (RR 0.75 [0.57-0.98], p=0.036).

Nezavisno od slabog resekcionog kvaliteta i TRG, preterapijska visoka peritumoralna CD8+ infiltracija (HR 2.36 [1.21-4.61], p=0.012), kratke intratumorske FoxP3+ do CD8+ udaljenosti ćelije od ćelije kod srednje CD8+ intratumorske gustine (HR 2.55 [1.00-6.52], p=0.050), pozitivna intratumorska PD-1 ekspresija (HR 1.92 [1.08-3.45], p=0.038), i niska intratumorska CD8+ gustina u kombinacija sa pozitivnom PD-L1 ekspresijom (HR 3.13 [1.12-8.33], p=0.042) bili su značajno nepovoljni prognostički faktori u multivarijantnoj analizi.

Zaključci: Procenjivani imunološki parametri pokazali su nezavisnu prediktivnu i prognostičku vrednost kod pacijenata sa adenokarcinomima ezofagusa.

Abstract in English Language

Background and purpose: Tumour infiltrating lymphocytes (TIL), tumour associated macrophages (TAM) and PD-L1/PD-1 expression play a key role in anticancer immunosurveillance. We studied their influence on response to neoadjuvant radiochemotherapy (RCT) and prognosis in patients with oesophageal adenocarcinoma (OAC).
Materials and methods: Between 10/2004 and 06/2018, pre-RCT biopsy-specimens were available from 76 patients with locally advanced, non-metastatic OAC scheduled for trimodality therapy. We evaluated intra- and peritumoural expression of FoxP3+, CD8+, CD68+, CD163+, and PD-L1/PD-1 to determine their influence on tumour regression grade (TRG) and survival.
Results: Poor tumour regression was detected for cN+ (RR 0.77 [95% CI 0.66-0.90], p=0.001), low intratumoural FoxP3+/CD8+ ratio (RR 0.75 [0.60-0.96], p=0.020), high peritumoural CD163+/CD68+ ratio (RR 0.77 [0.60-0.99], p=0.045), high intratumoural TAM density (RD -0.44 [-0.82 to -0.06], p=0.023), and negative peritumoural PD-L1 expression (RR 0.75 [0.57-0.98], p=0.036).
Apart from poor resection quality and TRG, pretherapeutic high peritumoural CD8+ infiltration (HR 2.36 [1.21-4.61], p=0.012), short intratumoural FoxP3+ to CD8+ cell-to-cell distances in middle ranged CD8+ density (HR 2.55 [1.00-6.52], p=0.050), positive intratumoural PD-1 expression (HR 1.92 [1.08-3.45], p=0.038), and low intratumoural CD8+ density combined with positive PD-L1 expression (HR 3.13 [1.12-8.33], p=0.042) were significant unfavourable prognostic factors in multivariate analysis.
Conclusions: The evaluated immunologic parameters showed independent predictive and prognostic value in patients with OAC.

Additional Tables and Figures

Table 3. Pretherapeutic variables with possible impact on favourable tumour regression after RCT (risk analysis of prognostic factors)

Parameters (n cases evaluated)	Threshold	RR (95 % CI)	p-value	RD (95 % CI)	p-value
All patients (n=58)					
Clinical data					
Age ≥ median (n=58)	≥63.80 (yr)	0.96 (0.75-1.23)	p=0.738	-0.03 (-0.24 to 0.17)	p=0.737
cT3&4 (n=58)	T3&4 vs. T1&2	1.02 (0.00-1.43)	p=0.907	0.02 (0.00 to 0.29)	p=0.906
cN+ (n=58)	N+ vs. N0	0.77 (0.66-0.90)	**p=0.001**	-0.23 (-0.35 to -0.11)	**p<0.001**
pG3 in pre-RCT biopsies (n=58)	G3 vs. G1&2	0.90 (0.71-1.15)	p=0.412	-0.08 (-0.28 to 0.11)	p=0.407
CD8+ and FoxP3+					
High FoxP3+/CD8+ ratio Tu (n=53)	≥1.00	1.33 (1.05-1.68)	**p=0.020**	0.23 (0.05 to 0.41)	**p=0.011**
High FoxP3+/CD8+ ratio pTu (n=46)	≥0.07	1.38 (0.88-2.17)	p=0.165	0.23 (-0.06 to 0.52)	p=0.117
Short FoxP3+ to CD8+ dist Tu (n=51)	<0.9*SRD (µm)	0.83 (0.67-1.04)	p=0.106	-0.16 (-0.34 to 0.03)	p=0.100
Short FoxP3+ to CD8+ dist pTu (n=43)	<0.9*SRD (µm)	1.08 (0.74-1.56)	p=0.700	0.06 (-0.23 to 0.34)	p=0.695
CD68+ and CD163+					
High CD163+/CD68+ ratio Tu (n=53)	≥2.23	0.79 (0.61-1.02)	p=0.070	-0.19 (-0.39 to 0.00)	p=0.055
High CD163+/CD68+ ratio pTu (n=47)	≥2.01	0.77 (0.60-0.99)	**p=0.045**	-0.21 (-0.42 to -0.01)	**p=0.036**
High TAM density Tu (n=53)	≥401.5 (mm^{-2})	0.49 (0.21-1.17)	p=0.108	-0.44 (-0.82 to -0.06)	**p=0.023**
PD-L1+ and PD-1+					
Positive PD-L1 expression Tu (n=52)	≥1%	0.91 (0.70-1.19)	p=0.484	-0.08 (-0.29 to 0.14)	p=0.480
Positive PD-L1 expression pTu (n=44)	≥1%	1.34 (1.02-1.76)	**p=0.036**	0.24 (0.03 to 0.44)	**p=0.023**
Positive PD-1 expression Tu (n=52)	≥1%	0.88 (0.66-1.15)	p=0.343	-0.11 (-0.32 to 0.11)	p=0.332
Positive PD-1 expression pTu (n=44)	≥1%	0.97 (0.72-1.30)	p=0.825	-0.03 (-0.26 to 0.21)	p=0.825

TRG tumour regression grade, *RCT* radiochemotherapy, *RR* risk ratio, *CI* confidence interval, RD risk difference, *yr* years, *Tu* in tumoural area, *pTu* in peritumoural area, *dist* cell-to-cell distance, *TAM* tumour associated macrophages (CD68+ plus CD163+)
Favourable TRG: Mandard 1-3 vs. Mandard 4-5
cN+: clinically positive lymph nodes pre-RCT
High ratio (density): The ratio of the cell densities (the density, respectively) in this case is equal to or higher than the threshold obtained by ROC-analysis of all cases.
Short cell to cell distance, SRD: The median cell distance is more than 10 % shorter than the simulated random distance *(SRD)* in this case.
Cell-to-cell distance analysis was omitted, if the count or the density of the markers was less than 2 or less than 2/mm², respectively.

Results of risk analysis, Pearson's chi-squared test and two tailed z-test. Bold marking for p<0.05

Table 4. Uni- and multivariate analysis of variables with possible impact on OS

Patient variables	OS					
			Univariate analysis		Multivariate analysis	
	Md (mo)	5 yr (%)	HR (95% CI)	p-value	HR (95% CI)	p-value
All patients (n=76)	21	30				
Gender						
Female (n=16)	19	24				
Male (n=60)	21	31	0.96 (0.49-1.86)	n.s.		
Age (median 65.35 yr)						
44.3 - <65.35 y (n=38)	26	39				
≥66.35 - 86.5 y (n=38)	21	17	1.44 (0.82-2.52)	p=0.209	1.28 (0.71-2.29)	p=0.409[1]
Clinical staging (UICC)						
II (n=4)	26	38		n.s. p=0.207		
III (n=70)	21	30		p=0.076		
IV (n=2)	7	0				
(cT)						
cT1 & cT2 (n=13)	26	42				
cT3 & cT4 (n=63)	21	28	1.11 (0.51-2.44)	n.s.		
(cN)						
cN0 (n=12)	40	44				
cN+ (n=64)	17	27	1.91 (0.95-3.87)	p=0.071	2.17 (0.86-5.47)	p=0.101[1]
Grading (biopsy)						
pG1 (n=3)	57	33				
pG2 (n=32)	21	39				
pG3 (n=41)	21	24		n.s.		
Localization				*comp. to AEG III*		
OAC without AEG (n=7)	40	0		**p=0.012**		n.s.[4]
AEG Siewert I (n=40)	16	32		**p=0.011**		n.s.[4]
AEG Siewert II (n=26)	26	33		**p=0.005**		n.s.[4]
AEG Siewert III (n=3)[#]	7	0		*all other n.s.*		
Surgery						
With surgery (n=58)	26	36				
Without surgery (n=18)	19	0	1.81 (0.87-3.76)	p=0.111	1.41 (0.72-2.76)	p=0.314[2]
Resection quality (n=58)						
R0 (n=51)	40	41				
R1 & R2 (n=7)	10	0	7.74 (2.06-29.1)	**p=0.003**	2.88 (1.19-6.98)	**p=0.020**[3]
TRG Mandard (n=58)						
1 & 2 & 3 (n=47)	40	41				
4 & 5 (n=11)	9	14	3.93 (1.45-10.6)	**p=0.006**	2.27 (1.06-4.85)	**p=0.034**[4]
1 & 2 (n=42)	40	40				
3 & 4 & 5 (n=16)	13	25	1.73 (0.80-3.79)	p=0.146		n.s.[4]

Pre-Tx immunologic parameters						
CD8+ low density Tu (n=36)	16	23				
CD8+ high density Tu (n=35)	26	34	0.63 (0.36-1.13)	p=0.125	0.69 (0.39-1.22)	p=0.196[5]
CD8+ low density pTu (n=28)	50	44				
CD8+ high density pTu (n=29)	16	17	2.29 (1.16-4.52)	**p=0.017**	2.36 (1.21-4.61)	**p=0.012[5]**
FoxP3+ low density Tu (n=36)	19	30				
FoxP3+ high density Tu (n=35)	21	27	1.06 (0.60-1.88)	p=0.838		n.s.[5]
FoxP3+/CD8+ low ratio Tu (n=36)	26	34				
FoxP3+/CD8+ high ratio Tu (n=35)	16	22	1.47 (0.83-2.62)	p=0.186	1.36 (0.77-2.41)	p=0.285[5]
FoxP3+ CD8+ short dist Tu (n=52)	21	22	1.61 (0.85-3.06)		1.59 (0.79-3.21)	
FoxP3+ CD8+ long dist Tu (n=17)	40	41	0.62 (0.33-1.18)	p=0.144	0.63 (0.31-1.27)	p=0.194[5]
FoxP3+ CD8+ short dist Tu* (n=30)	17	22	2.35 (1.06-5.21)		2.55 (1.00-6.52)	
FoxP3+ CD8+ long dist Tu* (n=12)	80	58	0.43 (0.19-0.95)	**p=0.036**	0.39 (0.15-1.00)	p=0.050[5]
CD68+ low density Tu (n=36)	19	18				
CD68+ high density Tu (n=35)	40	39	0.73 (0.41-1.30)	p=0.286	0.68 (0.38-1.21)	p=0.187[5]
TAM/CD8+ low ratio Tu (n=35)	43	37				
TAM/CD8+ high ratio Tu (n=35)	16	21	1.74 (0.97-3.12)	p=0.062	1.63 (0.91-2.91)	p=0.098[5]
PD-L1 neg Tu (n=30)	32	35				
PD-L1 pos Tu (n=39)	21	24	0.69 (0.39-1.23)	p=0.212		n.s.[5]
PD-L1 neg & CD8+ hi Tu (n=9)	n/a	53				
PD-L1 pos & CD8+ lo Tu (n=13)	11	15	0.32 (0.12-0.89)	**p=0.028**	0.31 (0.10-0.96)	**p=0.042[5]**
PD-1 neg Tu (n=31)	50	47				
PD-1 pos Tu (n=38)	19	16	0.52 (0.29-0.93)	**p=0.028**	0.53 (0.29-0.97)	**p=0.038[5]**
PD-1 neg & CD8+ hi Tu (n=11)	n/a	62				
PD-1 pos & CD8+ lo Tu (n=14)	14	13	0.25 (0.09-0.69)	**p=0.007**	0.25 (0.08-0.76)	**p=0.015[5]**
PD-L1 neg pTu (n=35)	43	41				
PD-L1 pos pTu (n=19)	19	25	0.71 (0.34-1.45)	p=0.343		n.s.
PD-L1 neg & CD8+ lo pTu (n=19)	57	50				
PD-L1 pos & CD8+ hi pTu (n=12)	13	17	0.33 (0.12-0.90)	**p=0.031**	0.39 (0.16-0.96)	**p=0.042[5]**
PD-1 neg pTu (n=24)	46	50				
PD-1 pos pTu (n=30)	21	22	0.50 (0.25-0.99)	**p=0.047**	0.50 (0.25-1.01)	p=0.054[5]
PD-1 neg & CD8+ lo pTu (n=14)	n/a	61				
PD-1 pos & CD8+ hi pTu (n=18)	16	17	0.29 (0.11-0.74)	**p=0.010**	0.25 (0.09-0.73)	**p=0.011[5]**

OS overall survival, *Md* median, *mo* months, *yr* years, *HR* hazard ratio, *CI* confidence interval, *UICC* International Union against Cancer, *OAC* oesophageal adenocarcinoma, *AEG* adenocarcinoma of the esophagogastric junction, *comp.* compared, *TRG* tumour regression grade, *n.s.* not significant (a p-value less than 0.05 was considered to be significant), *n/a* not achieved, *Tx* therapy, *Tu* tumoural area, *pTu* peritumoural area, *dist* cell-to-cell distance, *TAM* tumour associated macrophages (CD68+ plus CD163+), *neg* negative, *pos* positive, *hi* high density, *lo* low density

[#] 2 of the total number of 3 patients with Siewert III had only R1-resection.

* Lower and upper quintile of underlying CD8+ density were excluded from analysis.

Results of univariate analysis (Kaplan-Meier analysis and logrank [Mantel-Cox] test) and of multivariate analysis (Cox regression)

Cox regression adjusted for: [1] surgery, [2] age and cN, [3] TRG, [4] resection quality, [5] cN

Table 5. Uni- and multivariate analysis of variables with possible impact on DFS

Patient variables	DFS					
			Univariate analysis		Multivariate analysis	
	Md (mo)	5 yr (%)	HR (95% CI)	p-value	HR (95% CI)	p-value
All patients (n=76)	17	24				
Gender						
Female (n=16)	11	18				
Male (n=60)	19	27	0.84 (0.42-1.67)	n.s.		
Age (median 65.35 yr)						
44.3 - <65.35 y (n=38)	15	34				
≥66.35 - 86.5 y (n=38)	19	11	1.24 (0.71-2.16)	p=0.446	1.14 (0.62-2.03)	p=0.693[1]
Clinical staging (UICC)						
II (n=4)	19	n/a		n.s. p=0.107		
III (n=70)	17	25		p=0.063		
IV (n=2)	3	0				
(cT)						
cT1 & cT2 (n=13)	19	34				
cT3 & cT4 (n=63)	17	23	1.14 (0.55-2.37)	n.s.		
(cN)						
cN0 (n=12)	n/a	51				
cN+ (n=64)	13	20	2.01 (1.02-3.98)	**p=0.045**	2.38 (0.95-6.02)	p=0.065[1]
Grading (biopsy)						
pG1 (n=3)	57	33				
pG2 (n=32)	20	34				
pG3 (n=41)	15	19		n.s.		
Localization				*comp. to AEG III*		
OAC without AEG (n=7)	15	21		**p=0.008**		n.s.[4]
AEG Siewert I (n=40)	16	26		**p=0.001**		n.s.[4]
AEG Siewert II (n=26)	21	27		**p<0.001**		n.s.[4]
AEG Siewert III (n=3)[#]	5	0		*all other n.s.*		
Surgery						
With surgery (n=58)	17	29				
Without surgery (n=18)	16	n/a	1.49 (0.75-2.99)	p=0.256	1.26 (0.64-2.46)	p=0.505[2]
Resection quality (n=58)						
R0 (n=51)	22	33				
R1 & R2 (n=7)	6	0	14.3 (3.37-60.5)	**P<0.001**	3.28 (1.29-8.31)	**p=0.012**[3]
TRG Mandard (n=58)						
1 & 2 & 3 (n=47)	21	35				
4 & 5 (n=11)	10	0	4.40 (1.59-12.2)	**p=0.004**	2.17 (0.98-4.83)	p=0.056[4]
1 & 2 (n=42)	21	33				
3 & 4 & 5 (n=16)	10	21	1.90 (0.86-4.20)	p=0.114		n.s.[4]

Pre-Tx immunologic parameters						
CD8+ low density Tu (n=36)	15	15				
CD8+ high density Tu (n=35)	20	30	0.68 (0.39-1.21)	p=0.189	0.74 (0.42-1.30)	p=0.296[5]
CD8+ low density pTu (n=28)	38	33				
CD8+ high density pTu (n=29)	13	22	2.01 (1.05-3.87)	**p=0.036**	2.09 (1.11-3.94)	**p=0.023[5]**
FoxP3+ low density Tu (n=36)	17	27				
FoxP3+ high density Tu (n=35)	20	18	1.11 (0.63-1.95)	p=0.723		n.s.[5]
FoxP3+/CD8+ low ratio Tu (n=36)	21	33				
FoxP3+/CD8+ high ratio Tu (n=35)	16	13	1.47 (0.83-2.59)	p=0.185	1.34 (0.76-2.37)	p=0.304[5]
FoxP3+ CD8+ short dist Tu (n=52)	16	16	1.49 (0.79-2.80)		1.47 (0.74-2.92)	
FoxP3+ CD8+ long dist Tu (n=17)	22	35	0.67 (0.36-1.27)	p=0.219	0.68 (0.34-1.35)	p=0.270[5]
FoxP3+ CD8+ short dist Tu* (n=30)	15	17	2.08 (0.95-4.55)		2.26 (0.91-5.63)	
FoxP3+ CD8+ long dist Tu* (n=12)	56	50	0.48 (0.22-1.06)	p=0.068	0.44 (0.18-1.10)	p=0.080[5]
CD68+ low density Tu (n=36)	16	21				
CD68+ high density Tu (n=35)	21	25	0.75 (0.42-1.32)	p=0.317	0.69 (0.39-1.20)	p=0.189[5]
TAM/CD8+ low ratio Tu (n=35)	21	33				
TAM/CD8+ high ratio Tu (n=35)	13	14	1.66 (0.93-2.95)	p=0.085	1.53 (0.87-2.71)	p=0.142[5]
PD-L1 neg Tu (n=30)	22	32				
PD-L1 pos Tu (n=39)	15	23	0.68 (0.38-1.20)	p=0.180		n.s.[5]
PD-L1 neg & CD8+ hi Tu (n=9)	n/a	25				
PD-L1 pos & CD8+ lo Tu (n=13)	12	8	0.34 (0.12-0.92)	**p=0.034**	0.51 (0.14-1.82)	p=0.298[5]
PD-1 neg Tu (n=31)	38	34				
PD-1 pos Tu (n=38)	13	23	0.58 (0.33-1.04)	p=0.066	0.60 (0.33-1.07)	p=0.083[5]
PD-1 neg & CD8+ hi Tu (n=11)	n/a	55				
PD-1 pos & CD8+ lo Tu (n=14)	9	7	0.31 (0.12-0.81)	**p=0.016**	0.26 (0.11-0.87)	**p=0.026[5]**
PD-L1 neg pTu (n=35)	38	34				
PD-L1 pos pTu (n=19)	15	21	0.57 (0.28-1.18)	p=0.130		n.s.
PD-L1 neg & CD8+ lo pTu (n=19)	49	37				
PD-L1 pos & CD8+ hi pTu (n=12)	12	17	0.34 (0.13-0.91)	**p=0.032**	0.40 (0.17-0.95)	**p=0.039[5]**
PD-1 neg pTu (n=24)	49	46				
PD-1 pos pTu (n=30)	30	16	0.50 (0.26-0.97)	**p=0.042**	0.50 (0.25-0.99)	**p=0.048[5]**
PD-1 neg & CD8+ lo pTu (n=14)	n/a	53				
PD-1 pos & CD8+ hi pTu (n=18)	13	19	0.31 (0.12-0.77)	**p=0.012**	0.31 (0.11-0.81)	**p=0.018[5]**

DFS disease free survival, *Md* median, *mo* months, *yr* years, *HR* hazard ratio, *CI* confidence interval, *UICC* International Union against Cancer, *OAC* oesophageal adenocarcinoma, *AEG* adenocarcinoma of the esophagogastric junction, *comp.* compared, *TRG* tumour regression grade, *n.s.* not significant (a p-value less than 0.05 was considered to be significant), *n/a* not achieved, *Tx* therapy, *Tu* tumoural area, *pTu* peritumoural area, *dist* cell-to-cell distance, *TAM* tumour associated macrophages (CD68+ plus CD163+), *neg* negative, *pos* positive, *hi* high density, *lo* low density

[#] 2 of the total number of 3 patients with Siewert III had only R1-resection.

* Lower and upper quintile of underlying CD8+ density were excluded from analysis.

Results of univariate analysis (Kaplan-Meier analysis and logrank [Mantel-Cox] test) and of multivariate analysis (Cox regression)

Cox regression adjusted for: [1] surgery, [2] age and cN, [3] TRG, [4] resection quality, [5] cN

Table 6. Uni- and multivariate analysis of variables with possible impact on NED

Patient variables	NED					
			Univariate analysis		Multivariate analysis	
	Md (mo)	5 yr (%)	HR (95% CI)	p-value	HR (95% CI)	p-value
All patients (n=76)	38	42				
Gender						
Female (n=16)	21	29				
Male (n=60)	56	46	0.63 (0.27-1.50)	n.s.		
Age (median 65.35 yr)						
44.3 - <65.35 y (n=38)	38	45				
≥66.35 - 86.5 y (n=38)	31	36	0.98 (0.49-1.96)	p=0.959	0.81 (0.39-1.72)	p=0.591[1]
Clinical staging (UICC)						
II (n=4)	19	n/a		n.s.	p=0.107	
III (n=70)	49	44			**p=0.011**	
IV (n=2)	3	0				
(cT)						
cT1 & cT2 (n=13)	n/a	59				
cT3 & cT4 (n=63)	31	39	1.23 (0.50-3.00)	n.s.		
(cN)						
cN0 (n=12)	n/a	57				
cN+ (n=64)	31	39	1.69 (0.72-4.00)	p=0.228	1.79 (0.63-5.10)	p=0.276[1]
Grading (biopsy)						
pG1 (n=3)	n/a	67				
pG2 (n=32)	n/a	51				
pG3 (n=41)	22	33		n.s.		
Localization				*comp. to AEG III*		
OAC without AEG (n=7)	15	25		**p=0.007**		n.s.[4]
AEG Siewert I (n=40)	56	45		**p=0.019**		n.s.[4]
AEG Siewert II (n=26)	38	48		**p=0.003**		n.s.[4]
AEG Siewert III (n=3)[#]	7	0		*all other n.s.*		
Surgery						
With surgery (n=58)	56	45				
Without surgery (n=18)	21	n/a	1.78 (0.76-4.20)	p=0.187	1.70 (0.74-3.89)	p=0.208[2]
Resection quality (n=58)						
R0 (n=51)	n/a	52				
R1 & R2 (n=7)	10	0	33.3 (5.05-219)	**p<0.001**	4.78 (1.50-15.2)	**p=0.008**[3]
TRG Mandard (n=58)						
1 & 2 & 3 (n=47)	22	34				
4 & 5 (n=11)	7	0	4.50 (1.21-16.7)	**p=0.003**	2.10 (0.75-5.90)	p=0.158[4]
1 & 2 (n=42)	22	32				
3 & 4 & 5 (n=16)	10	21	2.26 (0.82-6.21)	p=0.113		n.s.[4]

Pre-Tx immunologic parameters						
CD8+ low density Tu (n=36)	22	25				
CD8+ high density Tu (n=35)	n/a	51	0.68 (0.34-1.36)	p=0.276	0.72 (0.36-1.44)	p=0.351[5]
CD8+ low density pTu (n=28)	n/a	53				
CD8+ high density pTu (n=29)	20	32	2.33 (1.03-5.24)	**p=0.041**	2.39 (1.08-5.28)	**p=0.032[5]**
FoxP3+ low density Tu (n=36)	56	44				
FoxP3+ high density Tu (n=35)	22	35	1.08 (0.54-2.15)	p=0.835		n.s.[5]
FoxP3+/CD8+ low ratio Tu (n=36)	38	47				
FoxP3+/CD8+ high ratio Tu (n=35)	22	31	1.18 (0.59-2.36)	p=0.642		n.s.[5]
FoxP3+ CD8+ short dist Tu (n=52)	22	35	1.46 (0.66-3.20)		1.46 (0.62-3.42)	
FoxP3+ CD8+ long dist Tu (n=17)	56	50	0.69 (0.31-1.51)	p=0.347	0.69 (0.29-1.61)	p=0.388[5]
FoxP3+ CD8+ short dist Tu* (n=30)	38	37	1.63 (0.63-4.21)		1.67 (0.59-4.70)	
FoxP3+ CD8+ long dist Tu* (n=12)	n/a	55	0.61 (0.24-1.58)	p=0.309	0.60 (0.21-1.67)	p=0.326[5]
CD68+ low density Tu (n=36)	21	35				
CD68+ high density Tu (n=35)	56	45	0.67 (0.33-1.35)	p=0.260	0.63 (0.31-1.26)	p=0.190[5]
TAM/CD8+ low ratio Tu (n=35)	n/a	57				
TAM/CD8+ high ratio Tu (n=35)	22	22	1.88 (0.93-3.77)	p=0.077	2.77 (0.88-3.60)	p=0.112[5]
PD-L1 neg Tu (n=30)	38	39				
PD-L1 pos Tu (n=39)	39	21	0.86 (0.43-1.73)	p=0.680		n.s.[5]
PD-L1 neg & CD8+ hi Tu (n=9)	n/a	56				
PD-L1 pos & CD8+ lo Tu (n=13)	12	19	0.48 (0.15-1.56)	p=0.224		n.s.[5]
PD-1 neg Tu (n=31)	56	46				
PD-1 pos Tu (n=38)	20	34	0.57 (0.28-1.14)	p=0.110	0.57 (0.28-1.16)	p=0.120[5]
PD-1 neg & CD8+ hi Tu (n=11)	n/a	71				
PD-1 pos & CD8+ lo Tu (n=14)	13	22	0.28 (0.09-0.92)	**p=0.035**	0.27 (0.07-1.02)	p=0.053[5]
PD-L1 neg pTu (n=35)	49	44				
PD-L1 pos pTu (n=19)	22	46	0.77 (0.33-1.83)	p=0.560		n.s.
PD-L1 neg & CD8+ lo pTu (n=19)	56	50				
PD-L1 pos & CD8+ hi pTu (n=12)	15	28	0.35 (0.11-1.11)	p=0.076		n.s.
PD-1 neg pTu (n=24)	n/a	64				
PD-1 pos pTu (n=30)	21	29	0.41 (0.19-0.92)	**p=0.030**	0.39 (0.16-0.95)	**p=0.038[5]**
PD-1 neg & CD8+ lo pTu (n=14)	n/a	66				
PD-1 pos & CD8+ hi pTu (n=18)	19	21	0.25 (0.09-0.69)	**p=0.008**	0.23 (0.07-0.74)	**p=0.014[5]**

NED no evidence of disease, *Md* median, *mo* months, *yr* years, *HR* hazard ratio, *CI* confidence interval, *UICC* International Union against Cancer, *OAC* oesophageal adenocarcinoma, *AEG* adenocarcinoma of the esophagogastric junction, *comp.* compared, *TRG* tumour regression grade, *n.s.* not significant (a p-value less than 0.05 was considered to be significant), *n/a* not achieved, *Tx* therapy, *Tu* tumoural area, *pTu* peritumoural area, *dist* cell-to-cell distance, *TAM* tumour associated macrophages (CD68+ plus CD163+), *neg* negative, *pos* positive, *hi* high density, *lo* low density

[#] 2 of the total number of 3 patients with Siewert III had only R1-resection.

* Lower and upper quintile of underlying CD8+ density were excluded from analysis.

Results of univariate analysis (Kaplan-Meier analysis and logrank [Mantel-Cox] test) and of multivariate analysis (Cox regression)

Cox regression adjusted for: [1] surgery, [2] age and cN, [3] TRG, [4] resection quality, [5] cN

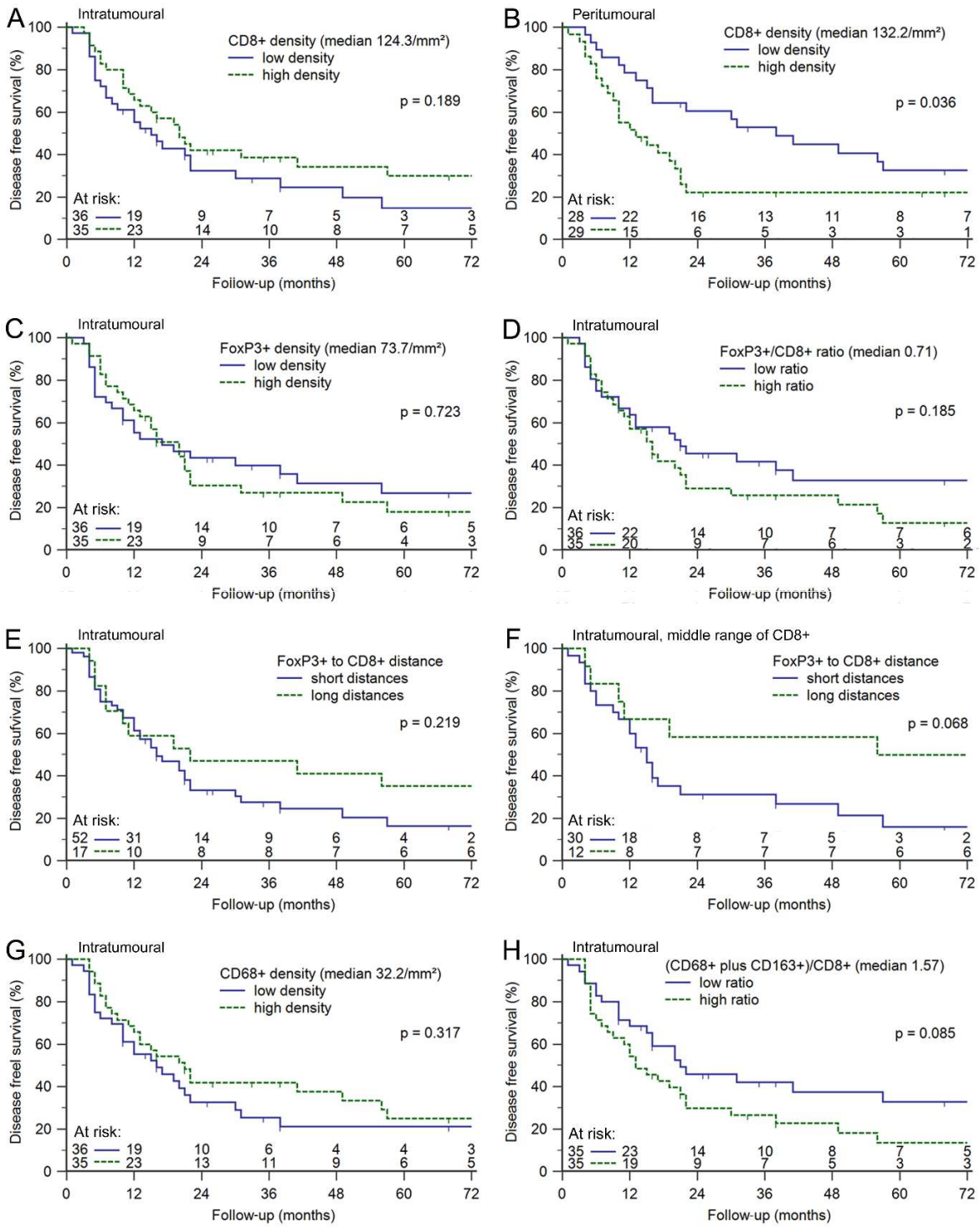


Figure 18. Influence of pretherapeutic immunologic parameters on DFS

A: Intratumoural CD8+ density, HR 0.68 (95% CI 0.39–1.21). **B:** Peritumoural CD8+ density, HR 2.01 (1.05–3.87). **C:** Intratumoural FoxP3+ density, HR 1.11 (0.63–1.95). **D:** Intratumoural FoxP3+/CD8+ ratio, HR 1.47 (0.83–2.59). **E:** Intratumoural FoxP3+ to CD8+ cell distance, HR 0.67 (0.36–1.27). **F:** Intratumoural FoxP3+ to CD8+ cell distance, lower and upper quintile of underlying CD8+ density excluded (≤53.5/mm^2 and ≥303/mm^2), HR 0.48 (0.22–1.06). **G:** Intratumoural CD68+ density, HR 0.75 (0.42–1.32). **H:** Intratumoural (CD68+ plus CD163+)/CD8+ ratio, HR 1.66 (0.93–2.95).

Results of logrank test

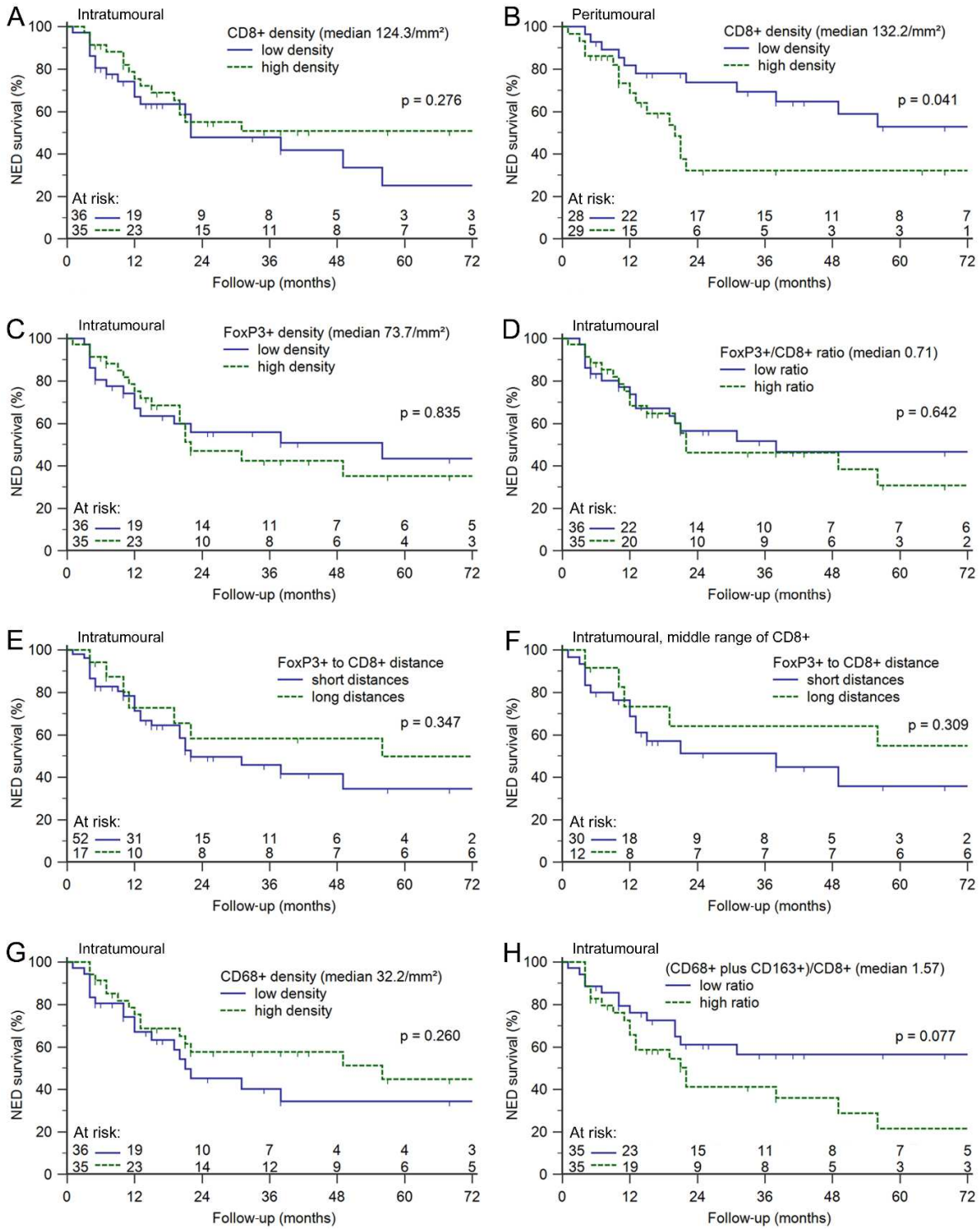


Figure 19. Influence of pretherapeutic immunologic parameters on NED survival

A: Intratumoural CD8+ density, HR 0.68 (95% CI 0.34–1.36). **B:** Peritumoural CD8+ density, HR 2.33 (1.03–5.24). **C:** Intratumoural FoxP3+ density, HR 1.08 (0.54–2.15). **D:** Intratumoural FoxP3+/CD8+ ratio, HR 1.18 (0.59–2.36). **E:** Intratumoural FoxP3+ to CD8+ cell distance, HR 0.69 (0.31–1.51). **F:** Intratumoural FoxP3+ to CD8+ cell distance, lower and upper quintile of underlying CD8+ density excluded (≤53.5/mm^2 and ≥303/mm^2), HR 0.61 (0.24–1.58). **G:** Intratumoural CD68+ density, HR 0.67 (0.33–1.35). **H:** Intratumoural (CD68+ plus CD163+)/CD8+ ratio, HR 1.88 (0.93–3.77).

Results of logrank test

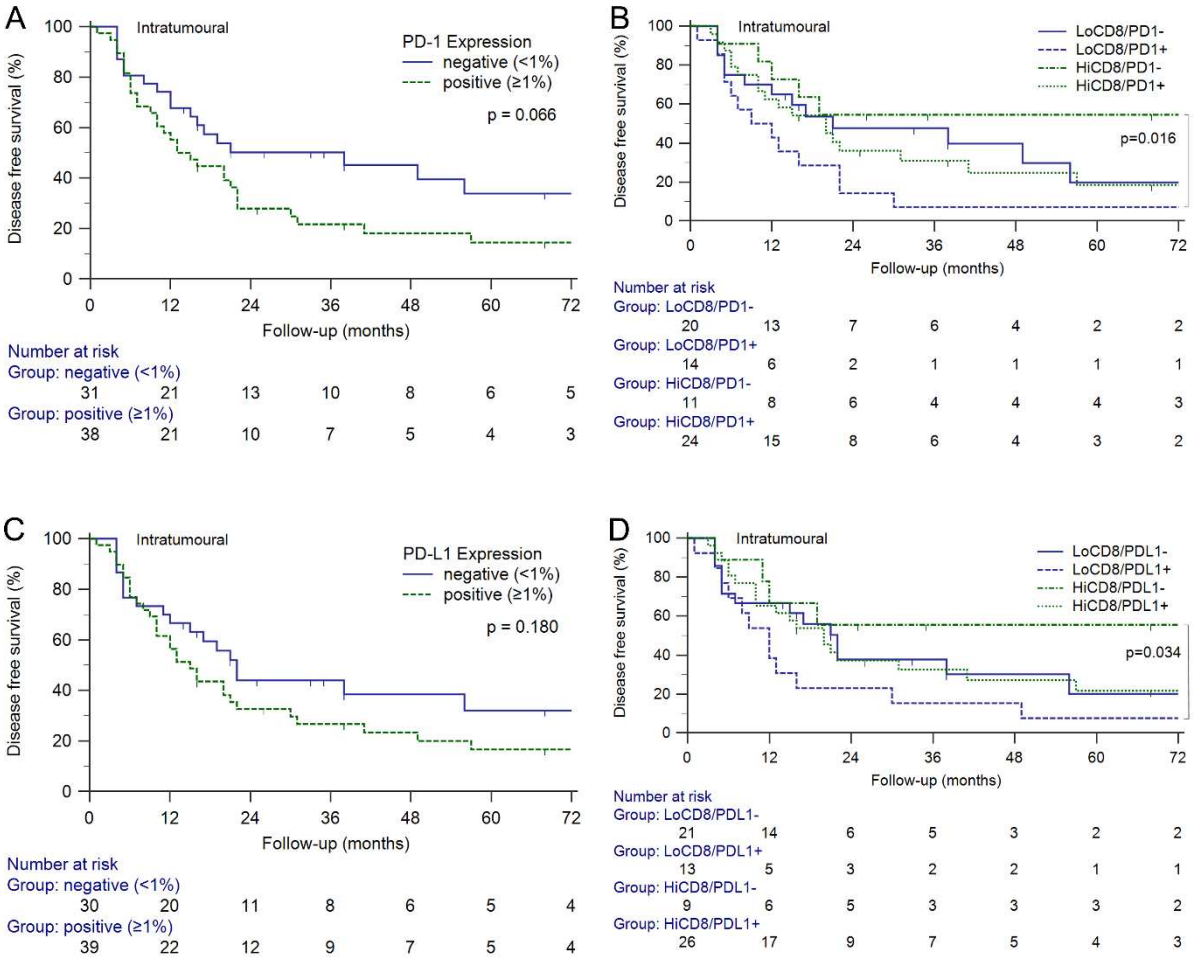


Figure 20. Influence of PD-1 and PD-L1 expression in tumoural area on disease free survival

A: PD-1 expression, HR 0.58 (95% CI 0.33–1.04). **B:** PD-1 expression combined with CD8+ density, LoCD8/PD1+ compared to HiCD8/PD1-: HR 0.31 (0.12–0.81). **C:** PD-L1 expression, HR 0.68 (95% CI 0.38–1.20). **D:** PD-L1 expression combined with CD8+ density, LoCD8/PDL1+ compared to HiCD8/PDL1-: HR 0.34 (0.12–0.92).

Results of logrank test

HiCD8/LoCD8 high/low CD8+ density (median 124.3/mm^2), *PD1-/PD1+* negative/positive PD-1 expression, *PDL1-/PDL1+* negative/positive PD-L1 expression (threshold 1%)

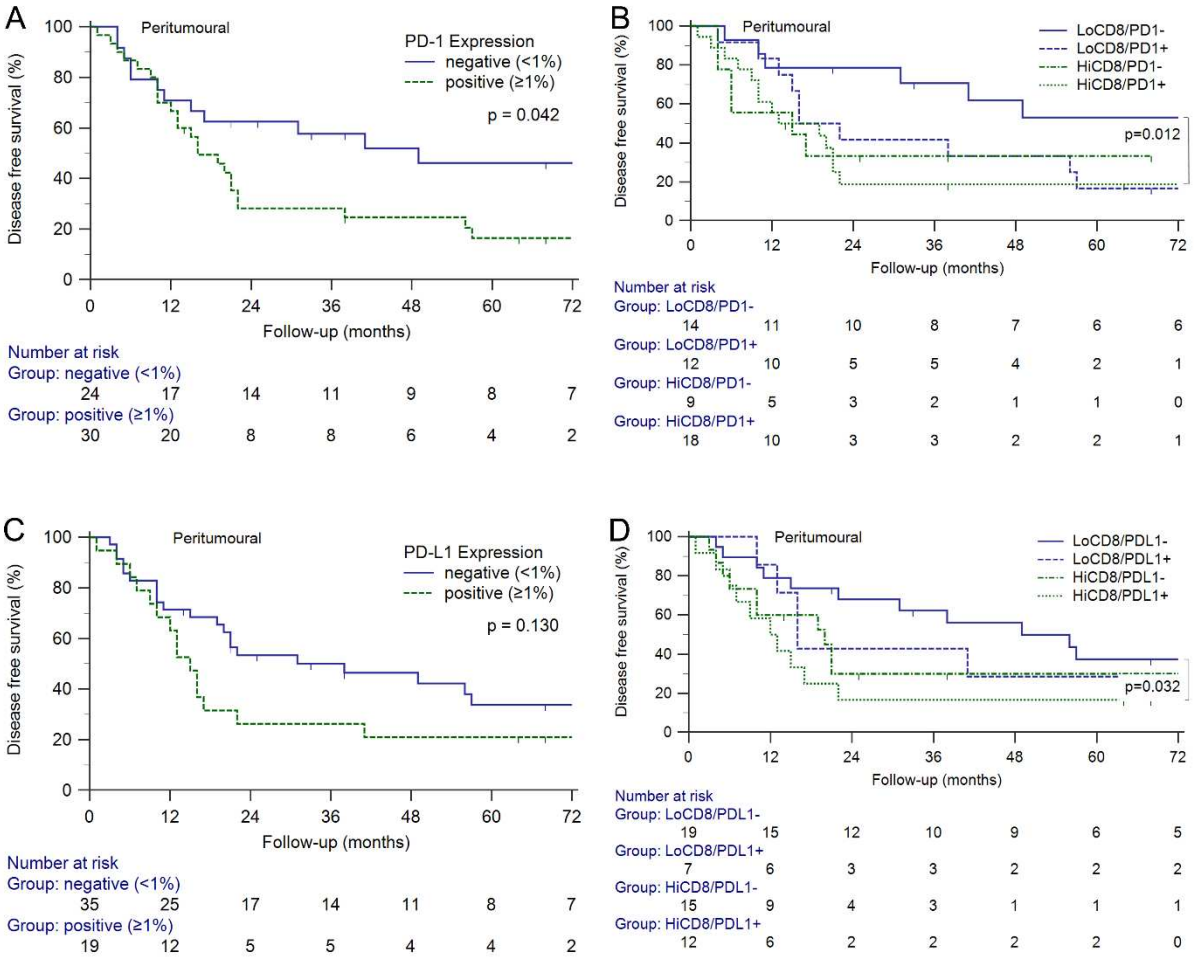


Figure 21. Influence of PD-1 and PD-L1 expression in peritumoural area on disease free survival

A: PD-1 expression, HR 0.50 (95% CI 0.26–0.97). **B:** PD-1 expression combined with CD8+ density, HiCD8/PD1+ compared to LoCD8/PD1-: HR 0.31 (0.12–0.77). **C:** PD-L1 expression, HR 0.57 (95% CI 0.28–1.18). **D:** PD-L1 expression combined with CD8+ density, HiCD8/PDL1+ compared to LoCD8/PDL1-: HR 0.34 (0.13–0.91).

Results of logrank test

HiCD8/LoCD8 high/low CD8+ density (median 132.2/mm^2), *PD1-/PD1+* negative/positive PD-1 expression, *PDL1-/PDL1+* negative/positive PD-L1 expression (threshold 1%)

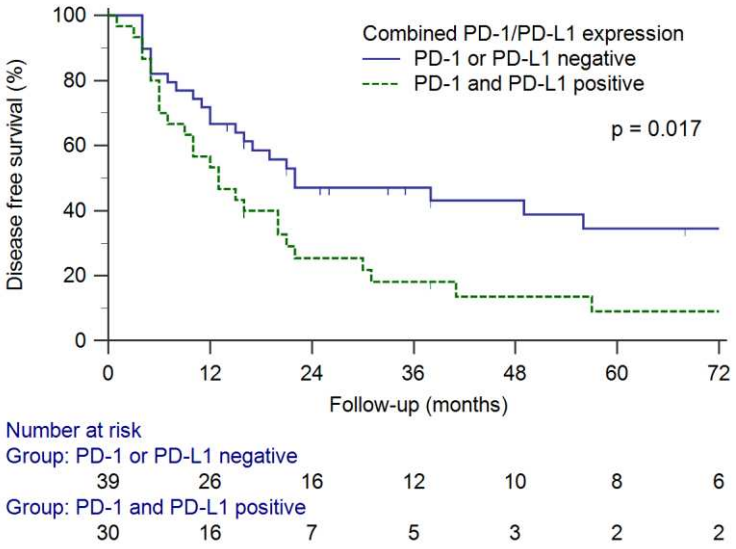


Figure 22. Influence of combined PD-1 and PD-L1 expression in tumoural area on disease free survival

PD-1 or PD-L1 negative compared to PD-1 and PD-L1 positive: HR 0.48 (0.27–0.88).

Results of logrank test, threshold of positive expression ≥1%

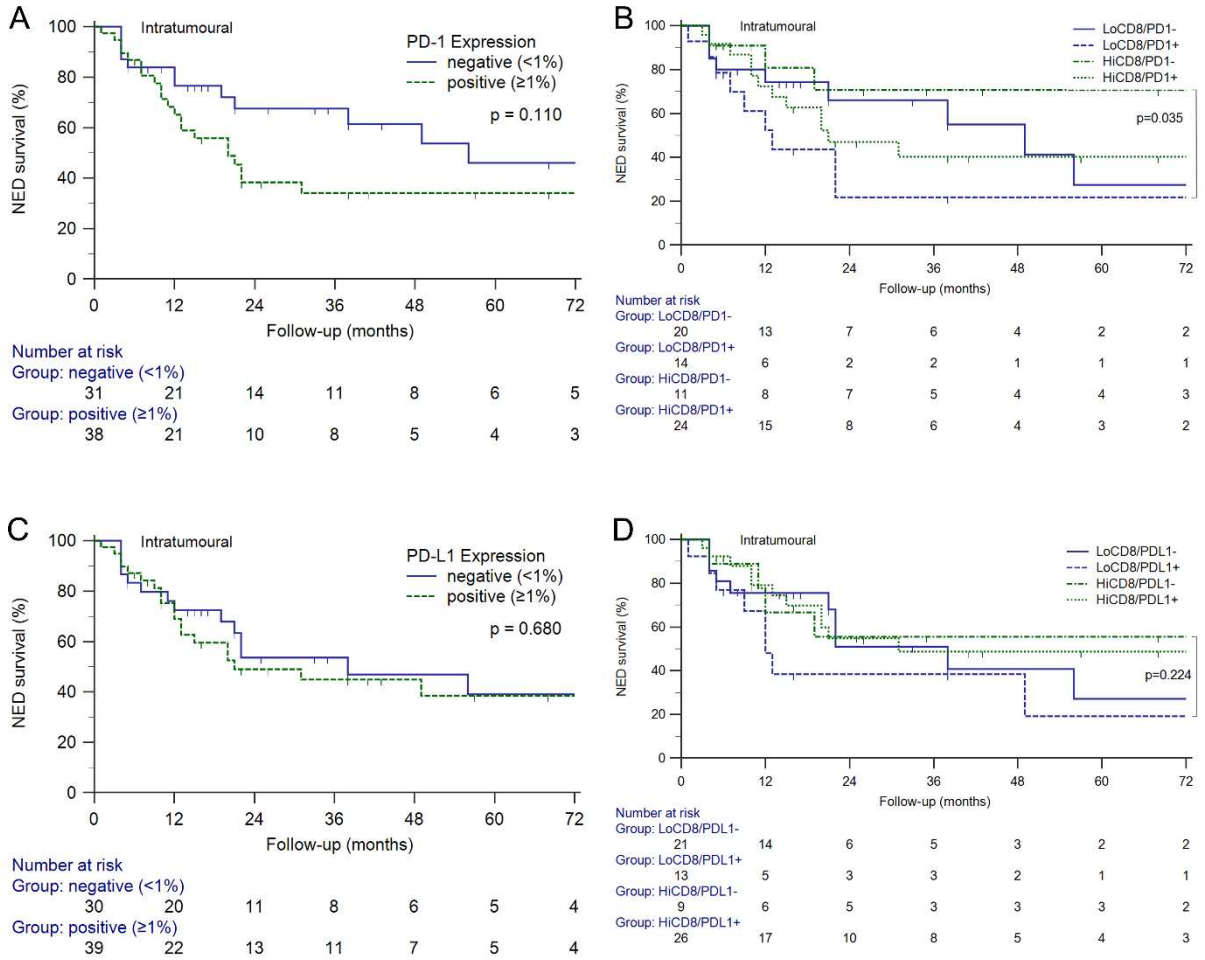


Figure 23. Influence of PD-1 and PD-L1 expression in tumoural area on NED survival

A: PD-1 expression, HR 0.57 (95% CI 0.28–1.14). **B:** PD-1 expression combined with CD8+ density, LoCD8/PD1+ compared to HiCD8/PD1-: HR 0.28 (0.09–0.92). **C:** PD-L1 expression, HR 0.86 (95% CI 0.43–1.73). **D:** PD-L1 expression combined with CD8+ density, LoCD8/PDL1+ compared to HiCD8/PDL1-: HR 0.48 (0.15–1.56).

Results of logrank test

NED No evidence of disease, *HiCD8/LoCD8* high/low CD8+ density (median 124.3/mm^2), *PD1-/PD1+* negative/positive PD-1 expression, *PDL1-/PDL1+* negative/positive PD-L1 expression (threshold 1%)

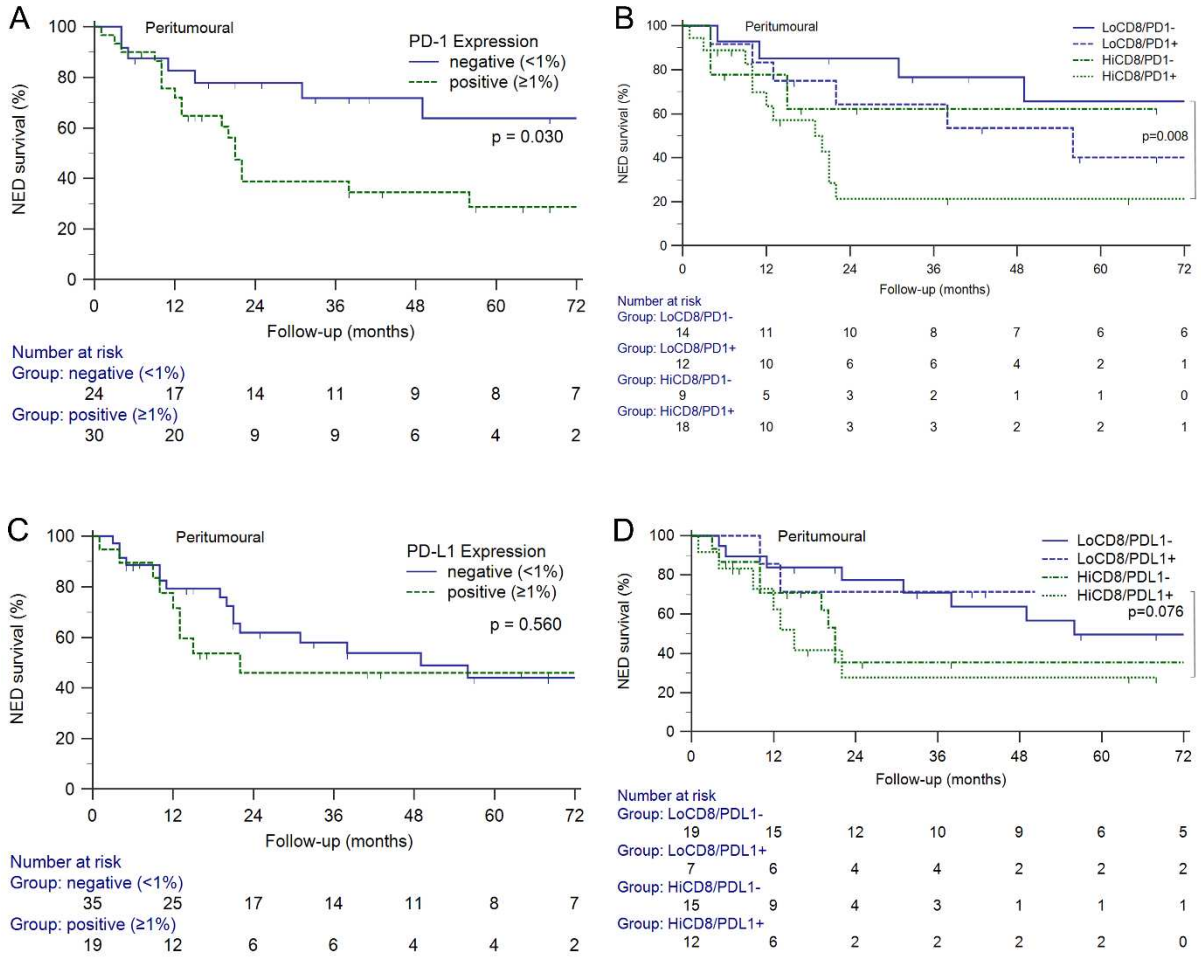


Figure 24. Influence of PD-1 and PD-L1 expression in peritumoural area on NED survival

A: PD-1 expression, HR 0.41 (95% CI 0.19–0.92). **B:** PD-1 expression combined with CD8+ density, HiCD8/PD1+ compared to LoCD8/PD1-: HR 0.25 (0.09–0.69). **C:** PD-L1 expression, HR 0.77 (95% CI 0.33–1.83). **D:** PD-L1 expression combined with CD8+ density, HiCD8/PDL1+ compared to LoCD8/PDL1-: HR 0.35 (0.11–1.11).

Results of logrank test

NED No evidence of disease, *HiCD8/LoCD8* high/low CD8+ density (median 132.2/mm^2), *PD1-/PD1+* negative/positive PD-1 expression, *PDL1-/PDL1+* negative/positive PD-L1 expression (threshold 1%)

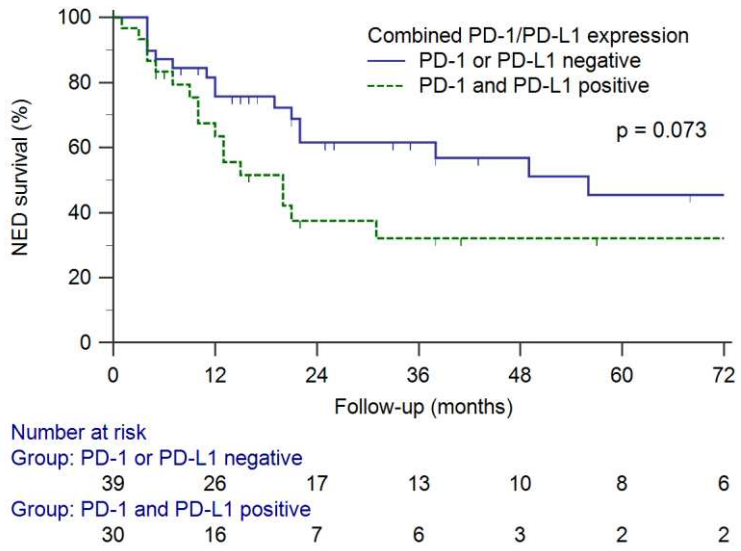


Figure 25. Influence of combined PD-1 and PD-L1 expression in tumoural area on NED survival

PD-1 or PD-L1 negative compared to PD-1 and PD-L1 positive: HR 0.52 (0.25–1.06).

Results of logrank test, threshold of positive expression ≥1%

NED no evidence of disease

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Biographical note

Personal Information	
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Date of Birth:	January 25th 1959
Place of Birth:	Coburg, Germany
Marital status:	Married, 3 children
Nationality:	German
Religion:	Roman catholic

School Education	
1969 – 1978	Gymnasium Ernestinum in Coburg
1978	German University entrance qualification examination (Abitur); grade point average 1.1

University Education	
May 1985	Promotion (M.D.) magna cum laude
October 1978 – October 1984	Human Medicine at the University of Erlangen, Germany
September 1980	First Section of the Physician's Examination,
September 1983	Second Section of the Physician's Examination
October 1983 - September 1984	Final Year Electives Gynaecology and Obstetrics, Staatl. Frauenklinik Bamberg Surgery, Klinikum Bamberg Internal Medicine, Klinikum Bamberg
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Work Experience	
Since March 2020	Medical director of Regiomed Klinikum Lichtenfels
May 2009	Medical specialist in Medical Treatment in Oncology
Since January 2003	Head physician, Department of Gastroenterology, REGIOMED Klinikum Lichtenfels

October 1998 to December 2002	Head physician, Department of Internal Medicine, Klinik Bad Windsheim
October 1998	Medical specialist in Diabetology
January 1994	Medical specialist in Gastroenterology with Radiology and Sonography
March 1993 to September 1998	Senior physician, Department of Gastroenterology, Klinikum Coburg
January 1992	Medical specialist in Internal Medicine with Radiology
April 1985 to February 1993	Assistant physician, Department of Internal Medicine, Klinikum Coburg

Languages	
German	Mother tongue
English	Oral and written
Italian	Basic
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