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Intra-patient comparison of microarchitecture of tumour negative lymph nodes from oesophageal cancer patients – Results from the MRC Oe02 trial

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ABSTRACT

Background: Regional lymph node (LN) status is a key prognostic factor in oesophageal cancer (OeC). Tumour-derived antigens can activate immune reactions in LNs, potentially reflecting the host's anti-tumour immune response. It remains unclear whether this response is homogeneous across all tumour negative LNs (LNneg) within individual OeC patients.

Purpose: To investigate the hypotheses: (1) the host anti-tumour immune response is similar in all LNneg from an individual OeC patient reflected in a similar microarchitecture in all LNneg; and (2) immune response measured in the largest LNneg can represent that of all LNnegs.

Methods: (y)pN0 patients from the Oe02 trial with at least two LNneg were included. Microarchitectural LN features (germinal centres (GermC), lymphocytes outside GermCs (lymphocytes), histiocytes) were morphometrically quantified. Linear mixed-effects models, intraclass correlation coefficients (ICC) and Bland-Altman plots were used to determine systematic bias, reliability/variability and agreement of LNneg microarchitecture measurements.

Results: Linear mixed-effects models showed no systematic bias in LNneg microarchitectural features within a patient. The ICC revealed moderate variability for lymphocytes (ICC: 0.39; 95 %CI: 0.01– 0.61, $p = 0.02$)) and GermC (ICC: 0.50; 95 %CI: 0.22–0.68, $p < 0.001$), and high variability for histiocytes (ICC: 0.07 (95 %CI: −0.45–0.40, $p = 0.38$). Bland-Altman plots showed that 5.0 % of GermC, 5.0 % of histiocytes and 8.5 % of lymphocyte measurements were outside the 95 % limits of agreement.

Conclusions: This is the first study to systematically assess agreement of microarchitectural features in LNneg within an individual (y)pN0 OeC patient. The absence of systematic bias supports using largest LNneg as surrogate for OeC patient's overall anti-tumour immune response.

1. Introduction

Oesophageal cancer (OeC) is the 6th most common cause of cancer-related death worldwide[1]. The median 5-year overall survival (OS) rate is between 25 % and 47 % if patients are diagnosed at an early disease stage, and less than 5 % if diagnosed with advanced disease stage

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[2]. The first randomised controlled phase III trial showing the superiority of neoadjuvant chemotherapy plus surgery over surgery alone in OeC patients was the UK MRC OE02 trial changing clinical practice [3].

A key factor determining survival of OeC patients is the status of their tumour-draining regional lymph nodes (LN), also known as N status according to the Tumour Node Metastasis (TNM) classification [4,5]. The UICC TNM classification 8th edition for OeC is based on the number of LN with metastases and distinguishes between N0 (absence of metastasis in regional LNs (LNneg)), N1 (1–2 LNs with metastasis (LNpos)), N2 (3–6 LNpos) or N3 (≥7 LNpos) [6].

Recent studies suggest that a high absolute number of LNpos, a low total number of resected LNs, as well as a high ratio of LNpos to the total number of resected LNs are all independent predictors of poor survival [7,8]. Furthermore, research suggests that OeC patients with a high density of tumour-infiltrating lymphocytes (TILs) in the primary tumour tend to have fewer LNpos and better overall survival than patients with a low density of TILs [9,10]. Our own research suggested that the prognostic significance of LNneg is not solely determined by its quantity; but that LNneg size and microarchitecture may be equally important [11–13]. Multiples studies, including our own, suggest that certain microarchitectural immune response patterns in regional tumour-draining LNs may provide a readout of the host's anti-tumour immune response which has been related to survival in patients with gastric, colorectal or head and neck cancer [14–18].

The tumour-derived antigen driven immune response is primarily executed in tumour-draining LN. The LN capsule encloses specific anatomical compartments such as the sinuses, cortex, paracortex, germinal centres and medulla, each with a special immunological function (see Fig. 1)[19]. The lymph fluid from the primary tumour contains tumour-derived antigens which are captured by antigen-presenting dendritic cells activating an immune response. This activation includes proliferation of lymphocytes and generation of anti-tumour primed T- and B-cells [20]. Lymphocyte proliferation may result in an increase in LN size and microscopic changes like follicular hyperplasia and/or paracortical hyperplasia [21]. It has been suggested that microscopic LN evaluation may reveal insights into the ability of the patient's immune system to mount anti-tumour responses [22].

Several studies have investigated the prognostic significance of LN microarchitecture in different cancer types in the past [13,14,26]. However, none of these studies outlined the criteria for selecting the LN to be examined from the numerous LNs typically found in a resection specimens. In our own previous study in OeC, we decided to use the

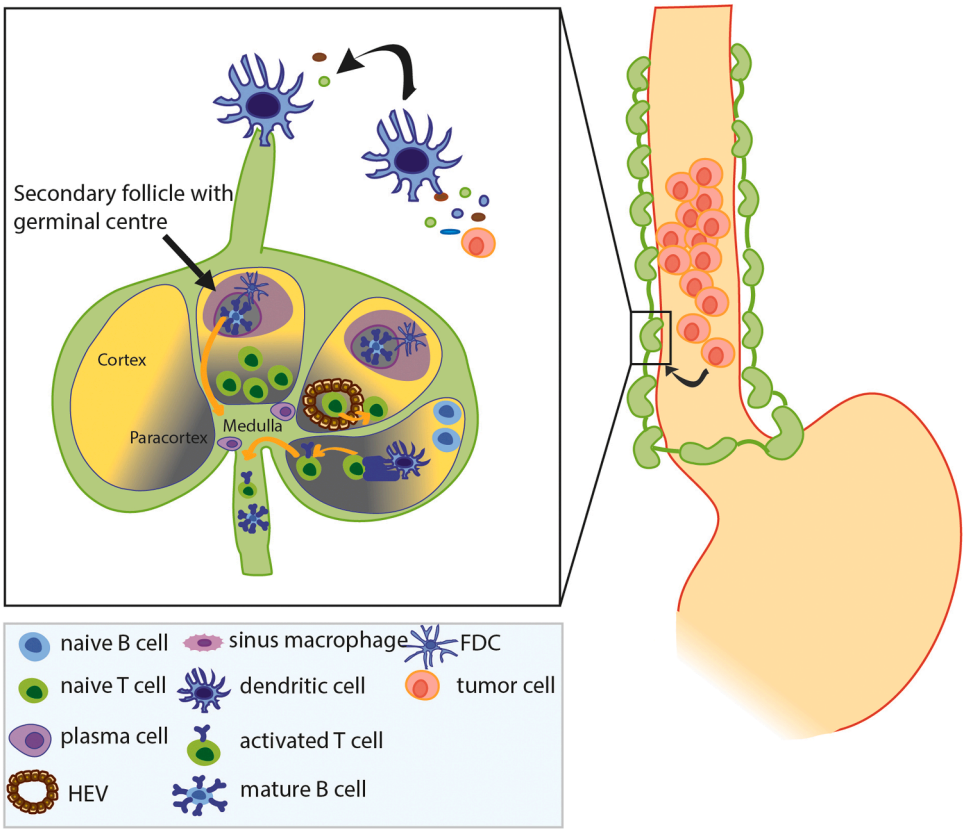

Fig. 1. Lymphatic drainage of the oesophagus and physiology within the lymph node. The different layers of the oesophageal wall get drained by different lymphatic vessels. Lymphatic drainage starts with lymphatic vessels just above the muscularis mucosae and in the submucosa whereby lymph fluid gets mainly drained in a longitudinal way. In the upper two thirds of the oesophagus, lymph flows in cranial direction, while in the lower third, drainage is in caudal direction. The lymph from the lower parts ultimately flows into the inferior paraoesophageal, subcarinal, parahiatal and left gastric nodes. Additionally, the middle third thoracic oesophagus gets drained to the superior and posterior mediastinal lymph nodes. The abdominal part of the oesophagus gets drained to the left gastric and left and right paracardial lymph nodes. The cervical oesophagus gets drained into the lower deep cervical, paraoesophaeal and paratracheal lymph nodes. The lymph fluid - including antigen-presenting cells (APC) and tumour-antigens - enters the LN via afferent lymphatic vessels to the subcapsular sinus, the space between LN capsule and LN parenchyma [23]. Macrophages, called histiocytes in this location, phagocytize incoming antigens and present them to B- and T-lymphocytes. From the subcapsular sinus, lymph drains to the outer part of the parenchyma, namely the cortex. The cortex mainly consists of immature B-cells which get stimulated by APC and form follicles with germinal centres - an expression of increased antibody-mediated immune response [24]. After flowing through the cortex, lymph reaches the paracortex - this is the area where T-cells are predominantly located. The T-cells mediate the cell-driven immune response which results in tumour killing by cytotoxic T-cells. After passing the paracortex, lymphocytes enter the medulla (innermost layer of the LN) and passes by medullary cords. Those mainly contain plasma cells, mature B-cells and histiocytes. From the medulla, lymph fluid including immune cells exits the LN via efferent lymphatic vessels into the lymphatic circulation and blood stream primed to specifically attack cancer cells [25].

largest LNneg following Grundmann et al. who had conducted animal studies into the LN microarchitecture and proposed that the characteristics of the largest LNneg are representative of all other (non-largest) LNnegs [27]. However, Grundmann et al. did not provide any details on the methods used to reach this conclusion.

The question whether the size or microarchitectural features of the largest LNneg are representative of all LNneg from the same patient has not been investigated at all. However, there is a clinical need to establish whether there is heterogeneity of the LNneg microarchitecture within OeC patients or whether the largest LNneg per patient can be used as surrogate for the host's anti-tumour immune response. This knowledge is particularly important for the planning of prospective studies into the prognostic and/or predictive value of the host anti-tumour immune response in LNneg.

We hypothesised that all LNneg from an individual OeC patient have a similar microarchitecture irrespective of their size. Our study aim was therefore to quantify the microarchitectural LN compartments of all available LNneg from OeC patients without LN metastasis (LN status pN0). Results from the largest LNneg were compared with the non-largest LNnegs per patient.

2. Methods

2.1. Patients

For the current cross-sectional study, tumour negative lymph nodes (LNneg) from (y)pN0 patients from the UK MRC OE02 trial were analysed. In this trial, patients with histologically or cytologically proven locally advanced resectable oesophageal cancer (OeC) were randomly allocated to treatment by neoadjuvant chemotherapy consisting of two cycles of 5-Fluorouracil and cisplatin followed by surgery (CS patients) or to treatment by surgery alone (S patients) [28]. Haematoxylin eosin (H&E)-stained slides and paraffin blocks from the resection specimen were retrospectively collected for previous studies [11]. Ninety-six OE02 patients with available slides were (y)pN0 patients, of which 81 patients had more than one analysable LNneg.

Clinicopathological data including outcome data had been extracted from relevant databases for previous studies [11]. Ethical approval of the study was granted by the South East Research Ethics Committee, London, UK, REC reference: 07/H1102/111.

2.2. Assessment of lymph nodes

Available H&E-stained slides of LNneg from 96 patients with (y)pN0 status in their resection specimen were analysed (43 S patients, 53 CS patients; Fig. 2) after whole slide scanning at 40x magnification using an Aperio XT-scanner. 81 of the 96 (y)pN0 patients (35 S patients, 46 CS patients) had two or more LNneg enabling intra-patient comparisons.

Manual outlines using ImageScope (Leica Biosystems, Nussloch) and quality control from a second independent investigator of outlines and LN status (confirming N0 status) were used from a previous study [11]. The largest LNneg diameter was used as surrogate for LNneg size. LNneg

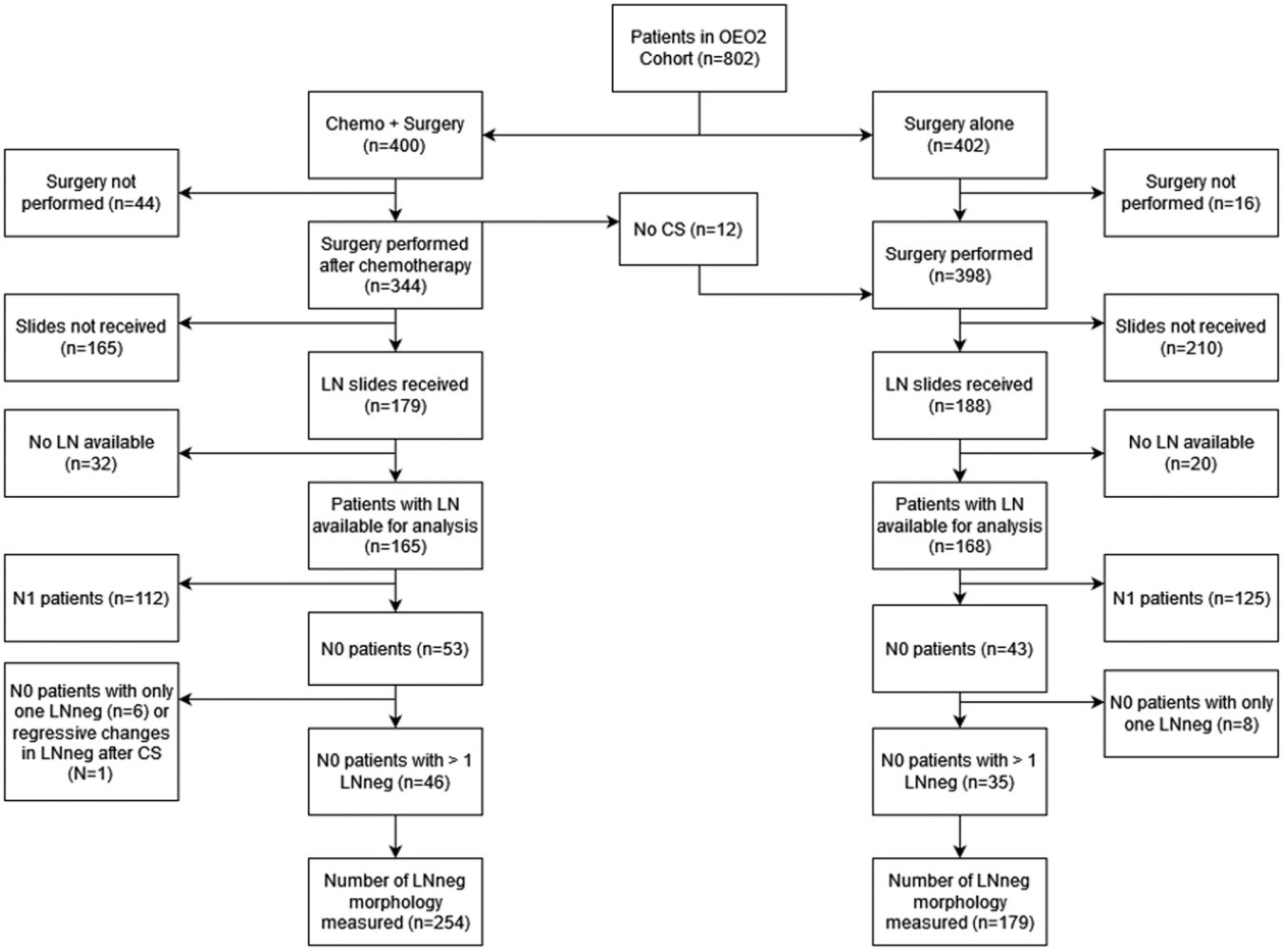

Fig. 2. Flow diagram showing the number of CS and S patients in the OE02 trial and number of (y)pN0 patients for whom lymph node microarchitecture was analysed from virtual histopathological slides. LNneg= tumour negative lymph nodes.

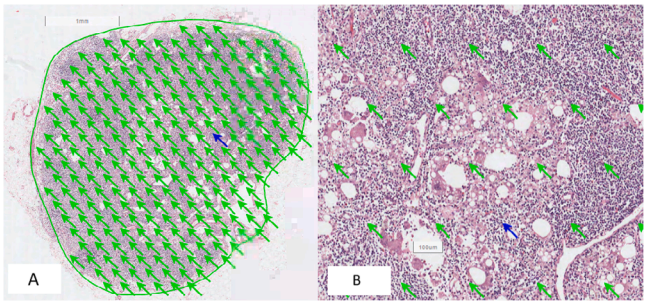

Fig. 3. Illustration of the point counting method. A) Two hundred and fifty measurement points (=arrows) were automatically distributed within the annotation of a LNneg. Magnification: x1.25. B) The tissue at the tip of the arrow was scored according to the five microarchitectural features. Magnification: x5.

with regressive changes after neoadjuvant chemotherapy were excluded from the analysis.

The investigators analysing LNs were blinded to any clinicopathological parameters.

LNneg microarchitectural features were quantified morphometrically using the manual point counting option in the Medical Image Manager software (HeteroGenious Limited, Leeds, UK) and calculated as [(100/area of the LN)*area with the feature] resulting in the %area of the LN covered with the feature, as described in our previous study [11]. We distinguished between [1] lymphocytes outside germinal centres (% area lymphocytes), [2] histiocytes outside germinal centres irrespective whether they are located within the sinus or in the parenchyma (%area histiocytes) and [3] germinal centres (GermC) (%area GermC), vessels (%area vessels) and other tissue including fat tissue, stromal cells and connective tissue (%area other tissue).

2.3. Statistical analyses

Statistical analyses were performed using SPSS statistics software (version 29, IBM). Baseline characteristics of the study patients were presented using median and range, or as counts and percentages, as appropriate.

We tested our hypothesis that the microarchitectural features (lymphocytes, germinal centres and histiocytes) of the largest LNneg are similar to that of the non-largest LNneg using three statistical methods.

(1) we used linear mixed-effects regression models to evaluate systematic bias (i.e. presence of mean differences) of microarchitectural features of LNneg within a patient comparing largest LNneg and non-largest LNnegs.
(2) we quantified reliability/variability of LNneg microarchitectural features within a patient with the intraclass correlation coefficient (ICC) for the largest LNneg and a randomly chosen non-largest LNneg of the same patient.
(3) we utilised Bland-Altman plots to analyse and visualise agreement between microarchitectural features of the largest LNneg and microarchitectural features of a randomly chosen non-largest LNneg.

These heterogeneity analyses were done for the whole trial population irrespective of treatment as stratification of analyses by treatment would have resulted in a group size which would have been too small for this type of analysis.

3. Results

3.1. Patient cohort

The median (range) age of patients (n = 81) included in our study was 61 years (40.3–76.7 years). Median (range) follow-up time was 34.1 months (0-158.16 months). Fifty-six patients (68 %) had died by the end of the study period. For a summary of clinicopathological data see Table 1.

3.2. Comparison of microarchitectural features of the largest LNneg with non-largest LNneg

Eighty-one (y)pN0 patients (35 S patients, 46 CS patients) had more than one LNneg. In total, 433 LNnegs were analysed (254 from CS patients (59 %) and 179 from S patients (41 %)). The median number of LNneg per patients was four, ranging from two to 20 LNneg. The median size of all LNneg was 4.7 mm (range: 0.4–17.5 mm), while the median size of the largest LNneg was 8.1 mm (range: 2.0–17.5 mm). Since the sample size within each treatment arm was insufficient to ensure adequate statistical power, the following comparative analyses of the microarchitectural features were conducted using the entire cohort rather than stratifying analyses by treatment arm.

For all LNneg of the patients, the median (range) area percentage of LNneg microarchitectural features was 55.5 % (6.1 – 94.0 %) for lymphocytes; 1.2 % (0.0 – 17.8 %) for germinal centres and 18.1 % (0.0 – 84.2 %) for histiocytes. The median(range) area percentage of the largest LNneg microarchitectural features was 54.1 % (10.3 – 84.6 %) for lymphocytes, 1.2 % (0.0 – 15.4 %) for germinal centres and 18.7 % (0.0 – 65.3 %) for histiocytes.

Table 1
Clinicopathological characteristics of all OeC patients with analysed negative lymph nodes (LNneg) in our cohort (n = 81).

		Number of patients	%
Sex	Male	31	38
	Female	50	62
Age at diagnosis (age groups)	< 55	21	26
	55–70	45	56
	> 70	15	18
Location of primary tumour	Lower	50	62
	Middle	26	32
	Upper	5	6
Histology of primary tumour	Adenocarcinoma	37	45
	Squamous cell carcinoma	32	40
	Other	12	15
(y)pT	T0*	6	8
	T1	10	12
	T2	12	15
	T3	51	63
	T4	2	2
Grade of differentiation	Moderate/Well	44	54
	Poor	27	33
	Unknown	10	13
Lymphatic invasion	No	65	80
	Yes	16	20
Blood vessel invasion	No	75	93
	Yes	6	7
Resection margin status	Negative	61	75
	Positive	13	16
	Unknown	7	9
Tumour regression grade (TRG) primary tumour	TRG 1–3	23	28
	TRG 4–5	56	69
	unknown	2	3

* T0: No residual tumour in the specimen.

3.3. Intra-patient reliability and agreement analysis

There was no evidence of any systematic bias of the three microarchitectural features. Regression coefficients (RC) of linear mixed-effects models were close to 0 and p-values were insignificant when comparing all LNneg of individual patients, see Table 2. An RC close to 0 means that the observed values of the microarchitectural features are due to random fluctuations and are not systematically biased.

In the reliability analysis, the %area with lymphocytes and the %area with germinal centres had a moderate intraclass correlation coefficients (ICC) of 0.39 and 0.50, respectively, confirming consistency across morphometric measurements of LN microarchitecture, see Table 3. However, for the %area with histiocytes, the reliability analysis seems to indicate lower consistency across measurements as the ICC was only 0.07.

Bland-Altman plots were used to investigate on the agreement between the largest LNneg and a randomly chosen LNneg. The highest agreement was observed for the %area with germinal centre and %area with histiocytes measurement: only 5.0 % of the measurements were outside of the 95 % limit of agreement for both parameters (see Fig. 4). For the %area with lymphocytes, 8.5 % of the measurements were outside of the 95 % limit of agreement.

4. Discussion

Research in oesophageal cancer (OeC) has primarily focused on the clinical impact of primary tumour characteristics or presence of metastatic lymph nodes (LNpos), whilst knowledge about the microarchitecture of tumour negative LN (LNneg) remains limited. Intra-patient variability of the microarchitectural changes in LNs as surrogate of the host's anti-tumour immune response has not been investigated in detail.

To the best of our knowledge, this is the first study to systematically assess the heterogeneity of the LNneg microarchitecture of OeC patients without LN metastases ((y)pN0) treated with neoadjuvant chemotherapy and surgery or surgery alone.

Our statistical analyses revealed that there were no signals for systematic bias of the assessment of largest LNneg microarchitecture compared to the non-largest LNneg examined in this cohort. In the subsequent reliability analysis, we found evidence indicating reasonable consistency of the LNneg microarchitecture in (y)pN0 OeC patients. Interestingly, our findings suggested that the variability for %area with histiocytes is much more pronounced compared to the %area with germinal centres or lymphocytes. Visual assessment of Bland-Altman plots showed that the agreement between intra-patient LNneg microarchitecture was acceptable.

Past studies investigating the microarchitecture of LNneg in gastrointestinal cancer patients have primarily focussed on the prognostic value of LNneg microarchitecture. However, there are barely any studies comparing LNneg microarchitecture within patients. Grundmann et al. have concluded from their studies in colorectal cancer that the largest LNneg can be taken as surrogate for other LNneg as they have comparable microarchitecture [29]. Although Grundmann et al. have not clarified their methods of LN analysis, their conclusion is supported by the results of our current study.

There are also studies showing different morphology of LN in healthy people depending on anatomical location of LN station within the body, showing that e.g. mesentery LN differ from axillary and inguinal LN as the former consists of more lymphocytes in the sinusses [30]. These findings are intriguing. However, in our study all analysed LNneg were located in the locoregional drainage area of the oesophagus and not in different anatomical locations, which reduces the likelihood of significant differences among LNneg. Within locoregional LNneg, proximity of the investigated LNneg to the primary tumour might influence their immunologic response to tumour-derived antigens, thereby contributing to variation of the morphology between LNs from the same patient, as outlined in a review by Cruz de Casas [31]. Ozawa et al.'s animal studies suggested that the first LN in a LN chain, connected by lymphatic vessels, harbours the highest concentration of reactive immune cells due to a filtering phenomenon [32]. The degree to which this phenomenon affects the different LN compartments and especially the histiocytes as 'the first line of defense', which showed a higher intra-patient variability in LNneg, remains unknown.

One limitation of our study is that the number of ypN0 patients was too small to allow for a comparison of lymph nodes between treatment arms. Nevertheless, in the current study we performed intra-patient comparisons and thus, we would not expect a bias by analysing both treatment arms together as the analysis is executed within patients and not comparing morphologies between patients. In our study, we classify immune cells in the LN based on Haematoxylin/eosin stained tissue sections. We are currently unaware whether a similar histological LN microarchitecture translates to analogous function of the LN. In future studies, additional immunohistochemistry stainings could provide more information on functional aspects of lymph node biology and reactions to cancer.

In conclusion, our results suggest that there is only a minimal degree of variation in LNneg morphology among LNneg in patients with oesophageal cancer (OeC). Therefore, in order to assess patient's host immune response, we consider it reasonable to use the largest LNneg from the resection specimen as a surrogate when investigating prognostic biomarker in OeC patients, as shown in a past research of our group [11]. In breast cancer and melanoma, the status of the first tumour draining LN, the so-called sentinel LN, is used in the routine clinical setting for treatment decisions [33]. This is not the case in OeC yet, due to a more complex lymphatic drainage system. However, derived from the findings in our study, we suppose to call the largest LNneg in the resection specimen the 'index LNneg' for the respective oesophageal cancer patient and use this a proxy of patients' immune response in the locoregional resected LNnegs. Using the largest LNneg as surrogate offers a way to simplify the analysis by reducing the need to examine a large number of LNneg, especially given that future studies are likely to yield an even higher number of LNneg than the current OE02 study.

With the information that largest LNneg is a good representator of overall LNneg microarchitecture and therefore patients' immunogenicity, this facilitates the identification of OeC patients who might have

Table 2
Results of mixed linear models of the respective LNneg microarchitectural features with regression coefficient, standard error, 95 % confidence interval (CI) and p-value indicate no sign for systematic bias.

Microarchitectural features of LNneg	Regression coefficient	Standard error	95 % CI lower	95 % CI upper	p-value
%area with lymphocytes	−0.004	0.0169	−0.037	0.029	0.80
%area with histiocytes	−0.010	0.0150	−0.039	0.020	0.51
%area with germinal centres	−0.001	0.003	−0.007	0.005	0.74

Table 3
Results of reliability analysis showing a moderate reliability for lymphocytes and germinal centres with intraclass correlation coefficients of 0.39 and 0.50 respectively, while histiocytes are less reliable with a coefficient of 0.07.

Microarchitectural features of LNneg	Intraclass correlation coefficient (ICC)	Lower bound of 95 % CI	Upper bound of 95 % CI	p-value
%area with lymphocytes	0.39	0.04	0.61	0.02
%area with histiocytes	0.07	−0.45	0.40	0.38
%area with germinal centres	0.50	0.22	0.68	< 0.001

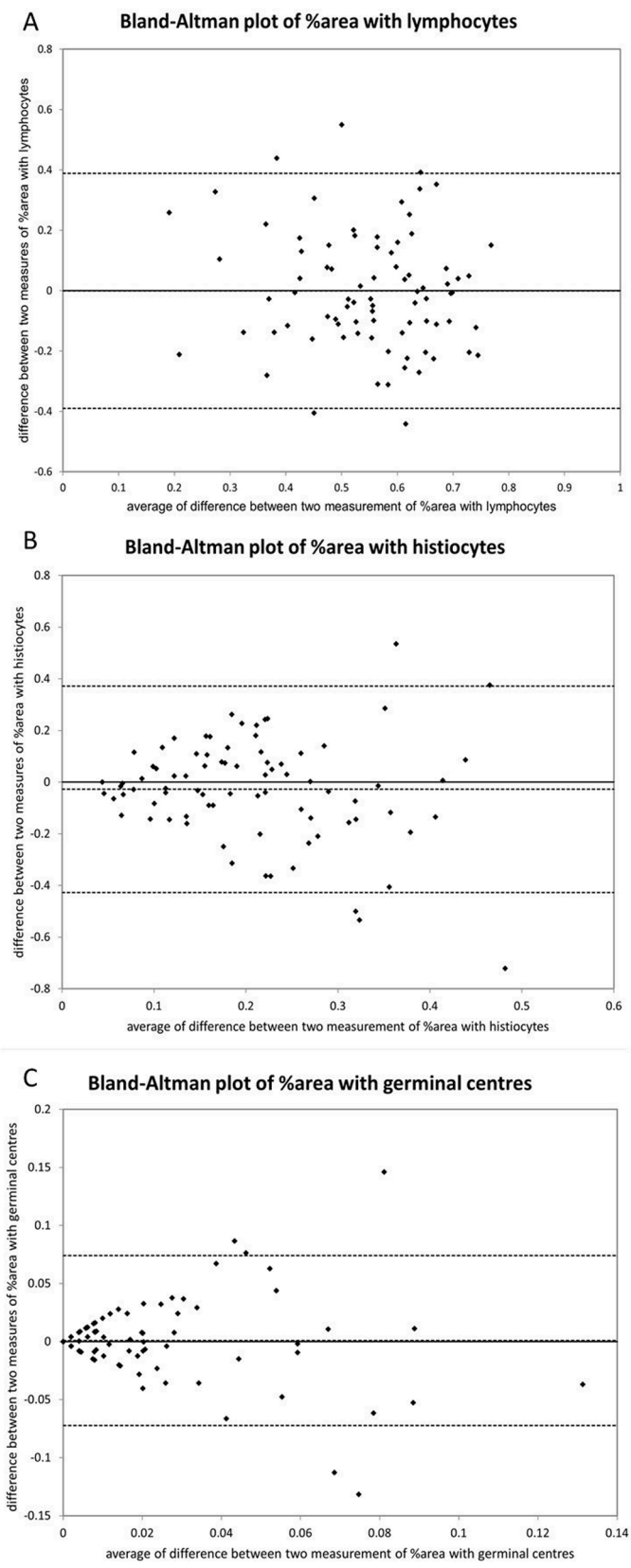

Fig. 4. Bland-Altman plot of %area with lymphocytes, %area with histiocytes and %area with germinal centres (panel A-C).

increased benefit of intensified adjuvant systemic (immune) therapy.

Further research of LNneg with knowledge of precise LN location would be helpful in order to understand locoregional differences in LN morphology. Moreover, it is essential to assess the prognostic significance of LNneg morphology in larger prospective trials, potentially utilizing artificial intelligence algorithms to validate these findings and guide post-surgery treatment decisions.

CRediT authorship contribution statement

David Cunningham: Conceptualization, Data curation, Funding acquisition, Investigation, Writing – original draft, Writing – review & editing. **Heike I. Grabsch:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. **Derek R. Magee:** Formal analysis, Methodology, Software, Writing – original draft, Writing – review & editing. **Gayatri Raghuram:** Data curation, Investigation, Writing – original draft, Writing – review & editing. **Sander M.J. van Kuijk:** Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Elzbieta Budginaite:** Conceptualization, Formal analysis, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Maximilian Kloft:** Conceptualization, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing, Formal analysis, Software, Validation. **Ruth E. Langley:** Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Matthew G. Nankivell:** Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **William H. Allum:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: HG has received honoraria from Astra Zeneca and BMS for scientific advisory board activities not related to the current study. MK has received honoraria from Takeda and BeiGene for scientific advisory board activities not related to the current study. DC has received grant funding from MedImmune, Clovis, Eli Lilly, 4SC, Bayer, Celgene, Leap and Roche not related to the current study.

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County Hospital, Royal Victoria Hospital, Belfast, South Cleveland Hospital, Southampton General Hospital, Southend General Hospital, St Albans City Hospital, St George's Hospital, London, St James's University Hospital, Treliske Hospital, Western Infirmary, Worthing Hospital, Wythenshawe Hospital, Midhurst Community Hospital.

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