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Characterization and dosimetric predictors for absolute lymphocyte count changes during neoadjuvant chemoradiotherapy with or without pembrolizumab for esophageal squamous cell carcinoma: an analysis of a prospective cohort

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Abstract

Aim To characterize the differences of dynamic changes for absolute lymphocyte count (ALC) among esophageal squamous cell carcinoma (ESCC) patients treated with neoadjuvant chemoradiotherapy (nCRT) with or without pembrolizumab, as well as to investigate the clinical and lymphocyte-related organs dosimetric parameters that would impact ALC nadir during nCRT.

Materials and methods A total of 216 ESCC patients who received nCRT (with pembrolizumab 144; without pembrolizumab: 72) were identified from a prospective cohort. Weekly and 1-month post-nCRT ALC were identified. lymphocyte-related organs at risk (LOARs) were delineated. linear and logistic regression analysis was used to analyze the association between G4 lymphopenia/lymphopenia nadir and clinical/DVHs factors. Receiver-operating characteristic curves were used to derive optimal dosimetric planning constraints. Grade 4 (G4) lymphopenia was defined as ALC $< 0.2 \times 10^9$/L during nCRT.

Results G4 lymphopenia was observed in 35 ESCC patients (16.2%) during neoadjuvant treatment. Compared to nCRT alone, the addition of pembrolizumab to nCRT significantly improve lymphopenia recovery in the 1-months

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after nCRT ($p=0.0003$), but the ALC at other time point during nCRT and ALC nadir was comparable between the two groups. A total of 198 patients finally received surgery. Of them, 98 patients archived pCR (49.5%), with 50.4% (68/135 patients) in nCRT with pembrolizumab and 47.6% (30/63) in nCRT alone($p=0.94$), respectively. The mean ALC nadir in the pCR group was significantly higher than those without ($p=0.0003$). Multivariable linear and logistic regression analysis indicated that TVB mean dose, TVB V5, TVB V10, TVB V20, mean cardiopulmonary dose, mean ribs dose, mean whole body dose, mean spleen dose, V5, V10, and V20 of spleen dose were significantly associated with developing grade 4 lymphopenia. Dosimetric analysis showed that lymphocyte-sparing photon or proton irradiation was feasible while did not compromise clinically acceptable objectives.

Conclusion The addition of pembrolizumab to nCRT improved lymphopenia recovery for ESCC after trimodality therapy. ALC nadir was significantly associated with pCR and RFS after nCRT. Sparing of LOARs using advanced radiation techniques might reduce the risk of developing lymphopenia and improve treatment response in the era of immunotherapy.

Keywords Lymphocyte-related organs at risk, Neoadjuvant chemoradiotherapy, Esophageal squamous cell carcinoma, Pembrolizumab

Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive cancers and surgery remains the main treatment for locally advanced ESCC [1]. However, surgery alone is usually accompanied by high recurrence, with a 5-year survival of 20% [2, 3]. Recently, long-term evidence from two large phase III studies of CROSS trial [4] and NEOCRTEC5010 trial [5] has indicated that survival of ESCC patients would benefit from neoadjuvant chemoradiotherapy(nCRT). Therefore, the standardized treatment approach for locally advanced carcinoma of esophagus and gastro-esophageal junction is a trimodality therapy consisting of nCRT followed by esophagectomy. Despite the survival rate of esophageal cancer has been substantially improved with the wide application of nCRT, approximately half of these patients would experience local regional recurrence or distant metastasis [5]. In order to reshape the landscape of local advanced esophageal cancer (LAEC) management, multiple researches have been successively launched in both neoadjuvant and adjuvant settings. Emerging evidence from these studies suggests the possibility of an improved pathological complete response (pCR) and overall survival rates obtained from the combined immune checkpoint inhibitors (ICIs) for LAEC. Until now, ICIs combined with chemotherapy has become the optimal first-line treatment for LAEC.

Since more and more evidence regarding the negative impact of lymphopenia on multiple cancer outcomes is increasing, radiotherapy-induced lymphopenia receives great attention for oncologists [6–10]. Prior to the immunotherapy era, multiple studies have been conducted to investigate the relationship between dose-volume to lymphocyte-related organs at risk (LOARs) and lymphopenia in ESCC patients. Fang P. et al. [11] demonstrated that a higher absolute lymphocyte count (ALC) level during nCRT was associated with a higher rate of pCR for esophageal cancer patients undergoing trimodality therapy. More recently, Tseng I. et al. [12] found that less irradiation to spleen and bone marrow plus lower estimated radiation doses to immune cells (EDIC) were jointly prone to reduce the incidence of G4 lymphopenia during definitive concurrent chemoradiotherapy for ESCC patients. In the era of immunotherapy, Cheng X. et al. [13] performed a retrospective study and found that the addition of ICIs to definitive chemoradiotherapy could mitigate the decline of ALC during radiotherapy and might prolong survival. In addition, the authors demonstrated that low ALC remained a significant prognostic factor for progression-free survival of ESCC. Nevertheless, few studies have been performed to compare the difference of dynamic ALC changes during nCRT with or without pembrolizumab, and the impact of ALC nadirs on pCR and survival of ESCC patients in the neoadjuvant settings. Moreover, there is still lack of high-quality evidence for dosimetric parameters influenced grade 4 (G4) lymphopenia during nCRT. Based on a prospective cohort, we conduct this study to characterize the changes of ALC during nCRT with or without pembrolizumab in ESCC patients. We aim to establish LOARs dose constraints for lymphocyte-sparing treatment in thoracic irradiation to reduced radiation-induced lymphopenia risks and maximize the immunomodulatory role of radiotherapy for ESCC in the era of immunotherapy.

Materials and methods

Patient selection

The study was designed as a prospective cohort from four prospective trials (NCT NCT03792347, NCT04435197, NCT04513418, NCT03990532) [14–16]. Medical records of ESCC patients who received nCRT with or without surgery between January 2019 and December 2023 form the prospective cohorts were retrospectively reviewed.

Data regarding surgical procedures, neoadjuvant therapy, and potential confounding clinical and demographic

data (sex, age, body mass index, medical history, smoking status, alcohol use, location of tumor, primary tumor length, radiotherapy modality, clinical TNM stage were manually extracted.

According to the treatment protocol, patients who met the following criteria were included in this analysis: (1) pathologically confirmed as stage II–IVa ESCC according to the eighth TNM staging system defined by the American Joint Committee on Cancer; (2) received nCRT combined with or without pembrolizumab of a total radiation dose 41.4 Gy, and concurrent chemotherapy consisting of weekly carboplatin (area under the curve of 2 mg/mL per min) and paclitaxel/nab-paclitaxel (50 mg/m2 of body surface area); and (3) had at least 3 weekly blood tests results available during nCRT and 1 month after nCRT. According to the trial protocol, all enrolled patients would receive two cycles of pembrolizumab on days 1 and 22 of the neoadjuvant therapy at a dose of 200 mg, which had been reported in our previous studies [17, 18]. Radiation planning was performed on the Eclipse Treatment Planning System 16.1 (Varian Medical Systems, Palo Alto, CA), patients were treated with intensity-modulated radiotherapy (IMRT) or Volumetric Modulated Arc Therapy (VMRT) utilizing 6-MV photons. The Institutional Review Board of our center approved this study, and informed consent obtained from all enrolled patients. Physical examination, standard blood tests, esophagogastroduodenoscopy (EGD) with endoscopic ultrasound and biopsies, X-ray esophagography, chest/abdominal computed tomography (CT), and/or positron emission tomography (PET) were completed in all patients prior to treatment.

ALC data

ALCs value were collected in one week before nCRT and weekly during nCRT and 1 month after completion of nCRT. According to CTCAE version 5.0, lymphopenia was defined as ALC less than 1.1×109 /L(lower limit of normal of ALC at our institution). The degree of lymphopenia is divided into 0–4 levels as follows:0 ($\geq 1.1 \times 10^9$ /L); 1 ($< 1.1 \sim 0.8 \times 10^9$ 2 (< 0.8–0.5×10^9/L), 3 (< 0.5–0.2×10^9/L), and 4 ($< 0.2 \times 10^9$/L). The lowest ALCs during nCRT were also identified for analysis. Normalized ALC were calculated by dividing the weekly lab value over the baseline pre-nCRT value.

Lymphocyte-related organs at risk contouring

The lymphocyte-related organs at risk (LOARs) can be separated into organs with abundant bloodstreams like heart, lung, and blood vessels and lymphoid structures and hematopoietic tissues, such as lymphatic vessels and lymph nodes as well as spleen and bone marrow [19]. For assessing the thoracic bone marrow, the thoracic vertebrae body (TVB) from T1 to T12, thoracic ribs (ribs 1–12), and sternum were contoured on planning computed tomography simulation scans. All LOARs were manually contoured by four physicians (C. Zhao, H.Li, X.Han and X.Li) and validated by two senior physicians (W.X Qi and S. Zhao). The DVH data were collected for TVB10-V40 (TVB V10-40), Ribs V10-V40 (RV10-40), and Sternum V10-V40 (SV10-40), in increments of 10%. The TVB V5, RV5, SV5, mean vertebral dose (MVD), mean rib dose (MRD), and mean sternum dose (MSD) were also collected. A DVH parameter of Vx was defined as the percentage of organ receiving x Gy of radiation. Mean cardiopulmonary dose (mCPD) and V5 of mCPD was calculated as mean dose to the volumetric sum of heart, lungs, entire aortic arch and Thoracic aorta (excluded marrow), as a surrogate for circulating blood pool (Fig. 1). The DVH data were collected for mCPD for V10-40 in increments of 10% were also collected. Volume of spleen receiving 5–40 Gy (SV5-40) was determined by DVH analysis.

Thoracic radiation treatment planning comparison

For each patient, we retrospectively replanned a new lymphocyte-sparing thoracic radiation plan and a new intensity modulated proton therapy (IMPT) plan. Target volume coverage constraints and dose constraints of LOARs and conventional ORAs are summarized in

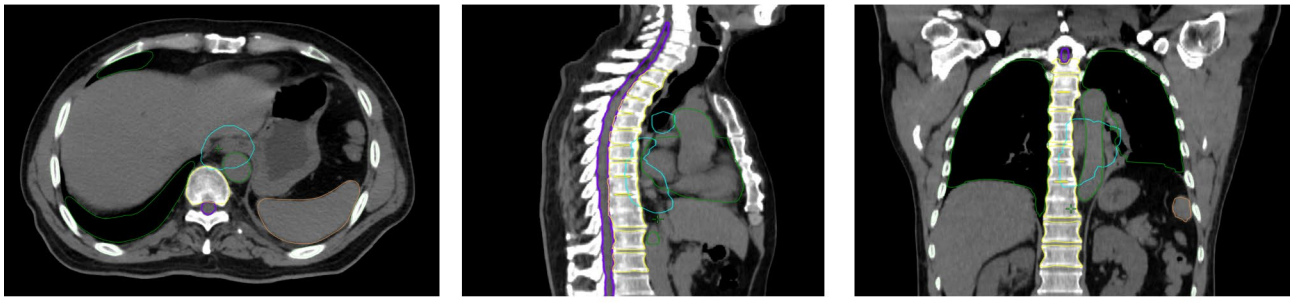

Fig. 1 Typical case for LOARs contouring. light blue: PTV; yellow: thoracic vertebrae body; Orange: spleen; light green: ribs; dark green: Mean cardiopulmonary dose; Purple: spinal cord; **A:** view of transverse plane; **B:** view of sagittal plane; **C:** view of coronal plane

Supplemental Table 5. A total of 41.4 Gy was prescribed in 23 fractions to at least 95% of the PTV for IMRT plan. The IMRT planning systems used was Eclipse (Varian Medical Systems, Palo Alto, CA). The IMPT plans were prescribed to 95% CTV with an RBE = 1.1. The IMPT plan was calculated by using Monte Carlo dose calculation algorithm to robustly optimize to the CTVs in RayStation treatment planning system (TPS) employing the APQ-SAPT-PS-01 (APACTRON particle equipment company, Shanghai, China) pencil beam scanning machine beam model.

Statistical analysis

Continuous variables were summarized by median and range, and categorical variables were expressed by frequency and proportion. Fisher's test, Student's t test, and Mann-Whitney U test were used to compare the distribution of clinicopathological and dosimetric features between the groups. Spearman analysis was used to assess the correlations between dosimetric parameters and lymphocytes at nadir. The Shapiro-Wilks test was performed to assess for normality of ALC values, and non-normally distributed values were log transformed.

Dose-volume histogram (DVH) parameters for the thoracic vertebrae body (TVB, mean dose, V5-V40), sternum (mean dose, V5-V40), cardiopulmonary (mean dose), spleen (mean dose, V5-V40), and ribs (mean dose, V5-V40) were assessed for associations with changes in ALC during the course of nCRT. Multivariable linear and logistic regression models controlling for age (continuous variable), body mass index (continuous variable), nCRT regimen (with pembrolizumab vs. without pembrolizumab), sex (female vs. male) and tumor length (continuous variable) were used to investigate the effect of dosimetric parameters on risk of G4 lymphopenia and lnALC nadir. For the dosimetric parameters that were significantly related to G4 lymphopenia, receiver operating characteristics (ROC) analysis was performed to identify ideal cutoff values in which equal weight was given to sensitivity and specificity. For comparisons in clinical target volume (CTV), PTV and LOARs doses were also conducted using paired sample sign test because data were not normally distributed. All statistical tests were performed using R version 3.6.1 software (The R Foundation for Statistical Computing, Vienna, Austria; http://www.r-project.org), and p value < 0.05 was considered as significant.

Results

Patient and treatment characteristics

A total of 216 eligible patients were enrolled in this study, including 144 treated with nCRT with pembrolizumab and 72 with nCRT alone. Among the all cohort, the mean age was 64.2 ± 7.6 years (ranging from 39.0 to 79.0 years), with 35 female and 181 male patients. There were 5 (2.3%) patients at stage II, 163 (75.5%) at stages IIIA–IIIB, and 48 (22.2%) at stage IVA. Most of whom (57.9%) had tumors in the middle segment of the esophagus. The mean tumor length was 4.6 ± 2.4 cm (ranging from 1.0 to 17.0). All of 216 completed 41.4 Gy radiation course, and 4 patients interrupted concurrent chemotherapy due to toxicities. Table 1 provided a summary of the patients and treatment characteristics. In addition, the baseline characteristics and dosimetric parameters between nCRT and nCRT with pembrolizumab were comparable ($p > 0.05$).

Dosimetric parameters

The mean values of the dosimetric parameters of LOARs were summarized in Table 1. The mean dose of CPD, TVB, ribs and sternum were 1026.7 cGy, 1841.8 cGy, 720.5 cGy and 1611.5 cGy. The mean V5 of CPD, TVB, ribs and sternum were 59.2%, 70.8%, 38.3% and 85.8% respectively. The V30 or V40 of TVB were higher than those of CPD, ribs and sternum, most likely because of its adjacent to the GTV.

Dynamic changes of ALC during nCRT with or without pembrolizumab

ALC steadily declined to nadir at week 5 throughout nCRT, and then quickly recovered (supplemental Fig. 1). We then divided the patients into two cohort according to the type of nCRT regimen, and found that the addition of pembrolizumab to nCRT significantly improved ALC and normalized ALC value recovery in the 1-months after nCRT (Fig. 2, $p = 0.0003$), while the ALC at other time point during nCRT and ALC nadir was comparable between the two groups (supplemental Fig. 2).

Correlation between ALC nadir and pCR

Of the 216 enrolled patients treated with nCRT with or without pembrolizumab, a total of 188 patients were finally received surgery. Among them, 86 patients archived pCR (45.7%). For 127 patients treated with nCRT with pembrolizumab and surgery, 60 patients (47.2%) archived pCR. For 61 patients treated with nCRT and surgery, 26 patients (42.6%) archived pCR. There was no significant difference of pCR between the two groups ($p = 0.71$). The mean ALC nadir in the pCR group was significantly higher than those without pembrolizumab ($p = 0.035$, Fig. 3).

We then explore the correlation between G4 lymphopenia and neoadjuvant treatment response. As for pCR, there was no significantly difference between G4 lymphopenia and G0-3 lymphopenia group (45.2% vs. 45.9%, $p = 0.90$). However, rate of ESCC patients archiving TRG 0–1 was significantly lower in G4 lymphopenia than

Table 1 Baseline characteristics and dosimetric comparison between nCRT with or without pembrolizumab among ESCC patients

name	levels	Overall cohort(*N* = 216)	without pembrolizumab (*N* = 72)	with pembrolizumab (*N* = 144)	*p*
age	Mean ± SD	64.2 ± 7.6	64.3 ± 8.1	64.1 ± 7.4	0.84
sex	female	35(16.2%)	13 (18.1%)	22 (15.3%)	0.74
	male	181(83.8%)	59 (81.9%)	122 (84.7%)	
BMI	Mean ± SD	22.5 ± 2.8	22.4 ± 2.9	22.5 ± 2.8	0.88
T stage	T2	45(20.8%)	19(26.4%)	26(18.1%)	0.36
	T3	165(76.4%)	51(70.8%)	114(79.2%)	
	T4	6(2.8%)	2(2.8%)	4(2.8%)	
N stage	N0	4(1.9%)	0(0%)	4(2.8%)	0.47
	N1	73(33.8%)	24(33.3%)	49(34%)	
	N2	92(42.6%)	30(41.7%)	62(43.1%)	
	N3	47(21.8%)	18(25%)	29(20.1%)	
Tumor location	Upper	11(5.1%)	3 (4.2%)	8 (5.6%)	0.09
	middle	125(57.9%)	35 (48.6%)	90 (62.5%)	
	Distal/GEJ	70(32.4%)	34 (47.2%)	46 (32.0%)	
Stage	IIA	5 (2.3%)	0(0%)	5(3.5%)	0.21
	IIIA	8 (3.7%)	1(1.4%)	7(4.9%)	
	IIIB	155 (71.8%)	53(73.6%)	102(70.8%)	
	IVA	48 (22.2%)	18(25%)	30(20.8%)	
Primary tumor length (cm)	Mean ± SD	4.6 ± 2.4	4.6 ± 2.2	4.6 ± 2.5	0.91
mCPD, cGy	Mean ± SD	1026.7 ± 277.4	1031.9 ± 231.6	1024.1 ± 298.4	0.83
V5	Mean ± SD	59.2 ± 15.3	60.1 ± 15.9	58.8 ± 15.0	0.56
V10	Mean ± SD	42.8 ± 12.5	43.1 ± 12.8	42.6 ± 12.4	0.79
V20	Mean ± SD	26.3 ± 14.3	26.7 ± 18.2	26.1 ± 11.9	0.83
V30	Mean ± SD	16.0 ± 11.1	15.0 ± 10.9	16.5 ± 11.2	0.35
V40	Mean ± SD	9.3 ± 9.7	8.8 ± 9.7	9.6 ± 9.7	0.57
Mean TVB dose, cGy	Mean ± SD	1841.8 ± 510.8	1835.6 ± 474.5	1845.0 ± 529.5	0.90
TVB V5	Mean ± SD	70.8 ± 17.6	70.3 ± 17.2	71.0 ± 17.8	0.77
TVB V10	Mean ± SD	66.1 ± 16.7	66.0 ± 16.4	66.1 ± 16.9	0.94
TVB V20	Mean ± SD	55.8 ± 16.9	55.2 ± 16.4	56.1 ± 17.2	0.72
TVB V30	Mean ± SD	39.4 ± 20.4	38.1 ± 21.9	40.0 ± 19.6	0.54
TVB V40	Mean ± SD	24.1 ± 24.4	23.5 ± 25.9	24.4 ± 23.7	0.79
Mean ribs dose, cGy	Mean ± SD	720.5 ± 198.1	739.5 ± 185.8	710.9 ± 203.9	0.32
RV5	Mean ± SD	38.3 ± 10.1	39.8 ± 11.7	37.5 ± 9.1	0.15
RV10	Mean ± SD	29.7 ± 7.7	30.4 ± 8.2	29.4 ± 7.4	0.37
RV20	Mean ± SD	18.9 ± 8.1	19.0 ± 8.7	18.8 ± 7.8	0.82
RV30	Mean ± SD	11.0 ± 9.1	10.8 ± 9.6	11.1 ± 8.8	0.85
RV40	Mean ± SD	6.2 ± 9.6	5.8 ± 8.5	6.4 ± 10.0	0.65
Mean Sternum dose, cGy	Mean ± SD	1611.5 ± 637.8	1643.2 ± 670.8	1595.6 ± 622.5	0.61
SV5	Mean ± SD	85.8 ± 19.4	85.1 ± 19.5	86.2 ± 19.4	0.69
SV10	Mean ± SD	73.7 ± 25.1	73.2 ± 26.2	74.0 ± 24.6	0.83
SV20	Mean ± SD	49.0 ± 26.6	46.9 ± 26.7	50.1 ± 26.6	0.41
SV30	Mean ± SD	26.7 ± 26.9	25.1 ± 27.1	27.5 ± 26.8	0.54
SV40	Mean ± SD	14.7 ± 23.6	13.6 ± 23.5	15.2 ± 23.7	0.64

Abbreviations: BMI: Body Mass Index; GEJ: gastroesophageal junction; SD: standard deviation; mCPD: mean cardiopulmonary dose; TVB: thoracic vertebrae body; RV: volume of ribs; SV: volume of sternum;

those in G0-3 lymphopenia patients (51.6% vs. 71.3%, $p = 0.052$, supplemental Table 1).

Factors associated with lnALC nadir

Since high ALC nadir was associated with improved pCR, we further performed univariate and multivariable linear regression analysis to identify the association between clinical and dosimetric parameters with lnALC nadir.

Univariate linear regression analysis indicated that greater mCPD, devised as a normalized, surrogate measure of dose to circulating blood pool, was significantly associated with lnALC nadir ($p < 0.0001$). Multiple parameters of thoracic vertebral body dosimetry

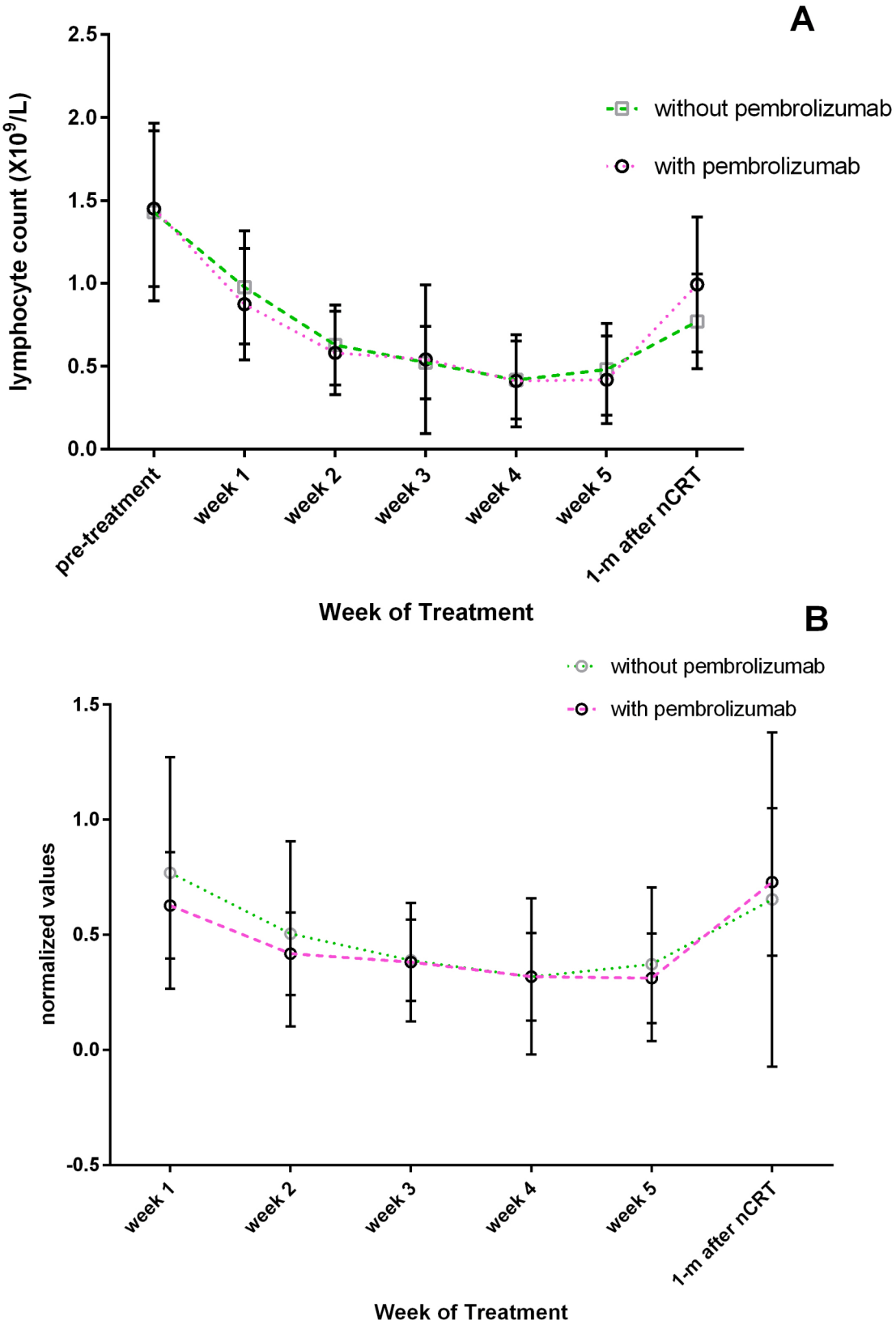

Fig. 2 Dynamic changes of lymphocytes during nCRT with or without pembrolizumab. (**A**) absolute lymphocyte count; (**B**) normalized values

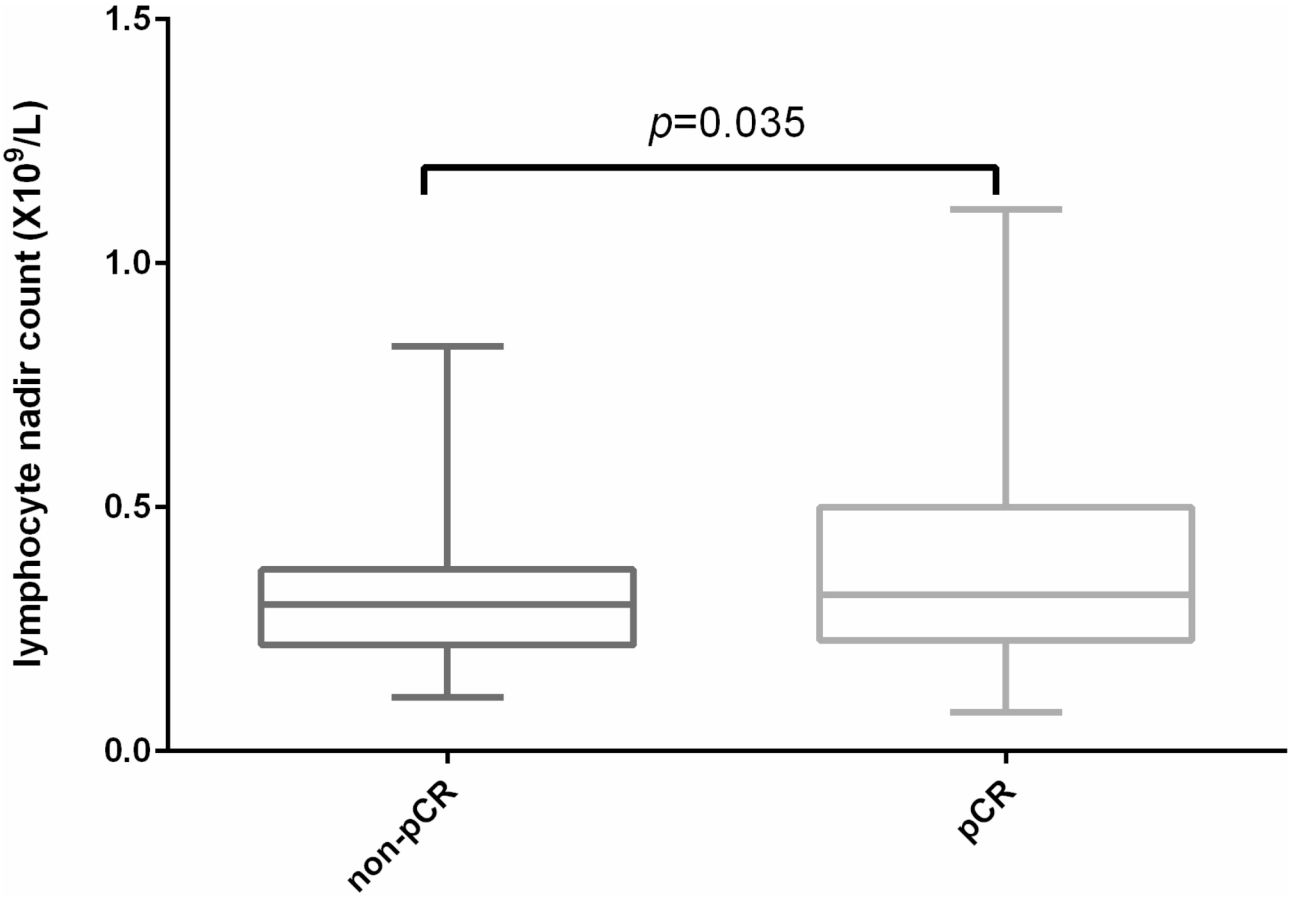

Fig. 3 Comparison the ALC nadir between pCR and non-pCR groups

(TVB) were associated with lnALC nadir on univariate analysis (Table 2). Mean TVB dose ($p < 0.0001$), TVB V5 ($p < 0.0001$), TVB V10 ($p < 0.0001$) and TVB V20 ($p = 0.0005$) were significantly associated with lnALC nadir. For dosimetric data of risbs, mean ribs dose($p < 0.0001$), RV5($p = 0.0053$), and RV10 ($p = 0.0049$) were significantly associated with lnALC nadir. Additionally, multiple parameters of spleen dosimetry were associated with lnALC nadir on univariate analysis. Mean spleen dose($p < 0.0001$), V5 of spleen ($p < 0.0001$), V10 of spleen($p < 0.0001$), V20 of spleen ($p = 0.0001$) and V30 ($p = 0.0088$) of spleen were significantly associated with lnALC nadir (supplemental Table 2).

On multivariable linear analysis accounting for age, sex, BMI, primary tumor length and nCRT regimen, mCPD remained significantly associated with lnALC nadir ($p < 0.0001$). Additional multivariable analyses demonstrated that mean TVB dose, TVB V5, TVB V10, TVB V20, mean ribs dose, RV5, mean spleen dose, V5 of spleen, V10 of spleen, V20 of spleen and V30 of spleen remained significantly associated with lnALC nadir (all $p < 0.05$, Table 2). Adjusted R2 was highest for models containing TVB V5 or V5 of spleen dose.

Factors associated with G4 lymphopenia

Then we performed univariate and multivariable logistic regression analysis to identify the association between clinical and dosimetric parameters with G4 lymphopenia. In consistent with univariate linear regression result, univariate logistic regression demonstrated that mCPD ($p = 0.00035$), mean ribs dose ($p = 0.0028$), and mean whole body dose ($p = 0.0073$) was significantly associated with G4 lymphopenia. Similarly, multiple parameters of TVB dosimetry such as mean dose, V5, V10, V20 and spleen dosimetry such mean dose, V5, V10, V20 were significantly associated with G4 lymphopenia (all $p < 0.05$, supplemental Table 3).

On multivariable logistic analysis accounting for age, sex, BMI, primary tumor length and nCRT regimen, mCPD ($p = 0.00083$) and mean whole body dose ($p = 0.028$) remained significantly associated with G4 lymphopenia. Additional multivariable analyses demonstrated that mean TVB dose, TVB V5, TVB V10, mean ribs dose, mean spleen dose, V5 of spleen, V10 of spleen, and V20 of spleen remained significantly associated with G4 lymphopenia (all $p < 0.05$, Table 3). Additionally, percentage of TVB V5-40 and spleen V5-40 in patients

Table 2 Multiple linear regression of factors associated with lnALC nadir

Variable	lnALC nadir		
	β(95%CI)	Adjusted R^2	*p*
Thoracic vertebrae body			
TVB V20	-0.0065	0.056	0.0005
TVB V10	-0.011	0.14	**< 0.001**
TVB V5	-0.012	0.16	**< 0.0001**
Mean TVB dose	-0.034	0.135	**< 0.0001**
Mean cardiopulmonary dose	-0.061	0.011	**< 0.0001**
Mean whole body dose	-0.1	0.063	**0.0002**
Ribs			
V40	0.0004	0.0001	0.90
V30	-0.0002	0.000	0.96
V20	-0.0034	0.0035	0.39
V10	-0.0082	0.018	0.049
V5	-0.0088	0.0358	**0.0053**
Mean ribs dose	-0.077	0.11	**< 0.0001**
Mean sternum dose	-0.003	0.0017	0.055
Spleen dose			
V40	-0.0056	0.013	0.094
V30	-0.0057	0.032	0.0088
V20	-0.0063	0.074	**0.0001**
V10	-0.0062	0.12	**< 0.0001**
V5	-0.0059	0.16	**< 0.0001**
Mean spleen dose	-0.031	0.15	**< 0.0001**

Abbreviations: TVB: Thoracic vertebrae body; BMI: body mass index

*Models included one dosimetric parameter and each of the following covariates: age (continuous), BMI (continuous), nCRT regimen, Sex and tumor length (continuous)

Table 4 Thresholds of LOARs for G4 lymphopenia

Variables	AUC	Optimal cut-off value
TVB		
V5	0.703	80.7%
V10	**0.674**	**76.87%**
V20	**0.602**	**69.54%**
Mean TVB dose	**0.654**	**19.04 Gy**
Mean Cardiopulmonary Dose	**0.696**	**11.10 Gy**
Mean Whole Body Dose	**0.642**	**3.07 Gy**
Mean ribs dose	**0.662**	**8.27 Gy**
Mean spleen dose	**0.747**	**4.31 Gy**
V5	**0.736**	**0.37%**
V10	**0.716**	**2.26%**
V20	**0.703**	**0.015%**

experiencing G4 lymphopenia was higher than those without G4 lymphopenia (supplemental Fig. 3).

Dose constrains of LOARs for reducing G4 lymphopenia

In order to identify thresholds for dosimetric planning, we analyzed the ROC curves for G4 lymphopenia for LOARs. Cutoffs to avoid G4 lymphopenia were mean TVB dose < 19.04 Gy, TVB V5 < 80.7%, TVB V10 < 76.87%, TVB V20 < 69.54%, mean cardiopulmonary dose < 11.01 Gy, mean whole body dose < 3.07 Gy, mean ribs dose < 8.27 Gy, mean spleen dose < 4.31 Gy, V5 of spleen < 0.33%, V10 of spleen < 2.22%, and V20 of spleen < 0.015% (Table 4). Development of grade 4 lymphopenia was more likely in patients with mean TVB dose ≥ 19 Gy (36.5% vs. 9.8%, $p = 0.0005$), TVB V5 ≥ 81%(33.3% vs. 9.2%, $p = 0.0007$), TVB V10 ≥ 77%(36.5% vs. 9.8%, $p = 0.0005$),

Table 3 Multiple logistic regression for factors associated with grade 4 Lymphopenia

Variable	Grade 4 Lymphopenia			
	OR(95%CI)	SE	AIC	*p*
Thoracic TVB				
Thoracic vertebrae V20	1.02(0.99–1.04)	0.011	-	0.18
Thoracic vertebrae V10	1.03(1.01–1.06)	0.012	**188.8**	**0.005**
Thoracic vertebrae V5	1.03(1.02–1.06)	0.012	**185.5**	**0.0012**
Mean thoracic vertebrae dose	1.11(1.03–1.20)	0.038	**189.4**	**0.0069**
Mean cardiopulmonary dose	1.32(1.12–1.56)	0.084	**185.0**	**0.00083**
Mean whole body dose	1.41(1.04–1.92)	0.16	**192.8**	**0.028**
Ribs				
V5	1.03(0.99–1.04)	0.012	-	0.15
Mean ribs dose	1.33(1.09–1.63)	0.1	**189.9**	**0.0055**
Spleen dose				
V30	1.02(0.99–1.04)	0.012	-	0.12
V20	1.02(1.00-1.03)	0.008	**192.6**	**0.017**
V10	1.02(1.01–1.03)	0.006	**188.9**	**0.003**
V5	1.02(1.01–1.03)	0.006	**184.7**	**0.0045**
Mean spleen dose	1.11(1.05–1.18)	0.03	**185.6**	**0.00042**

Abbreviations: SE: standard error; OR: odds ratio; TVB, Thoracic Vertebral Body;

*Models included one dosimetric parameter and each of the following covariates: age (continuous), BMI (continuous), nCRT regimen, Sex and tumor length (continuous)

TVB V20 ≥ 70%(33.3% vs. 12.8%, $p = 0.026$), mean cardiopulmonary dose ≥ 11 Gy(25.5% vs. 9.0%, $p = 0.01$), mean whole body dose ≥ 3 Gy (24.2% vs. 9.4%, $p = 0.021$), mean ribs dose ≥ 8 Gy(27.5% vs. 9.6%, $p = 0.0069$), mean spleen dose ≥ 4 Gy(32.5% vs. 6.1%, $p < 0.0001$), V5 of spleen ≥ 0.33%(27.8% vs. 3%, $p < 0.0001$), V10 of spleen ≥ 2.22%(29.1% vs. 4.4%, $p < 0.0001$), and V20 of spleen ≥ 0.015% (28% vs. 6%, $p = 0.0004$, supplemental Table 4).

Feasibility of LOARs-constrain treatment planning

We further explored if the above LOARs dose constrains could have been achieved in patients who were treated clinically with plans that did not meet these thresholds, while did not compromise clinically acceptable objectives (supplemental Table 5). As a result, we re-planned ten patients whose initial radiation plan exceeded the aforementioned LOARs thresholds by using LOARs-sparing IMRT or proton therapy. We found there was no significant difference in target coverage of PTV and traditional OARs including lungs and hear dose in original plans and lymphocyte-sparing IMRT plans ($p = 0.62$), while optimizing lymphocyte-sparing IMRT plans significantly decreased dose of LOARs such as the mean TVB dose, mean ribs dose, mean spleen dose. In comparison the initial plan and lymphocyte-sparing IMRT plans, significant improvement was observed for all LOARs structures and traditional OARs in optimized lymphocyte-sparing thoracic proton plans ($p < 0.05$, Table 5). Figure 4 showed the optimized lymphocyte-sparing proton plan. And supplemental Fig. 4 showed the dose-volume histogram for the original, optimized IMRT and optimized proton plans.

Discussion

Prior to the present study, several studies had shown an association between lymphopenia and poor prognosis in thoracic cancer patients undergoing definitive CRT with or without immunotherapy, especially grade 4 lymphopenia [11, 13, 19–22]. However, patients in most of these studies were treated with different nCRT or immunotherapy regimen, which would significantly impact the changes of ALC. In addition, the dynamic changes for ALC among ESCC patients in neoadjuvant setting with or without pembrolizumab, as well as the clinical and lymphocyte-related organs dosimetric parameters associated with ALC nadir during nCRT remained unknown. Based on a large ESCC prospective cohort from our institution, we found that the dynamic trends of ALC changes in ESCC patients during nCRT with or without pembrolizumab was comparable. ALC steadily declined to nadir at week 5 throughout nCRT in both groups, and then quickly recovered. However, we found that the addition of pembrolizumab to nCRT significantly improved the lymphopenia recovery in 1-month after nCRT

Table 5 Median values of target and LOARs dose between original and lymphocyte-sparing thoracic IMRT or proton radiotherapy

Structures		Planning median			Significance		
Name	**Objectives**	**Original (*n* = 10)**	**lymphocyte-sparing IMRT (*n* = 10)**	**lymphocyte-sparing thoracic IMPT (*n* = 10)**	**Original vs. lymphocyte-sparing IMRT**	**Original vs. lymphocyte-sparing thoracic IMPT**	**lymphocyte-sparing IMRT vs. IMPT**
PTV	**V95 ≥ 95%**	99.7%	99.8%	-	0.62		
CTV	**V95 ≥ 95%**	-	-	99.99%			
TVB							
Mean TVB dose	**19 Gy**	24.8 Gy	22.5 Gy	18.7 Gy	0.0014	0.0018	0.012
TVB V5	**81%**	93.5%	90.8%	77.0%	0.66	0.0002	0.00091
TVB V10	**77%**	85.4%	78.6%	65.38%	0.005	0.0013	0.0052
TVB V20	**70%**	67.5%	58.3%	47.2%	0.76	0.004	0.045
Mean CPD	**11 Gy**	11.9 Gy	12.0 Gy	6.1 Gy	0.015	< 0.0001	< 0.0001
Mean ribs dose	**8 Gy**	8.05 Gy	7.99 Gy	5.0 Gy	0.034	< 0.0001	< 0.0001
Mean spleen dose	**4 Gy**	7.43 Gy	3.87 Gy	3.77 Gy	0.0026	0.0018	0.29
Lungs							
Mean dose	**15 Gy**	9.78 Gy	9.83 Gy	5.24 Gy	0.14	< 0.0001	< 0.0001
V5	**60%**	56.45%	56.5 Gy	24.3%	0.35	< 0.0001	< 0.0001
V20	**30%**	14.15%	14.45%	9.38%	0.13	0.0019	0.002
V30	**20%**	3.25%	3.5%	4.14%	0.005	0.38	0.81
Heart							
Mean dose	**15 Gy**	17.56 Gy	18.4 Gy	7.17 Gy	0.013	< 0.0001	< 0.0001
V30	**40%**	15.6%	17.7%	11.24%	0.04	0.0013	0.0012
Spinal cord	**Dmax < 45**	37.7 Gy	36.5 Gy	34.3 Gy	0.20	0.013	0.021

Abbreviations: PTV: plan treatment volume; CTV: clinical treatment volume; mCPD: mean cardiopulmonary dose; TVB: thoracic vertebrae body; IMRT: intensity modulated radiation therapy; IMPT: intensity modulated proton therapy;

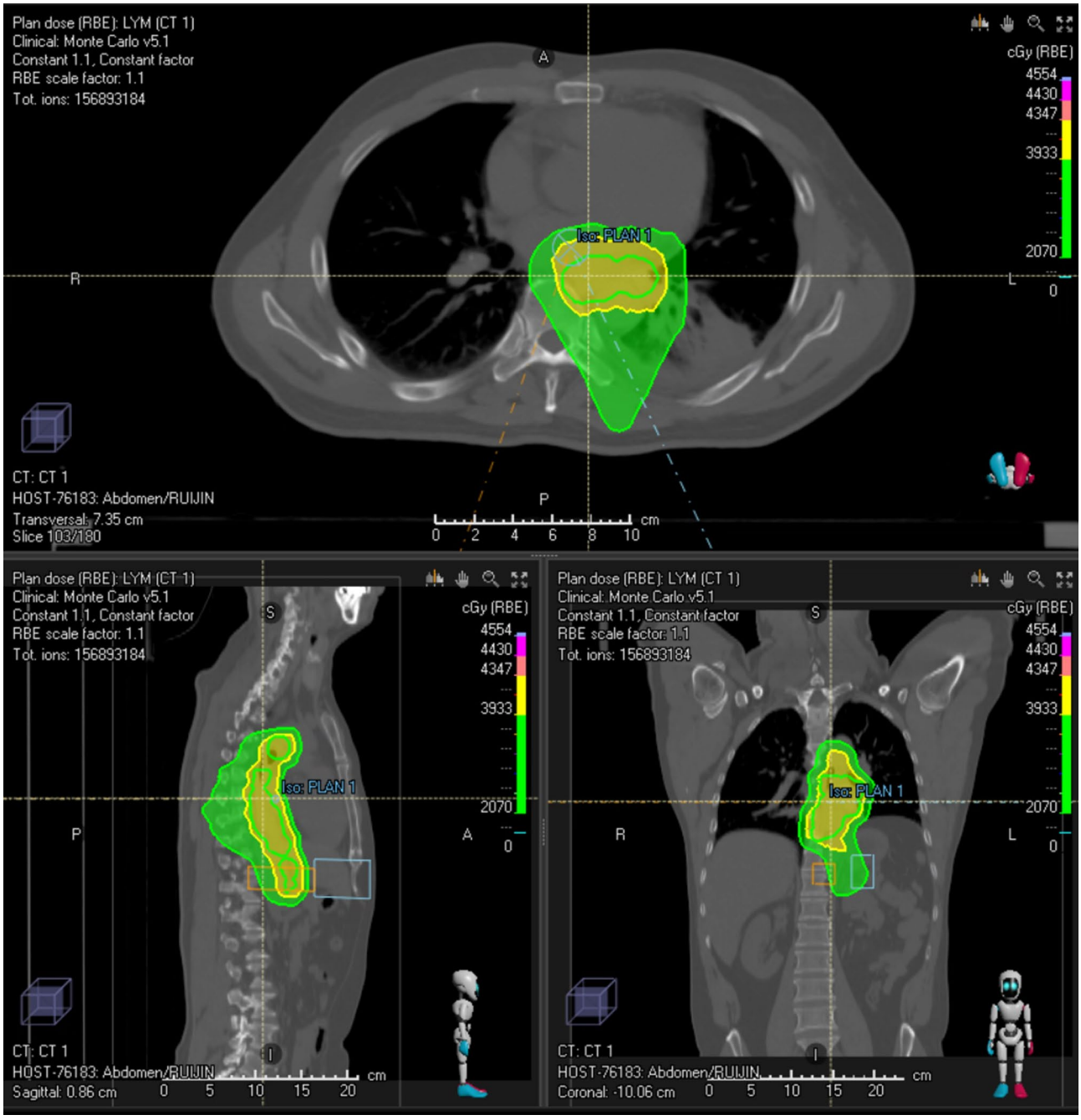

Fig. 4 Typical case of optimized lymphocyte-sparing proton plan

($p = 0.0003$), which suggested that pembrolizumab had a protective effect on lymphocytes and improved the synergistic effect of radiation and pembrolizumab [23]. We also found that the mean ALC in pCR group was significantly higher than those in non-pCR group ($p = 0.035$), which was in consistent with Fang P. et al.'s study. In that study, the authors demonstrated that median ALC nadir was significantly higher than those without (0.35×103/uL vs. 0.29×103/uL, $p = 0.007$) [11]. In addition, we found that patients experiencing grade 4 lymphopenia was associated with poor treatment response to neoadjuvant treatment (TRG 0–1: 51.6% vs. 71.3%, $p = 0.052$), but not for pCR ($p = 0.90$). Based on our findings, a higher ALC nadir during nCRT was associated with a higher rate of pCR for ESCC patients undergoing trimodality therapy, and ESCC patients experiencing grade 4 lymphopenia was associated with a significantly decreased major pathological response for ESCC patients.

As more and more evidence indicated that radiation-induced lymphopenia was related to poor treatment

response and survival outcomes in malignancies, we then investigated the dosimetric parameters that associated with G4 lymphopenia or lnALC nadir during nCRT. Multivariable linear regression indicated that mCPD, mean whole body dose, mean TVB dose, TVB V5, TVB V10, TVB V20, mean ribs dose, RV5, mean spleen dose, V5 of spleen, V10 of spleen, V20 of spleen and V30 of spleen were all independent predictors for lnALC nadir during nCRT by adjusting age, sex, BMI, tumor length and nCRT regimen. In consistent with linear regression results, multiple logistic regression indicated that mCPD, mean whole body dose, mean TVB dose, TVB V5, TVB V10, mean ribs dose, mean spleen dose, V5 of spleen, V10 of spleen, and V20 of spleen were independent predictor for developing G4 lymphopenia by adjusting age, sex, BMI, tumor length and nCRT regimen. We then used ROC curve to identify thresholds for significant parameters of LOARs in both multivariable linear and logistic regression. Our result was in consistent with previous report by Anderson et al. [24], the authors also found that the relative volumes of the spleen, TVB, and MBD, as well as the mean cardiopulmonary dose for predictors of ALC nadir in EC patients treated with neoadjuvant or definitive chemoradiotherapy. In order to establish a lymphocyte-sparing thoracic radiotherapy for ESCC patients, we used ROC to archive the optimal cutoffs by avoiding G4 lymphopenia and found that mean TVB dose < 19.04 Gy, TVB V5 < 80.7%, TVB V10 < 76.87%, TVB V20 < 69.54%, mean cardiopulmonary dose < 11.01 Gy, mean whole body dose < 3.07 Gy, mean ribs dose < 8.27 Gy, mean spleen dose < 4.31 Gy, V5 of spleen < 0.33%, V10 of spleen < 2.22%, and V20 of spleen < 0.015%.

With the improvement of treatment techniques, the LOARs-spearing thoracic radiotherapy was considered an effective method to protect lymphocytes to enhance the synergistic effect of radiation and immunotherapy. Prior to the present study, two studies had confirmed that bone marrow or LOARs-sparing pelvic irradiation was feasible [25, 26]. However, the feasibility of LOARs-spearing thoracic radiotherapy to minimize lymphopenia for ESCC patients remained unknown due to lack of established LOARs constrain. In the present study, we re-planned ten patient whose initial radiation plan exceeded the established LOARs thresholds and found that LOARs thresholds objectives were successfully achieved after optimization while maintained acceptable target and traditional OARs dose constrains. In comparison to photon radiotherapy, proton radiotherapy could further reduce LOARs and traditional OARs dose in comparison with LOARs-spearing IMRT plan while maintaining acceptable target, which was consistent with Zhang Y. et al.' work [27]. The authors also found that achieve lower doses for VBV 10 Gy, VBV 20 Gy, and MVD than VMAT and static IMRT plans while achieving the same target coverage. Overall, we successfully demonstrated the feasibility of lymphocyte-sparing treatment planning in thoracic irradiation, and our team had initiated a prospective trial to confirm the clinical impact of LOARs-spearing thoracic radiotherapy on lymphocyte and survival outcomes of ESCC treated with nCRT with or without pembrolizumab, the trial had been under recruiting (NCT06596954).

In our previous PALACE-1 study [17], preoperative pembrolizumab in combination with concurrent CRT in ESCC archived a pCR of 50.6% and an acceptable safety. In consistent with this result, among the largest sample size of 188 ESCC patients received surgery after neoadjuvant treatment, the present study showed that 60 patients (47.2%) archived pCR among 127 patients treated with nCRT with pembrolizumab. For those treated with nCRT alone, 26 of 61 patients (42.6%) archived pCR. The addition of pembrolizumab to nCRT improved approximately 5% pCR. However, long-term outcomes were still needed to be confirmed that whether the improved pCR rate would transfer into survival benefit.

Several limitations were needed to be concerned. First of all, the study design was a retrospective study from a single-center, therefore, a risk of selection bias could not be excluded. Secondly, flow cytometry was only applied to a limited part of patients, thus we were unable to analyze the relationship between lymphocyte subtypes and treatment response. Thirdly, long-term survival could not be available due to limited follow-up. Despite the above limitations, the present study had the following strengths: firstly, this was the first and largest sample size study to characterize the dynamic changes of absolute lymphocyte count for ESCC patients in neoadjuvant setting. Secondly, this was a retrospective analysis from a prospective cohort, therefore, all included patients treated with standardized nCRT regimen with or without pembrolizumab. According to trial protocol, the radiotherapy regimen was 41.4 Gy/23Fx, and concurrent chemotherapy regimen was same and consisted of carboplatin (area under the curve of 2 mg/mL per min) and paclitaxel or nab-paclitaxel (50 mg/m2 of body surface area). which reduced the impact of heterogeneity from nCRT and immunotherapy on the dynamic changes of ALC. Further studies were recommended to externally validate our findings among ESCC patients treated with the same nCRT regimen.

Conclusions

The addition of pembrolizumab to nCRT improved lymphopenia recovery in 1-month after nCRT. The ALC nadir and G4 lymphopenia was significantly associated with treatment response after nCRT. Sparing of LOARs using advanced radiation techniques might reduce the risk of developing lymphopenia and improve treatment

response by maximizing the synergistic effects of nCRT and immunotherapy.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Supplementary Material 7

Supplementary Material 8

Supplementary Material 9

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None.

Author contributions
Principal investigator: J.C. Y.Z and S.Z. Drafting of the protocol manuscript: W.X.Q. Conceptualization: C.X., G.C., S.Z., J.C. and W.X.Q.; Project administration: C.L., S.Z., S.L. and H.L. data analysis, acquisition, and interpretation: H.L, X.H., C. Z., X.L., W.X.Q., H.J.; manuscript preparation: W.X.Q., S.L., H.L., S.Z. Final approval of manuscript: all authors.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval
This study was approved by the Ethics Committee of Ruijin hospital, Shanghai Jiao Tong University School of Medicine.

Competing interests
The authors declare no competing interests.

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