

Prognostic significance of negative lymph node long axis in esophageal cancer - results from the randomized controlled UK MRC OE02 trial

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Mini abstract:

This study examines negative lymph node (LNneg) sizes in esophageal cancer patients after surgery. We find that (y)pN0 patients with large LNnegs have a better survival compared to patients with small LNnegs. LNneg microarchitecture analysis suggests a link between LNneg size and activated anti-tumor immune response.

Abstract

Objective: To analyze the relationship between negative lymph node (LNneg) size as a possible surrogate marker of the host anti-tumor immune response and overall survival (OS) in esophageal cancer (EC) patients.

Summary background data: Lymph node (LN) status is a well-established prognostic factor in EC patients. An increased number of LNnegs is related to better survival in EC. Follicular hyperplasia in LNneg is associated with better survival in cancer-bearing mice and might explain increased LN size.

Methods: The long axis of 304 LNnegs was measured in Hematoxylin-Eosin stained sections from resection specimens of 367 OE02 trial patients (188 treated with surgery alone (S), 179 with neoadjuvant chemotherapy plus surgery (C+S)) as a surrogate of LN size. The relationship between LNneg size, LNneg microarchitecture, clinicopathological variables and OS was analyzed.

Results: Large LNneg size was related to lower pN category ($p=0.01$) and lower frequency of lymphatic invasion ($p=0.02$) in S patients only. Irrespective of treatment, (y)pN0 patients with large LNneg had the best OS. (y)pN1 patients had the poorest OS irrespective of LNneg size ($p<0.001$). Large LNneg contained less lymphocytes ($p=0.02$) and had a higher germinal centers/lymphocyte ratio ($p=0.05$).

Conclusions: This is the first study to investigate LNneg size in EC patients randomized to neoadjuvant chemotherapy followed by surgery or surgery alone. Our pilot study suggests that LNneg size is a surrogate marker of the host anti-tumor immune response and a potentially clinically useful new prognostic biomarker for (y)pN0 EC patients. Future studies need to confirm our results and explore underlying biological mechanisms.

Keywords: Esophageal cancer, lymph nodes, immune system, survival, neoadjuvant chemotherapy

INTRODUCTION

Esophageal cancer (EC) is the 6th most common cause of cancer-related death worldwide with 604,100 new cases and 544,076 deaths in 2020 ^{1, 2}. Standard of care for patients with resectable (cT2N0 or higher) EC is currently neoadjuvant chemo(radio)therapy followed by surgical resection ^{3, 4}. The UK MRC OE02 trial was the first phase III trial showing the superiority of neoadjuvant chemotherapy followed by surgery over surgery alone, changing clinical practice⁵.

When determining the prognosis for EC patients, the N status (number of lymph nodes (LN) with metastatic disease) is one of the most important prognostic factors ^{6, 7}. An increasing number of regional LN metastases ('positive' LNs (LNpos)) has been associated with a poorer prognosis in EC patients and patients with other cancer types ⁸⁻¹⁰. There is also evidence to suggest that the LN ratio (number of LNpos/total number LNs) has prognostic value in EC patients ¹¹. Furthermore, it has been proposed recently that an increased number of lymph nodes without metastasis ('negative' LNs (LNneg)) is associated with improved overall survival (OS) in patients with esophageal squamous cell cancer (SCC) ¹². While most studies in the past focused on evaluating the prognostic value of the number of LNpos or the LN ratio ¹³, there are only few studies investigating the prognostic value of the LNpos size in EC patients with SCC, most based on radiological imaging in patients with metastatic disease ^{14, 15}. EC patients with larger LNpos seem to have a poorer survival than those with smaller LNpos ¹⁴⁻¹⁸.

It has been suggested that regional, primary tumor draining LNs have a key role in the host anti-tumor immune response and that increasing LNneg size might be related to a better prognosis ¹⁹. Indeed, a recent study in colorectal cancer patients suggested that the presence of large LNneg is related to a longer progression free survival ^{20, 21}. A study in Dukes B rectal cancer patients suggested that large LNneg are related to increased recurrence free survival and increased histologic anti-tumor response²². Furthermore, follicular hyperplasia in LNneg has been associated with better survival in mice with cancer and might explain increased LN size ²³. These findings were confirmed in colon cancer patients indicating that an increased number of large LNneg is related to an increase in primary tumor infiltrating lymphocytes²⁴.

A recent CT imaging-based study suggested that chemotherapy effects LNpos size in EC patients and the extent of downsizing may be related to patients long-term prognosis ²⁵. However, the current literature is still controversial regarding the effect of chemotherapy on LNneg, and it is not clear whether chemotherapy increases or decreases LNneg size ^{26, 27}. Mice studies suggest that the size of LNneg changes depending on primary tumor regression or progression ²³. The histopathological characterization of LNneg in a small group of EC patients treated by surgery alone found patterns of increased immunosuppression in LNnegs of pathological (p)N1 patients compared to pN0 patients²⁸.

To the best of our knowledge there has been no study investigating the relationship between histologically measured LNneg size and survival in EC patients treated with either surgery alone (S patients) or neoadjuvant chemotherapy followed by surgery (C+S patients).

We hypothesized that EC patients with large LNneg at the time of resection have a better survival regardless of treatment modality.

The aim of the present pilot study was to measure the long axis of LNnegs as a surrogate of LNneg size in the resection specimens of 367 EC patients from the OE02 trial and investigate the relationship between LN size, clinicopathological variables including treatment and patient overall survival.

METHODS

In the UK MRC OE02 trial, 802 patients with histologically or cytologically confirmed, locally advanced resectable esophageal cancer (EC) were randomized to treatment by surgery alone (S patients) or neoadjuvant chemotherapy consisting of two cycles of 5-Fluorouracil (5-FU) and cisplatin followed by surgery (C+S patients) between 1992 and 1998^{3, 4}. For inclusion and exclusion criteria as well as details about patient allocation and precise treatment schedules see publication of the clinical results³. In total, 344 C+S patients and 398 S patients proceeded to surgical resection in the trial. Hematoxylin/Eosin (HE) slides and paraffin blocks from the resection specimen were collected retrospectively. Slides with LNs were available from 179 C+S patients and 188 S patients for analyzes (see figure 1). This represents 48% of the OE02 trial patients who had a resection. Cases were not pre-selected, all available resected LNs were used for analyzes. The REMARK checklist was used for reporting of the methods and the results²⁹ (see supplement, table s-1, <http://links.lww.com/SLA/D443>).

Clinicopathological data of this subset of OE02 trial patients such as histological tumor type, grade of tumor differentiation, tumor regression grade (TRG) according to Mandard classification³⁰, depth of invasion (y)pT category) and LN status ((y)pN category) were established during central histopathology review or extracted from the original pathology reports (tumor size, tumor location, number of LNs, resection margin status) and classified according to UICC TNM 6th edition³¹. Clinical outcome data were extracted from the UK MRC OE02 clinical trial database. The study was approved by the South East Research Ethics Committee, London, UK, REC reference: 07/H1102/111.

The clinicopathological data from our study cohort were compared to the OE02 trial patients who had a resection in order to confirm the representativeness of our subset.

Measurement of lymph node long axis

HE stained slides with tumor free regional lymph nodes ('negative' LN (LNneg)) were available from 159 C+S patients and 145 S patients. Regional lymph nodes with tumor

metastasis ('positive' LN (LNpos)) were available from 110 C+S patients and 126 S patients (see figure 1).

Slides were scanned at 40x magnification using an Aperio XT Scanner and reviewed via a web interface using ImageScope (Aperio ImageScope v11.2.0.780, Leica, Milton Keynes, United Kingdom).

A LN was defined as any size of lymphoid tissue with a clearly identifiable capsule or as an aggregate of lymphoid tissue without capsule measuring >3mm in long axis according to the LN definition of UICC TNM classification 5th ed.³². An irregular border was also regarded as the outline of the LN. Two aggregates of lymphoid tissue were considered as one LNs if they were less than 1mm apart or were present in the same piece of fatty tissue on the same slide (see figure s-1, <http://links.lww.com/SLA/D443>). LNnegs with signs of tumor regression such as fibrosis or mucin lakes without viable tumor cells and therefore must have been tumor positive before treatment (n=12), were excluded from analysis.

The LN border was manually annotated along the outer border in ImageScope using a pen tool and touch screen (Wacom Cintiq 15x pl-550 15 LCD tablet, Krefeld, Germany). Length measurement lines were placed manually using the 'ruler tool' and measurements in mm were provided by the software. The investigator drawing the LN outlines and placing the measurement lines was blinded to any clinicopathological parameters including patient treatment details. The LN status (positive versus negative), outlines and long axis lines were quality controlled by a second independent investigator.

Assessment of negative lymph node microarchitecture

In order to better understand the relationship between LNneg size and survival in EC patients without LN metastasis, we decided to explore the LN microarchitecture features in more detail in these patients. We quantified the LN specific microarchitectural features using point counting with random systematic sampling, a well-established technique for morphometric object quantification³³. We used 250 measurement points $\pm 5\%$ tolerance for each LN (figure 3). Each measurement point was manually reviewed and the tissue type at the tip of the arrow was categorized as lymphocytes (outside of a germinal center), germinal center (GC), histiocytes, vessels, other tissue (fat, connective tissue) or non-informative (artifacts, arrow outside LN capsule etc.) at 5x magnification (see supplement, figure s-2, <http://links.lww.com/SLA/D443> and s-3, <http://links.lww.com/SLA/D443>). For each LNneg, the percentage of area (%area) covered with a particular microarchitecture feature was calculated as follows:

$$\%area = \frac{100}{\text{total number of informative points}} * \text{number of points with a particular feature}$$

Additionally, ratios of particular microarchitectural features were calculated per LNneg: %area of GC/%area of lymphocytes, %area of histiocytes/%area of lymphocytes, %area of GC/%area of histiocytes.

Statistical analyzes

Statistical analyzes were performed using SPSS statistics software (version 25, IBM, Hampshire, England) and R (version 3.5.3)³⁴. The length of the LN long axis was used as a surrogate of LN size. The largest LNneg and the largest LNpos per patient were used for statistical analyzes. All available samples were included. In case of missing data of LNneg or LNpos size, patients were excluded from the respective analyzes.

As we were also interested in a potential effect of neoadjuvant chemotherapy on LN size, LNpos size and LNneg size were compared within treatment arms as well as between treatment arms. Similarly, the relationship between LN size, histological tumor type (adenocarcinoma (AC) versus squamous cell carcinoma (SCC)), predominant grade of tumor differentiation according to WHO classification, depth of invasion ((y)pT), LN status ((y)pN), and resection margin status was investigated for LNneg and LNpos separately per treatment arm. TNM staging was calculated according to TNM classification 6th ed. as this was the TNM classification valid at the time of the original trial reporting. The Kruskal-Wallis test was used to determine if statistically significant differences were present between two or more groups.

As there were no data published which cut-off to use when analyzing the histological LN size, we initially used the radiological LN size cut-off of 10mm for categorizing LNs as being large ($\geq 10\text{mm}$) versus small ($< 10\text{mm}$).

In order to assess the relationship of LNneg size and survival, we fitted a restricted cubic spline (RCS) as described by Gauthier et al³⁵ to the data for each treatment arm, using the R package, Hmisc³⁶. Splines model the relationship between size of largest LNneg and hazard ratio of 5-year overall survival (OS) with individual piecewise curves drawn between a selected number of knot points. The number of knots is chosen based on the number of data points available. Knot points are selected based on percentiles of LNneg size in order to ensure that each curve is informed from an even amount of data. The 'restricted' element comes from restricting the individual curves so that they must meet at the knot points to form one smooth curve across LNneg size. From this curve it can be assessed whether there is an increased or decreased hazard ratio of 5-year OS across different values of LNneg size.

For this study, a three-knot placement for LNneg size was selected to maximize the information used to create the spline curves. These knot points would be utilized as cut-points to categorize LNneg size within the multivariate and univariate model if the relationship was found to be non-linear. The plot resulting from this analysis allowed us to visually assess whether there was a sufficiently large difference in the relationship between LNneg size and survival between treatment arms which would make it necessary to add an interaction term to the multivariate survival model. We chose a per protocol analysis to accurately assess the effects of the different treatments.

The primary endpoint in this study was 5-year OS. Five-year OS was calculated from the date of surgery to the date of death or last follow up using the Kaplan-Meier method. Survival differences were assessed using log-rank statistics. As there was no survival difference between C+S and S patients in our cohort (see result section), survival analyzes were performed for the whole cohort initially.

Multivariate survival analysis was performed using a Cox proportional hazard model including the covariates final treatment (surgery alone versus neoadjuvant chemotherapy followed by surgery), (y)pT category, (y)pN status, and LNneg size. For additional analysis, the variable of the cubic spline approach and interaction treatment-LNneg size were included as covariates in the Cox proportional hazard model. Proportional hazards were tested using the Kaplan-Meier plots.

In addition, we explored whether a potential survival benefit of the LNneg size could be related to LN status ((y)pN0 versus (y)pN1). For this analysis we combined LNneg size dichotomized at the median (LNneg size $<7.41\text{mm}$ or $\geq 7.41\text{mm}$) and (y)pN status and compared OS of patients with (y)pN0+LNneg $<$ median vs (y)pN0+LNneg \geq median vs (y)pN1+LNneg $<$ median vs (y)pN1+LNneg \geq median) using the Kaplan-Meier method. The median LNneg size of all patients (including (y)pN0 and (y)pN1 patients) was 7.41mm, which was used as a cut-off point.

In the (y)pN0 patient subcohort, LN microarchitectural features (%area and ratios) were compared between patients with large LNneg (\geq median LNneg size of N0 patients (8.13 mm)) and small LNneg (<8.13 mm) using Kruskal-Wallis test. A cut-off point of 8.13 mm was used as this was the median LNneg size of (y)pN0 patients. Boxplots were created to visualize the difference of the respective LN microarchitectural feature between groups of LNneg size.

P-values of <0.05 were considered significant. For an overview of the performed analyzes, see the REMARK profile, table s-2, <http://links.lww.com/SLA/D443>. For an overview of used cut-offs see table s-3, <http://links.lww.com/SLA/D443>.

RESULTS

The clinicopathological parameters such as age, sex, (y)pT and (y)pN stage and overall survival (OS) of patients included in the current study were similar to those of all OE02 trial patients who had a resection (see supplement, tables s-4, <http://links.lww.com/SLA/D443> and s-5, <http://links.lww.com/SLA/D443>, figure s-4, <http://links.lww.com/SLA/D443>). Therefore, patients included in our study cohort were considered representative of the OE02 trial population who had a resection.

The median (range) age of patients (n=304) included in the current study was 62.5 years (30-83.1 years). Median (range) follow up time from surgery was 16.9 months (0.06-158.1 months). Median (range) number of resected LNs per patient was 10 (1-67).

Three hundred patients (81.7%) had died at the end of the study period. For a summary of clinicopathological data at the time of randomization and from the resection specimen stratified by LNneg size see table 1, and table s-6, <http://links.lww.com/SLA/D443> for LNpos.

Lymph node size and relationship with clinicopathological parameters

This pilot study included 367 patients in total, 179 C+S patients and 188 S patients (figure 1). Of these 367 patients, 176 (48%) had LNpos and LNneg available for measurement, 63 (17%) had only LNpos available and 128 (35%) had only LNneg available. In total, we measured 2058 LNs, of which 1041 were from C+S patients and 1017 were from S patients.

The largest LNneg size was similar between C+S patients and S patients (median (range) C+S: 7.53mm (1.54-20.43mm) versus S: 7.35 mm (1.38-24.49mm), $p=0.5$).

In S patients, a larger LNneg size was related to a lower number of LNpos ($p=0.018$), lower pN status ($p=0.01$), and lower frequency of lymphatic invasion ($p=0.02$, table 1). Largest LNneg size was not related to primary tumor location, histological tumor type (SCC versus AC), grade of tumor differentiation or blood vessel invasion (all p -values > 0.05).

In C+S patients, largest LNneg size was not related to any of the clinicopathological characteristics (all p -values > 0.05 , table 1).

The size of the largest LNpos differed significantly between C+S patients and S patients (median (range) C+S: 8.7mm (2.43-25.56mm) versus S: 10.97mm (2.24-29.91mm), $p=0.003$). Largest LNpos size was not related to any of the clinicopathological characteristics neither in S patients nor in C+S patients (all p -values > 0.05 , table s-6, <http://links.lww.com/SLA/D443>).

Clinicopathological parameters and relationship with 5-year overall survival

Significant prognostic factors in univariate survival analysis were age at diagnosis ($p<0.001$), (y)pT category ($p=0.001$), (y)pN status ($p<0.001$), grade of primary tumor differentiation ($p=0.01$) and lymphatic invasion ($p<0.001$) (see table 2).

Negative lymph node size and relationship with 5-year overall survival

Using the radiologically used LN size cut-off of 10mm, there was no survival difference between patient with LNneg size ≥ 10 mm and LNneg size < 10 mm (HR: 1.21, 95%CI: 0.91-1.62; $p=0.19$) (see supplement, figure s-5, <http://links.lww.com/SLA/D443>).

Using the restricted cubic spline approach with three internal knots (see methods), we modelled the relationship between risk of death and maximum LNneg size per treatment arm. Figure 2 shows the spline curves for the two treatment arms including the respective confidence intervals. The curve shows a steadily decreasing risk of death with increasing

LNneg size in both treatment arms approximately up to the median LNneg size of all patients (7.41mm). For illustration, a Kaplan Meier graph of the whole patient cohort was plotted to compare OS between patients with LNneg size stratified at the median (figure 4). This illustrates the increased OS probability for patients with large LNneg (HR: 0.73; 95% CI: 0.56-0.94, p=0.017).

For LNneg size greater than the median, the spline curves (figure 3) begin to level off indicating no change in risk of death by increasing LNneg size before possibly slightly raising after LNneg size of 13mm. There is a suggestion from this restricted cubic spline approach, that the relationship between LNneg size and OS may be non-linear.

While we appreciated that both treatments showed somehow similar trends in their splines with respect to survival and despite confidence intervals were largely overlapping, the distance between the curves of S and C+S patients was felt to be sufficiently big enough to justify including an interaction term for treatment within the multivariate model. The three internal knots were used as cut-points to categorize the LNneg size variable into three groups within the multivariate survival models to reflect the difference across the knot groups.

Multivariate analysis

Multivariate analysis with the categorical covariates treatment, (y)pT status, (y)pN status, LNneg size groups by spline cut-points and treatment interaction of LNneg groups by spline cut-points confirmed (y)pN status (HR: 1.56 (1.16-2.15), p=0.006) and (y)pT status (HR: 1.87 (1.29-2.87), p=0.002) as independent prognostic variables. None of the other variables including LNneg size were significant in multivariate analysis, see table 2. There was no significant treatment interaction.

Exploratory overall survival analysis combining (y)pN status and negative lymph node size

We explored whether the LNneg size parameter provides additional information to the LN status and categorized patients according to their LN status and LNneg size into 4 groups: (y)pN0+LNneg<median; (y)pN0+LNneg≥median; (y)pN1+LNneg<median; (y)pN1+LNneg≥median. Univariate survival analysis showed a significant survival difference between groups (p<0.001, see figure 4). (y)pN0 patients with large LNneg had the best OS, followed by (y)pN0 patients with small LNneg, whereas (y)pN1 patients had the poorest survival irrespective of LNneg size. Interestingly, the survival of the (y)pN1 patients seems to be better during the first 2 years in the presence of large LNneg, whereas the survival of (y)pN1 patients seems to be no longer related to LNneg size after 2 years.

Negative lymph node microarchitecture of (y)pN0 patients

To find out whether microarchitectural changes of the largest LNneg in the (y)pN0 patients might be related to the better survival of some of the ypN0 patients, we analyzed the LN microarchitecture features of (y)pN0 patients and their relationship with LNneg size.

In (y)pN0 patients with small LNneg (LNneg size <8.13mm), the median percentage of LN area (%area) with lymphocytes was 61.1% (range: 22.6%-84.6%) compared to 50.9% (range: 10.3%-75.3%) in (y)pN0 patients with large LNneg (LNneg size ≥8.13mm), $p=0.02$, see figure 5a-c. The %area of germinal centers was higher in (y)pN0 patients with large LNneg, although not reaching statistical significance (median (range) 1.6% (0%-15.4%) vs 1.2% (0%-8.7%), $p=0.14$). The %area containing histiocytes did not differ between patients with small and large LNneg (13.2% (2.3%-63.1%) vs 20.1% (0-73%)); $p=0.2$). The germinal centers/lymphocytes ratio was higher in patients with large LNneg with borderline significance (0.03 (0-0.3) vs. 0.02 (0-0.14); $p=0.05$), suggesting that large LNnegs may have increased formation of germinal center structures compared to small LNnegs, see figure 5d-f.

DISCUSSION

Research aiming to identify new potential prognostic or predictive biomarker has mainly focused on the primary tumor or on the number of positive lymph nodes (LN), while the potential prognostic impact of features that characterize LNs without metastasis ('negative' LNs (LNneg)) has not been investigated in detail in patients with esophageal cancer (EC). Earlier studies in colorectal cancer suggested that the size of the LNneg could be an indicator of increased host anti-tumor immune response and could be related to a survival advantage^{21, 22, 24}.

The current study aimed to explore the relationship between the LN size and prognosis in (EC patients from the UK MRC OE02 trial treated with surgery alone (S patients) or neoadjuvant chemotherapy followed by surgery (C+S patients). We used the length of the long axis of a LN as surrogate of LN size. Overall, the LN sizes in our study were similar to that found by others³⁷. We found that patients with large LNneg in the resected specimen survived significantly longer than patients with small LNneg. Thus, this is the first study to suggest a potential prognostic role of LNneg size in EC patients irrespective of treatment modality or histological tumor type. Our EC results are concordant with previous findings reported in colorectal and gastric cancer³⁷⁻³⁹.

Lymph node status (pN) is known to be one of the most important prognostic factors in EC patients, more important than primary tumor regression grade as we showed in a previous study in the OE02 trial patients⁷. Although LNneg size was not an independent prognostic marker in multivariate analysis, our exploratory analysis combining the LNneg size with the LN status revealed that (y)pN0 EC patients with large LNneg have a better survival than (y)pN0 EC patients with small LNneg. This could potentially suggest that the LNneg size might be a clinically useful marker to identify (y)pN0 patients who may benefit from further adjuvant treatment.

Size changes in regional LNneg in pancreatic cancer and colorectal cancer patients have been related to an increased host anti-tumor immune response resulting in follicular hyperplasia with the formation of germinal centers, proliferation of lymphocytes in the medullary or paracortical area and/or sinus histiocytosis due to incoming tumor derived antigens^{40, 41}. It

has been shown that secondary follicles not only become hyperplastic but may also develop germinal centers and enlarge their underlying dendritic network possibly explaining the overall changes in LN size during immune response activation⁴²⁻⁴⁴. Our pilot study of the LN microarchitecture found that large LNnegs in (y)pN0 patients have significantly less lymphocytes located outside of germinal centers and a higher ratio of germinal centers to lymphocytes confirming studies showing immune response leading to LN size changes^{45, 46}. This could possibly provide an explanation for the improved overall survival of (y)pN0 patients with large LNneg.

A retrospective study in colorectal cancer suggested that a higher number of LNneg might be related to an increased lymphocyte infiltration in the primary tumor and better survival⁴⁷. Unfortunately, LN size measurements were not included in this study, so results cannot be directly compared with our findings⁴⁷. In the current study in EC patients, we found a relationship between large regional LNneg and lower number of LNpos. However, related to the relatively small number of patients in subgroups, we are unable to distinguish between a survival benefit related to enlarged LNneg (e.g., due to augmented immune activation in regional tumor draining LN) and a survival benefit due to overall lower metastatic burden.

To the best of our knowledge, the effect of chemotherapy on the histological size of regional LNneg has not been investigated in EC resection specimens. As the OE02 trial had a surgery alone arm and a chemotherapy plus surgery arm, we were able to assess whether neoadjuvant chemotherapy induces a change in the size of the LNneg and whether the prognostic value of the LNneg size was different between treatment arms. To our surprise, neither LNneg size nor prognostic value of large LNneg size were different between treatment arms. We had expected that due to the chemotherapy induced immunogenic cell death, LNneg size would increase⁴⁸. Indeed, LNneg size was found to be increased in rectal cancer patients treated with chemoradiotherapy, compared to patients treated with surgery alone⁴⁹. In contrast, Schröder et al did not find a difference in LNneg size when comparing LNneg size in a small series of EC SCC patients treated with either surgery alone or chemoradiotherapy⁵⁰. It should be emphasized that the aforementioned two studies combined chemotherapy with radiotherapy which makes direct comparison to our findings difficult.

The current study has some limitations. We used retrospectively collected material from a trial which recruited EC patients between 1992 and 1998. The OE02 trial protocol did not include specific guidance for the surgeons, regarding the type of LN dissections nor for the pathologist, on handling the resection specimen. We therefore had to assume that it was common practice to cut through the center of the LN at the time of specimen cut up by pathologists. Reassuringly, the LN sizes measured in the current study are comparable to those reported by others in more recent studies³⁷. LN sizes can also increase in case of an infection, clinical data to this effect is unavailable but presence of an infection could have influenced our results. The total number of resected LNs found per patient would be considered relatively low compared to current standards. This could be related to the LN dissection by the surgeon and/or pathologist, as the type of lymphadenectomy is unknown but also to the unavailability of the slides to us after such a long time of material storage. We

chose to analyze the largest LN size when patients had multiple LNs assuming that large LNs are more likely resected and found in the resected specimen by the pathologist than small ones. A larger number of LNs for all patients might have allowed a more detailed analysis of the effect of number of large LNs or combination of large LNneg with LNpos sizes. Unfortunately, data on the LNneg location was not available consequently we could not relate this to the tumor location. Future analysis in larger groups of patients including LNpos is needed.

In summary, this pilot study using resection material from EC patients randomized to the OE02 trial provides first insights into the potential importance of assessing LNneg size to predict prognosis in EC patients and in particular to identify EC patients with (y)pN0 status with a poorer prognosis who might benefit from additional post-surgical therapy. Our results require validation in an independent cohort and further studies to better understand the underlying biological mechanism of increased LNneg size in some patients. Based on our results, we hypothesize that large regional LNneg size identified at the time of diagnosis might be a potential clinically useful marker for the identification of highly immunogenic EC. Thus, there is a clinical need to improve radiological staging of LNs and in particular recognition of LNneg. This might be achieved in the first instance by combining pathology-based LN size studies with matched radiological imaging studies at the time of surgery to translate histological findings into patient management decisions in the future and personalize risk-stratification in EC patients.

ADDITIONAL INFORMATION

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Albans City Hospital, St George's Hospital, London, St James's University Hospital, Treliiske Hospital, Western Infirmary, Worthing Hospital, Wythenshawe Hospital, Midhurst Community Hospital

Ethics approval and consent to participate

The study was approved by the South East Research Ethics Committee, London, UK, REC reference: 07/H1102/111. The study was performed in accordance with the Declaration of Helsinki.

Consent for publication

Consent for publication was not required in this study as it does not contain individual person's data in any form (including individual details, traceable images or videos).

Data availability

Data (e.g. images) and metadata are available upon request.

Competing interests

All authors have declared no conflicts of interest.

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Figure legends

Figure 1. Flow diagram showing the number of patients originally randomized in the OE02 trial to either neoadjuvant chemotherapy and surgery or surgery alone and for whom lymph node sizes could be determined from virtual histopathological slides.

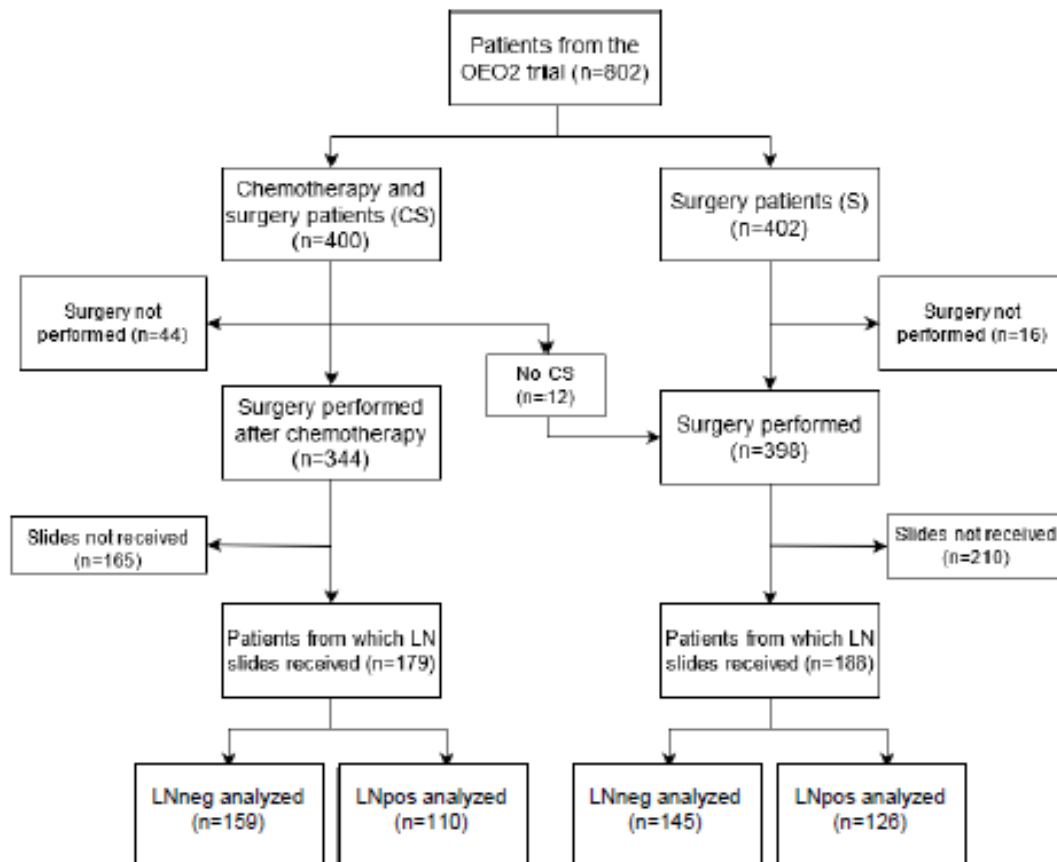


Figure 2. Restricted cubic spline plot illustrating the relationship between risk of death (Hazard ratio) and negative lymph node size separately for S patients (red line) and CS patients (blue line). The arrows mark the knots identified with the restricted cubic spline approach at 3.52mm, 7.41mm and 12.83mm. The dashed lines and shaded areas highlight that the confidence intervals for the two treatment groups are largely overlapping. Note that the function used to create the curves does not utilize the lower and upper 5% of data points as extreme values can have a large distorting effect on the curve leading to potential misinterpretation. This results in the surgery only curve being shorter than the chemotherapy plus surgery curve. In both treatment arms, the risk of death decreases with increasing LNneg size up to a lowest point around the second knot at 7.41mm. The effect of the LNneg size on the risk of death appears to be larger in the S patients. However, due to the overlapping confidence intervals and probably related to relatively small sample size, this difference is not significant.

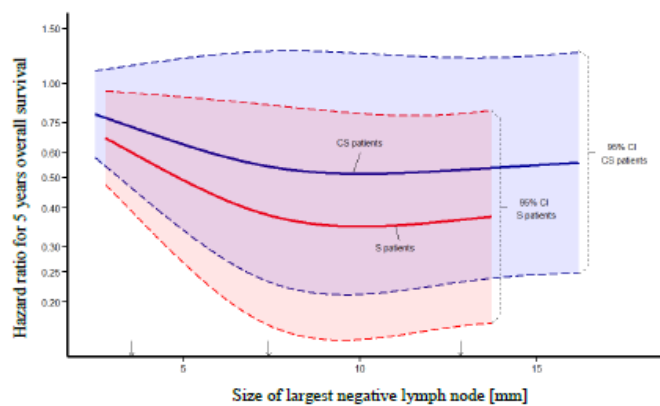


Figure 3. 5-year overall survival (OS) stratified by size of the negative lymph node (LNneg). LNneg size was dichotomized at the median (7.41mm). The Kaplan Meier plot shows that esophageal cancer patients with larger LNneg survive significantly longer than those with smaller LNneg. HR: 0.73, 95% CI: 0.56-0.94, p=0.017. 5-year OS 28% vs 19%.

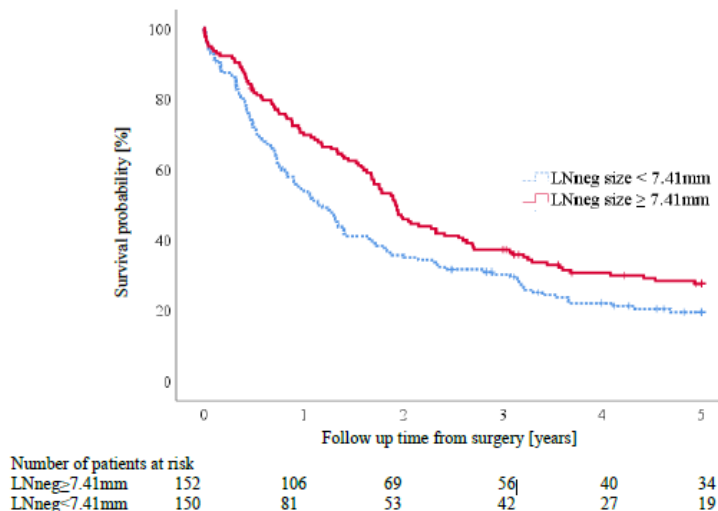


Figure 4. 5-year overall survival (OS) stratified by combined groups of (y)pN status and LNneg size. The Kaplan Meier plot shows that the survival of EC patients differs significantly depending on the stratified group ($p < 0.001$). N0+LNneg<median: HR: 1.64, 95%CI: 0.97-2.76; $p = 0.062$; N1+LNneg<median: HR: 2.46, 95% CI 1.62-3.72, $p < 0.001$; N1+LNneg \geq median: HR: 2.07, 95% CI 1.36-3.17, $p = 0.001$ (reference group: N0+LNneg \geq median). 5-year OS: N0-small LNneg:26,9%, N0-large LNneg: 42,4%, N1-small LNneg: 16,5%, N1-large LNneg: 18,6%.

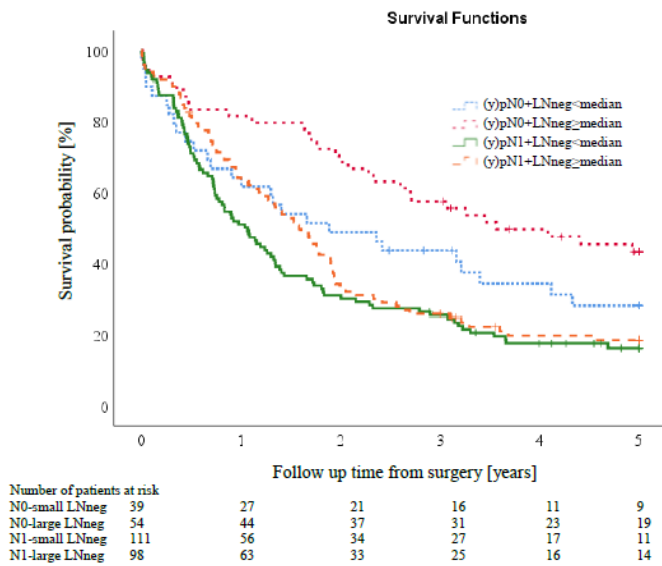


Figure 5a-f. Boxplots showing the difference in microarchitecture between large and small LNneg in the N0 subgroup of the OE02 cohort (n=93). Panel a, b and c showing percentage of area (%area) covered with lymphocytes, germinal centers and histiocytes respectively. Panel d, e and f showing ratios of germinal centers to lymphocytes, histiocytes to lymphocytes and ratio of germinal centers to histiocytes, respectively. Box represents 25th to 75th percentile, line in the box indicates the median, whiskers indicating lower and upper quartiles.

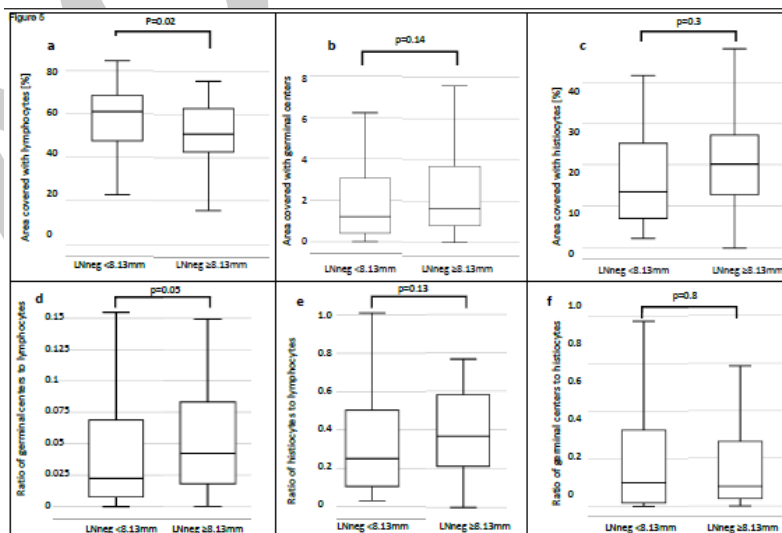


Table 1. Clinicopathological characteristics for chemotherapy plus surgery and S patients stratified by LNneg size (cut-off point= median 7.41mm).

Characteristics		All patients with LNneg		Chemo+surgery				Surgery alone					
		n	%	LNneg size <7.41mm		LNneg size ≥ 7.41mm		p-value	LNneg size <7.41mm		LNneg size ≥ 7.41mm		p-value
				n	%	n	%		n	%	n	%	
Sex	Male	222	73	59	75	65	81	0.36	56	77	42	58	0.05
	Female	82	27	20	25	15	19		17	23	30	42	
Age at diagnosis (median)		62.3		63.3		59.6		0.12	64.1		59.2		0.08
Location of primary tumor	Lower	199	64	55	69	56	70	0.54	45	61	43	60	0.79
	Middle	70	23	18	23	15	19		18	25	19	26	
	Upper	35	13	6	8	9	11		10	14	10	14	
Histology of primary tumor	AC	213	70	57	72	18	23	0.89	52	23	21	29	0.41
	SCC	73	24	17	22	57	73		17	71	47	65	
	Other	18	6	5	6	5	4		4	6	4	6	
(y)pT	T0*	8	3	5	6	3	4	0.12	0	0	0	0	0.75
	T1	19	6	3	4	9	10		5	7	2	3	
	T2	30	10	6	8	11	14		5	7	8	11	
	T3	238	78	61	78	57	71		60	82	60	85	
(y)pN	T4	8	3	4	4	0	0	3	4	1	1	0.82	
	N0	93	31	26	33	27	34	13	18	27	37		0.01
Grade of differentiation	N1	211	69	53	67	53	66	60	82	45	63	0.53	
	Moderate/Well	154	51	40	51	47	59	32	44	35	48		0.34
	Poor	134	44	32	41	27	34	40	56	35	48		
Lymphatic invasion	Unknown	16	5	7	8	6	7	1	1	2	4	0.96	
	No	199	64	58	73	59	74	33	45	49	67		0.02
Blood vessel invasion	Yes	105	36	21	27	21	26	39	55	24	33	0.98	
	No	166	55	73	92	74	92	58	79	61	85		0.37
Resection margin status	Yes	36	45	6	6	6	8	15	21	9	15	0.15	
	Negative	199	65	50	64	59	74	46	63	44	61		0.85
	Positive	80	26	24	30	15	18	20	27	21	29		
Tumour regression grade primary tumour	Unknown	45	9	5	6	6	8	7	10	7	10	0.67	
	TRG 1-3	39	25	21	27	18	23						
	TRG 4-5	135	75	58	73	62	77						

AC: Adenocarcinoma SCC: Squamous cell carcinoma *T0: No residual tumour in the specimen

Location primary tumor refers to lower, middle and upper thirds of the esophagus

Table 2. Univariate and multivariate overall survival analysis in the study cohort.

	Univariate		Multivariate	
	HR (95% CI)	p-value	HR (95%CI)	p-value
Sex				
<i>Male versus female</i>	1.23 (0.94-1.63)	0.14		
Age at diagnosis	1.03 (1.01-1.04)	<0.001		
Treatment				
<i>Chemotherapy plus surgery versus surgery alone</i>	1.14 (0.9-1.44)	0.28	0.98 (0.48-2.25)	0.96
Location primary tumor				
<i>Middle versus lower</i>	1.2 (0.89-1.62)	0.24		
<i>Upper versus lower</i>	0.67 (0.43-1.05)	0.08		
Histology primary tumor				
<i>AC versus SCC</i>	0.9 (0.69-1.19)	0.46		
<i>Other versus SCC</i>	0.84 (0.42-1.17)	0.62		
(y)T status				
<i>T3/T4 versus T0-T/T2</i>	1.5 (1.22-1.84)	<0.001	1.87 (1.29-2.87)	0.002
(y)N status				
<i>N1 versus N0</i>	2.01 (1.5-2.68)	<0.001	1.56 (1.16-2.15)	0.006
Grade of differentiation				
<i>Poor versus Moderate/well</i>	1.28 (0.98-1.67)	0.07		
Lymphatic invasion				
<i>Positive versus negative</i>	1.82 (1.38-2.4)	<0.001		
Blood vessel invasion				
<i>Positive versus negative</i>	1.96 (1.33-2.88)	0.001		
Resection margin status				
<i>Positive versus negative</i>	2.01 (1.56-2.59)	<0.001		
Tumour regression grade primary tumour				
<i>TRG 45 versus TRG 123</i>	1.45 (0.99-2.11)	0.06		
LNneg size				
<i>LNneg ≥ 7.41mm versus LNneg</i>	0.73 (0.57-0.95)	0.019		

<7.41mm				
Restricted cubic splines of LNneg size				
<i>Spline group 2 versus group 1</i>			0.79 (0.45-1.62)	0.46
<i>Spline group 3 versus group 1</i>			0.67 (0.38-1.37)	0.22
<i>Spline group 4 versus group 1</i>			0.93 (0.48-2.3)	0.87
Treatment interaction of LNneg size splines				
<i>Treatment*spline group 2</i>			1.18 (0.48-2.78)	0.71
<i>Treatment*spline group 3</i>			0.95 (0.36-2.13)	0.9
<i>Treatment*spline group 4</i>			0.74 (0.21-2.1)	0.6

Spline groups refer to cut-point defined groups of the restricted cubic spline approach.

Group 1: <3.52mm, group 2: 3.52-7.41mm, group 3: 7.41mm-12.83mm, group 4: >12.83mm.