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Large negative lymph nodes – a surrogate for immune activation in rectal cancer patients?

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ABSTRACT

Aim: The size of regional, tumor draining lymph nodes without metastasis (LNneg) found in rectal cancer resection specimens varies and seems to be related to patient survival. Yet, the histopathological features influencing LNneg size in rectal cancer have not been studied in detail. Our pilot study focused on investigating the relationship between lymph node (LN) size and LNneg microarchitecture in rectal cancer (RC) resection specimens.

Method: In this retrospective cohort study, resection specimens from 146 RC patients, treated with either surgery alone (n = 29) or neoadjuvant therapy followed by resection (n = 117), were included in the study. Histology of LNnegs was reviewed to establish number of lymphoid follicles and presence of intranodal fat. Longest long axis and area of each LN were measured digitally.

Results: 1830 LNnegs were measured. The microarchitecture was analyzed in a subset of 680 LNnegs. 153 (22.5 %) LNnegs contained intranodal fat. After neoadjuvant treatment, presence of intranodal fat was related to smaller LNneg area (median (range) area of LNneg without intranodal fat: 4.51 mm^2 (0.15-46.89 mm^2), with intranodal fat: 3.46 mm^2 (0.12-27.22 mm^2), $p = 0.048$). A higher number of lymphoid follicles was related to a larger LNneg area in both patient groups ($p < 0.001$).

Conclusion: Our pilot data suggest that in rectal cancer the presence of large regional LNnegs may reflect increased immune activation due to tumor related antigens. Further studies are warranted to investigate whether histologically visible microarchitectural features of LNnegs such as lymphoid follicles translate to particular features in radiological images and hence could potentially help to identify LNneg with more certainty at the time of pre-treatment disease staging.

1. Introduction

The number of lymph nodes (LNs) with metastasis (LNpos) is an important prognostic factor for colorectal cancer patients and determines the N status within the TNM staging [1]. A number of studies proposed that the absolute total number of LNs retrieved from the resection specimen and/or LN ratio (LNpos/LNs) are related to prognosis in rectal cancer (RC) patients [2–4].

TNM staging is currently the main clinical tool to determine a patient's prognosis and treatment plan. The N status refers to the number of regional LNpos and is one of the most important prognostic markers with therapeutic consequences [4]. LNs without metastasis (LNnegs) are increasingly being studied in patients with colorectal cancer [2,5–11] and it has been suggested that larger LNnegs in patients with pT1 or pT2 colon cancer might be related to a favorable outcome [12]. Murphy et al. found a better five-year survival in Dukes stage 3 RC patients with a higher number or a larger size of LNnegs [3]. A high LN yield in RC resection specimens has been related to a better prognosis [13]. It has also been shown that the longer the LN long axis, the higher the LN yield in colon cancer resection specimens [14] and that the total number of resected LNs is an independent prognostic factor [2].

Variations in regional LN size have been demonstrated in RC patients for LNpos and LNneg [5,6,10]. There is evidence to suggest that the LN microarchitecture changes depending on the status of the immune system of the host [15]. LN size differences have been related to prognosis and immune activation in previous studies [16,5,12]. There is

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also evidence suggesting LN size may be related to the extent of intranodal connective tissue present without evidence of prior infection or cancer [17,18], and smaller LNs may reflect a reduced function of the immune system in the elderly [19]. Increased LN size has been related to reactive hyperplasia within the LN, e.g. increased number of immune cells in LNs draining infections or neoplasm and thus were related to the immunological response by the patient [20,16]. In RC patients, large LN size was found to be independent of tumor size [5]. Other morphological changes, like presence of intranodal fat have been previously described in LNs and may influence LN size [17]. Chemoradiotherapy (CRT) had no effect on LNneg size in RC according to Kim et al. [21], whereas Heijen et al., although not distinguishing between size of LNpos and LNneg, reported a decrease in LN size after CRT [22]. However, the microarchitecture of LNnegs retrieved from RC resection specimens and its relationship with LNneg size has not been studied in detail until now.

We hypothesized that LNneg size variation in RC patients is related to the variation in the number of primary and secondary lymphoid follicles and the presence of intranodal fat.

In this pilot study, we aimed to investigate the relationship between LNneg microarchitecture (number of primary and secondary lymphoid follicles, presence of intranodal fat), LNneg size (largest diameter), LNneg area (mm^2) and clinicopathological characteristics (age, sex, LN location, depth of invasion (pT) and lymph node status (pN)) in 146 RC patients treated by neoadjuvant therapy and surgery or surgery alone.

2. Material and method

This retrospective cohort study included 1951 lymph nodes (LNs), both with and without metastasis, from 146 consecutive patients with rectal cancer (RC) with (y)pT0 to (y)pT4 disease who were treated with neoadjuvant therapy followed by surgery (n = 89) or surgery alone at the Department of Surgery, Maastricht University Medical Center+ (MUMC+), Maastricht, NL between 2008 and 2015 (Fig. 1). Patients with recurrent rectal cancer were excluded. Clinicopathological data was retrieved retrospectively from a departmental database, surgery reports and pathology reports. The study was approved by the local regulatory body (METC 2018−0672).

2.1. Data collection

All diagnostic Haematoxylin/Eosin (H&E) stained slides from the RC resection specimens were retrieved from the local pathology archive and slides containing LNs were scanned with a HP flatbed scanner (N6310 HP Scanjet) set at 600ppi resolution. If multiple slides with LNs of the resection specimen were missing, the patient was excluded from the study. For microarchitecture analysis, scans were imported into image analysis software (Medical Image Manager (MIM), HeteroGenius Ltd. Leeds, UK). The pathology was done according to best practice and the pathology reports were used to identify LNs containing metastasis. The largest section of each LNnegs and LNpos was manually outlined using the curve tool at 10x magnification. The LN capsule was included in the LN outline, fatty tissue and vascular structures outside of the LN capsule were excluded (Fig. 2). The MIM software calculated the area and longest axis after appropriate calibration.

In a randomly selected subset of 51 RC patients, with similar sex and treatment compared to the full dataset, LNnegs were reviewed using a conventional light microscope with a 2.5x objective to count primary lymphoid follicles, secondary lymphoid follicles and establish whether fat was present inside the LN. The slide review and measurements were done blinded to clinicopathological parameter. A lymphoid follicle was classified as primary follicle if no germinal center was visible and as a secondary follicle if a germinal center was clearly visible within the follicle (Fig. 3) [15]. The number of primary and secondary lymphoid follicles was initially counted separately in 4 categories: 0 lymphoid follicles, 1–3 lymphoid follicles, 4–9 lymphoid follicles and ≥10 lymphoid follicles. Subsequently, the primary and secondary lymphoid follicle categories were combined for a final lymphoid follicle score as follows: if the sum of the primary lymphoid follicle score and the secondary lymphoid follicle score was 0, the final lymphoid follicle score was 1; if the sum was 1, the final lymphoid follicle score was 2; if the sum was 2, the final lymphoid follicle score was 3; if the sum was 3, the final lymphoid follicle score was 4. If the sum was > 3, the final lymphoid follicle score was 5. Intranodal fat was categorized as being present if one or more adipocytes were visible within the H&E stained LNneg. Adipocytes outside the capsule or LN tissue were not considered.

Five percent of the examined LNnegs were randomly selected for re-evaluation by the primary observer and a second observer to assess intra- and interobserver agreement of the measurement.

Information on the location of individual LNnegs within the circumferential mesorectal fat was included in the analyses if available from patient records according to the Beets-Tan protocol [23]. For resection specimens which were dissected using the Beets-Tan protocol, the rectum including surrounding fat was sliced perpendicular to its longitudinal axis and the pathologist recorded the location of the lymph node in the mesorectal fat using a clock face with 12 o'clock being located anterior.

Findings from the randomly selected 51 patients were compared between those who had neoadjuvant treatment followed by surgery and those treated with surgery alone (S).

2.2. Statistical analysis

Statistical analysis was performed using SPSS statistics software (version 23, IBM, Hampshire, England). Kruskal-Wallis test and Mann Whitney-U test were used to investigate the relationship between LN long axis and the patients' sex, (y)pT, (y)pN and LN location in the mesorectal fat. Kruskal-Wallis test and Mann Whitney-U test were also used to analyze the relationship between LNneg area, lymphoid follicle score and presence of intranodal fat. Results are reported either as absolute values or median (range). Two-sided p-values less than 0.05 were considered significant.

3. Results

The current study included 97 (66 %) males and 49 females with a median age of 68 years (range: 42–86 years). 118 (80.8 %) patients had neoadjuvant treatment before surgical resection. Of the patients with neoadjuvant treatment, 116 (98.3 %) patients received radiotherapy, 40 (33.9 %) patients received 5 × 5 Gy and 76 (64.4 %) patients received 5-flourouracil (5-FU) long course chemoradiotherapy. 66 (55.9 %) patients were given adjuvant treatment with capecitabine, 16 (13.6 %) patients were treated with capecitabine and oxaliplatin and 24 (20.3 %) patients with only radiotherapy. Details of adjuvant chemotherapy were unknown for 14 (11.7 %) patients. 106 (72.7 %) patients underwent a lower anterior resection with total mesorectal excision, 17 (11.6 %) patients had an abdominoperineal rectum extirpation and the remaining patients underwent a Hartmann procedure (n = 8, 5.5 %) or pelvic exenteration (n = 4, 2.7 %). Type of surgery was unknown for 11 (7.5 %) patients. In total, 1951 LNs were retrieved from the 146 resection specimens (Table 1). A median (range) of 12 LNs (3–38 LNs) and a median (range) of 11 LNnegs (3–37 LNnegs) were retrieved per specimen. 121 (6.2 %) LNs contained tumor metastasis and 1820 (93.8 %) were negative (Table 1). The long axis of LNnegs was significantly shorter than that of LNpos (median long axis (range) LNneg 2.8 mm (0.42–27.78 mm) *vs.* LNpos 5.3 mm (1.53–22.96 mm), $p < 0.001$).

The microarchitecture was assessed in detail in LNnegs from 51 RC patients (Fig. 3). The microarchitecture of LNpos was not investigated due to the overall relatively low number of LNpos (n = 60) available in this subcohort. From the 51 patients, 31 (60.7 %) were males, the median (range) age was 69 years (42–86 years), and 34 (66.7 %) had

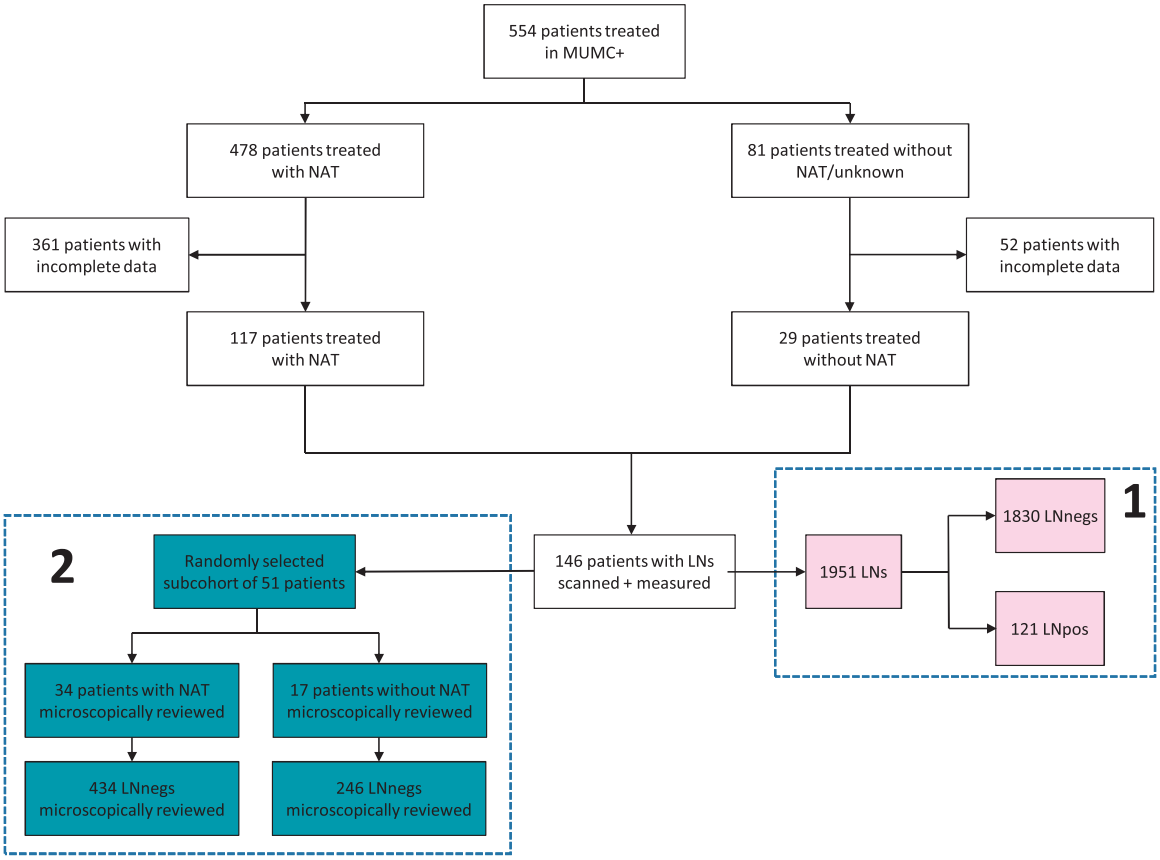

Fig. 1. Consort diagram showing the inclusion process of the lymph nodes (LNs). Selection 1 was used for the large cohort analysis. Selection 2 was used for the subcohort analysis of histological characteristics. NAT = neoadjuvant treatment, LN = lymph node, LNneg = negative lymph node, LNpos = positive lymph node.

neoadjuvant treatment before surgical resection. In this subcohort, 14 (27.5 %) patients received neoadjuvant treatment with 5 × 5 Gy radiotherapy, 4 (7.8 %) patients with 5 × 5 Gy radiotherapy combined with chemotherapy and 16 (31.4 %) patients with 5-FU long course chemoradiotherapy. A total of 680 LNnegs was retrieved from these 51 patients with a median (range) of 11 (2–37) LNnegs per patient. The median (range) area of this subset of LNnegs was 4.40mm^2 (0.12-51.43 mm^2). The LNneg area was smaller after neoadjuvant treatment (median (range) after neoadjuvant treatment 4.24 mm^2 (0.12-46.89 mm$^{2)}$ versus 4.67 mm^2 (0.39-51.43 mm^2) without neoadjuvant treatment (p = 0.026)).

3.1. Relationship of lymph node long axis length and patient characteristics

The LNneg long axis was longer in males compared to females in the cohort of 148 patients (Table 2). The length of the LNneg long axis was related to the depth of invasion ((y)pT) and lymph node status ((y)pN). The LNneg long axis of patients with (y)pT0 RC was shorter than in patients with (y)pT4 RC (median (range) LNneg long axis (y)pT0 (n = 92): 2.24 mm (0.79–9.25 mm) *vs.* (y)pT4 (n = 258): 3.38 mm (0.72–9.98 mm), p < 0.001). Median (range) of the LNneg long axis in patients with (y)pN0 (number of LN n = 1139) was 2.73 mm (0.42–15.49 mm); (y)pN1 (n = 520): 3.04 mm (0.43–27.78 mm) and (y)pN2 (n = 171) 3.12 mm (0.56–11.23 mm), respectively, p < 0.001). There was no relationship between (y)pT category and length of the

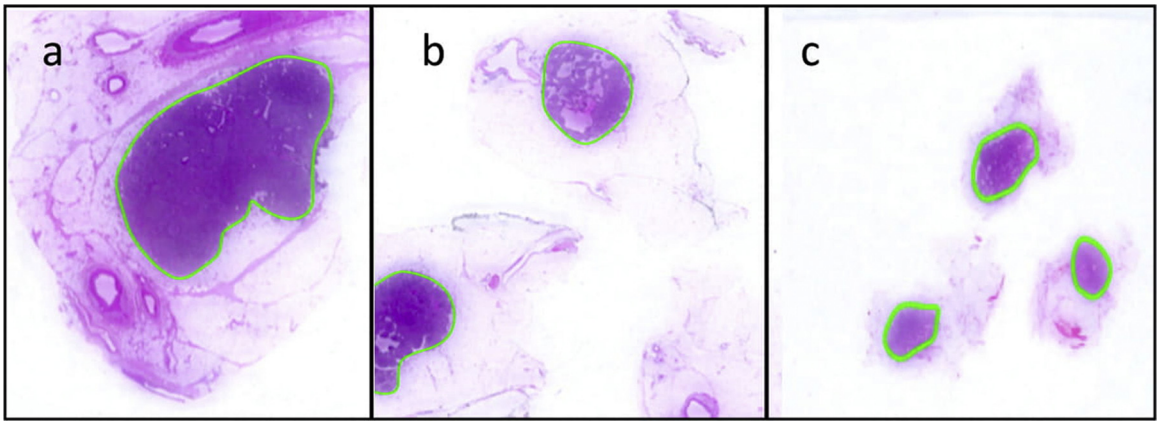

Fig. 2. Examples of manual outlining of lymph nodes using MIM image analysis software after scanning of Haematoxylin/Eosin stained sections on a flatbed scanner at 600 ppi.

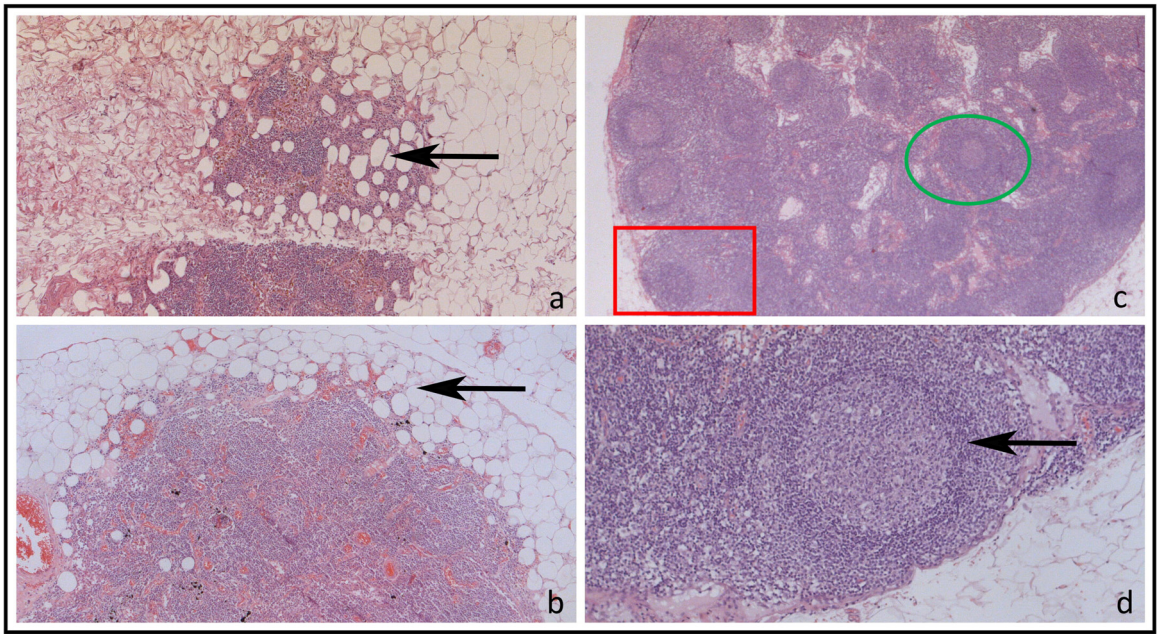

Fig. 3. Representative images of lymphoid follicles and intranodal lipomatosis. a. shows intranodal fat (arrow). b. shows lipomatosis around the LN and absence of LN capsule (arrow). c. shows a LN with multiple primary lymphoid follicles (for example see square) and secondary lymphoid follicles (see ellipse). d. shows a secondary lymphoid follicle at higher magnification (arrow).

LNpos long axis.

For 775 (79.2 %) LNs of the subcohort, the location within the mesorectal fat was known using a clock face with 12 o'clock being located anteriorly according to the Beets-Tan protocol [23]. There was no significant relationship between the length of the long axis of LNnegs (n = 727) and location within the mesorectal fat (median (range) LNneg largest diameter of 3.2 mm (0.50–9.4 mm) posterior and 3.7 mm (0.50–5.2 mm) anterior; p = 0.686).

3.2. Negative lymph node microarchitecture and relationship to lymph node size

LNneg microarchitecture was assessed based on number of lymphoid follicles and presence of intranodal fat in 51 patients. LNneg size was related to the total follicle count ($p < 0.001$) irrespective whether patients had neoadjuvant treatment (n = 434 LNnegs) or not (n = 246 LNnegs). In neoadjuvant treatment patients, 161 LNnegs with

Table 2
Relationship between largest diameter of positive or negative lymph nodes and sex.

	Median largest long axis (range) in mm		
	Males (n = 97)	Females (n = 49)	p-value
LNpos	5.33 (1.88–22.96)	5.12 (1.53–16.56)	0.327
LNneg	2.92 (0.43–27.78)	2.61 (0.42–9.39)	< 0.001

Table 1
Relationship between number of lymph nodes and clinicopathological variables.

	Lymph nodes full patient cohort (n = 1951)				LNnegs patient subcohort[a] (n = 680)			
					After neoadjuvant treatment (n = 434)		No neoadjuvant treatment (n = 246)	
	LNpos (n)	%	LNneg (n)	%	LNneg (n)	%	LNneg (n)	%
	121	100	1830	100	434	100	246	100
(y)pT category [1]								
T0	1	0.8	92	5.0	28	6.5	21	8.5
T1	2	1.7	79	4.3	45	10.6	29	11.8
T2	24	19.8	284	15.5	98	22.6	106	43.1
T3	85	70.3	1117	61.0	253	58.3	53	21.5
T4	9	7.4	258	14.1	10	2.3	37	15.0
(y)pN category								
N0	0	–	1139	62.2	207	47.7	162	65.9
N1	52	43.0	520	28.4	202	46.5	63	25.6
N2	69	57.0	171	9.3	26	6.0	21	8.5
Sex								
Male	79	65.3	1166	63.7	220	50.7	113	45.9
Female	42	34.7	664	36.3	214	49.3	133	54.1
Age								
≤64	59	48.8	665	36.3	164	37.8	63	25.6
65−79	53	43.8	974	53.2	176	40.6	175	71.1
80+	9	7.4	191	10.4	95	21.9	8	3.3

[1] Gospodarowicz MK, Brierley, J. D., & Wittekind, C. TNM classification of malignant tumours. John Wiley & Sons. (2017) TNM classification of malignant tumours. John Wiley & Sons.

[a] The subcohort consists of 51 randomly slected patients from whom the LN microarchitecture was assessed.

Table 3
Relationship between negative lymph node microarchitecture and neoadjuvant treatment in the subcohort of 51 rectal cancer patients.

	Lymph nodes after neoadjuvant treatment (n = 434)					Lymph nodes without neoadjuvant treatment (n = 246)				
	LNneg (n)	%	median area (mm^2)	range (mm^2)	p-value	LNneg (n)	%	median area (mm^2)	range (mm^2)	p-value
Intranodal fat										
With intranodal fat	101	23.3	3.46	0.12 - 27.22	0.05	52	21.1	3.73	0.44 - 23.11	0.16
Without intranodal fat	333	76.7	4.51	0.15 - 46.89		194	78.9	4.96	0.39 - 51.43	
Combined lymphoid follicle score										
Group 1	161	37.0	2.32	0.12 - 46.89	< 0.001	32	13.0	2.15	0.39 - 23.98	< 0.001
Group 2	126	29.0	3.75	0.40 - 29.69		54	22.0	2.28	0.45 - 10.34	
Group 3	72	16.6	5.82	0.79 - 20.10		52	21.1	4.04	1.46 - 32.07	
Group 4	50	11.5	7.50	1.24 - 34.27		50	20.3	6.96	1.61 - 51.43	
Group 5	25	5.7	9.73	3.24 - 27.22		58	23.6	9.00	2.35 - 50.91	

a combined follicle score 1, e.g. no primary or secondary follicles, had the smallest LN area (median (range) 2.32 mm^2 (0.12 – 46.89 mm^2)), the 25 LNnegs with follicle score 5, e.g. at least 10 follicles (primary and secondary combined), had the largest LN area (median (range) 9.73 mm^2 (3.24 – 27.22 mm^2)) (Table 3). Similarly, in surgery alone treated patients, the 32 LNnegs with a combined follicle score 1 had the smallest LN area (median (range) 2.15 mm^2 ((0.39 – 23.98 mm^2), and the 58 LNnegs with combined follicle score 5 had the largest LN area (median (range) 9.00 mm^2 (2.35 – 50.91 mm^2) (Table 3).

Patients without neoadjuvant treatment (n = 17) had more often LNnegs with more lymphoid follicles. In patients without neoadjuvant treatment, 23.6 % of all LNnegs had a combined follicle score 5 *vs*. 5.8 % of all LNnegs with a combined follicle score 5 in patients with neoadjuvant treatment (Table 3). The intra- and interobserver concordance in classifying follicles as primary or secondary and counting the number of follicles was 72.0 % and 66.3 % for primary lymphoid follicles and 75.0 % and 58.8 % for secondary lymphoid follicles, respectively. Most differences were found between grading '0′ primary or secondary lymphoid follicles and 1–3 primary or secondary lymphoid follicles.

In LNnegs after neoadjuvant treatment (n = 434), a relationship between LNneg area and presence of intranodal fat was seen (p = 0.048). LNneg area seemed to decrease when intranodal fat was present (median (range) LNneg area of LNs without intranodal fat (n = 333): 4.51mm^2 (0.15-46.89 mm^2) versus 3.46 mm^2 (0.12-46.89 mm^2) of LNs with intranodal fat (n = 101)), p = 0.048. In patients without neoadjuvant treatment (n = 246) there was a similar trend (median (range) LNneg area of LNs without intranodal fat (n = 194); 4.96 mm^2 (0.39-51.43 mm^2) versus 3.73 mm^2 (0.44-23.11 mm^2) of LNs with intranodal fat (n = 52)), p = 0.163. The intra- and interobserver concordance in determining the presence or absence of intranodal fat was 93.0 % and 83.0 %, respectively. The frequency of intranodal fat presence was comparable between patients irrespective of treatment (78.9 % without intranodal fat and 76.7 % without intranodal fat respectively).

4. Discussion

This retrospective single-center pilot study explored the relationship between lymph node (LN) area and LN microarchitecture (number of lymphoid follicles, presence of intranodal fat) in LNs without metastasis (LNnegs) in resection specimens from rectal cancer (RC) patients. The LN diameters in the current study were consistent with those reported previously [6], as was the location of LNs within the mesorectal fat [6] and the fact that there were fewer LNs retrieved from the specimen after neoadjuvant treatment [7,8]. Our study suggests a relationship between sex, LNneg largest diameter and total number of resected LNnegs. According to our findings, LNnegs are smaller in females with RC than in males. A recent study suggested that females with colon cancer are diagnosed at a later stage [24]. If this is also the case for females with rectal cancer, then one could speculate that the smaller LNnegs in females might be a reflection of an 'exhausted' immune system despite the suggestion from the literature, that females have a generally stronger immune response [25].

The number of LNnegs with intranodal fat was similar irrespective of treatment suggesting that neoadjuvant treatment has no influence of intranodal fat. However, LNneg largest diameter was smaller after neoadjuvant treatment in our study confirming a previous report [26]. Interestingly, we only saw a relationship between LNneg area and presence of intranodal fat in RC patients who were treated with neoadjuvant therapy. It has been previously suggested that the presence of intranodal fat could be a sign of LN atrophy after therapy [27]. Alternatively, work by Huber et al. suggested that intranodal fat may have a role as energy resource for immune cell metabolism, activation and differentiation [28,29]. Thus, one could speculate that LNnegs without intranodal fat might be larger due to previous intensive lymphoid cell proliferation or differentiation which utilised all the available intranodal fat.

A relation between higher number of lymphoid follicles and increased LNneg area was found in the current study. This relationship between area and number of follicles supports our hypothesis and that of other investigators [18] that increased area of LNnegs is a surrogate of a more activated immune system in the regional, tumor draining LNs. Matsuno et al. reported that antigens of the tumor can lead to the activation of an immune response via follicular hyperplasia, proliferation of lymphocytes or sinus histiocytosis in pancreatic cancer [30]. As follicular hyperplasia was related to larger LNneg area in our study, the area of a LN may reflect the extent of immune activation. Kolotova et al. suggested that treatment of RC patients with a combination of radiotherapy and chemotherapy results in stimulation of lymph nodes by tumor antigens leading to lymph nodes with lymphoid follicles that almost all contain activated germinal centers [10]. Architectural changes other than follicular hyperplasia or intranodal fat which may potentially influence the area of LNnegs besides were not observed in the current study. Previous studies showed fibrosis in LNs after CRT [31,32], a parameter which was not apparent and hence not considered in the current study.

The current study has some limitations. It is a retrospective observational pilot study in rectal cancer patients from a single center which could have led to selection bias. As this is a hypothesis generating study, we consider the effect of this bias as minimal. As expected in rectal cancer patients, 80 % of study patients underwent neoadjuvant treatment, so any results from comparing LNs from patients with and without neoadjuvant treatment need to be interpreted with caution. The overall number of patients with lymph node metastases included in the current study was very small and made subgroup analysis by disease stage unfeasible. The architecture of LNpos was not examined as we hypothesized this would reflect a different immune status due to direct contact between immune cells and tumor cells. Unfortunately, survival data and patient outcome was not yet mature enough to be included in the analysis. Future prospective studies need to include a protocol for surgeons and pathologists to facilitate

standardized LN collection from the specimen as well as standardized preparation of lymph nodes for histological assessment.

In summary, our pilot study suggests that in rectal cancer patients the area of LNnegs is related to the presence of follicular hyperplasia as well as the presence of intranodal fat. As large LNnegs seemed to have larger number of lymphoid follicles, we hypothesize that measuring LNneg area could be of potential clinical value as surrogate for the immunogenicity of the primary tumor and/or successful activation of a host's immune response to tumor antigens. Future studies are warranted to a) investigate underlying biological mechanisms and b) to investigate whether histologically visible microarchitectural features of LNnegs can be related to particular radiological findings and hence potentially help to improve LN assessment at the time of diagnosis as well as be useful to determine patient's treatment. To assess the potential clinical importance of our findings, a study cohort with longer follow up time would be valuable. Larger prospective multidisciplinary studies need to be performed potentially using image analysis tools to increase inter/intra observer accuracy and to allow studying large number of LNs including positive LNs.

Ethical approval for research

Maastricht Ethics Committee approval obtained, reference number METC 2018-0672.

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CRediT authorship contribution statement

Ruisch JE: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Visualization, Writing - original draft, Writing - review & editing. **Kloft M:** Conceptualization, Investigation, Visualization, Writing - review & editing. **Fazzi GE:** Data curation, Methodology, Software. **Melenhorst J:** Conceptualization, Data curation, Methodology, Supervision, Writing - review & editing, Validation. **Magee DR:** Methodology, Software, Writing - review & editing. **Grabsch HI:** Conceptualization, Funding acquisition, Methodology, Software, Supervision, Visualization, Writing - review & editing, Validation.

Declaration of Competing Interest

None.

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