British Journal of Cancer www.nature.com/bjc

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Cellular and Molecular Biology

Immune microenvironment and lymph node yield in colorectal cancer

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BACKGROUND: Lymph node (LN) harvesting is associated with outcomes in colonic cancer. We sought to interrogate whether a distinctive immune milieu of the primary tumour is associated with LN yield.
METHODS: A total of 926 treatment-naive patients with colorectal adenocarcinoma with more than 12 LNs (LN-high) were compared with patients with 12 or fewer LNs (LN-low). We performed immunohistochemistry and quantification on tissue microarrays for HLA class I/II proteins, beta-2-microglobulin (B2MG), CD8, CD163, LAG3, PD-L1, FoxP3, and BRAF V600E.
RESULTS: The LN-high group was comprised of younger patients, longer resections, larger tumours, right-sided location, and tumours with deficient mismatch repair (dMMR). The tumour microenvironment showed higher CD8+ cells infiltration and B2MG expression on tumour cells in the LN-high group compared to the LN-low group. The estimated mean disease-specific survival was higher in the LN-high group than LN-low group. On multivariate analysis for prognosis, LN yield, CD8+ cells, extramural venous invasion, perineural invasion, and AJCC stage were independent prognostic factors.
CONCLUSION: Our findings corroborate that higher LN yield is associated with a survival benefit. LN yield is associated with an immune high microenvironment, suggesting that tumour immune milieu influences the LN yield.

British Journal of Cancer (2023) 129:917–924; https://doi.org/10.1038/s41416-023-02372-1

INTRODUCTION

Lymph node (LN) yield is a core element for adequate staging, risk assessment, and triaging patients for adjuvant chemotherapy in colon cancer. Surgical pathologists frequently face the dilemma of: "how many LNs are adequate?" While current guidelines, including the American Joint Committee of Cancer (AJCC) and National Comprehensive Cancer Network (NCCN), require at least 12 LNs to adequately evaluate for metastatic disease [1–4], other studies endorse thresholds as low as 9 [5]. At the same time, the optimal number for many pathologists tends to follow the gestalt of 'as many as I can find,' which is endorsed by multiple studies [6, 7].

Recent studies have shown that increased LN harvest correlates positively with disease-free survival and overall survival in stage I-III colon cancers, regardless of LN metastasis. Indeed, while 12 LNs may be sufficient for adequate staging, Trepanier and colleagues found ≥24 LNs had better survival across all N stages in a cohort evaluating over 260,000 cases [8]. Other studies similarly predicted favourable outcomes with higher yields [9–11], necessitating a purpose to harvest LNs beyond tumour staging. This finding has segued into a number of studies that have found several factors, clinical, pathologic, and surgical measures that influence a higher LN yield including age [12–15], right-sided colon cancer [13, 15–17], larger tumour [15, 18, 19], specimen length [13, 15], and higher histological grade [12–15], among others. Hypothetically, several of these associations may, at least in part, be due to the complex dynamic between the tumour and host immune response.

We speculate that crosstalk between the tumour and the host immune microenvironment may influence LN yield. There have been few prior studies to explore this relationship. High tumour-associated inflammatory cell infiltrates, mainly CD8+ cells, were associated with greater LN retrieval [20, 21]. Tumours with microsatellite instability, which characteristically have a higher mutational burden and a greater host immune response, were also shown to have a greater LN yield [22]. More recently, Lal and colleagues [23] evaluated transcriptomic changes and found an enrichment of genes associated with an immune response with adaptive and dendritic cell response in tumours with higher LN yield. Collectively, these studies suggest that a prominent immune response in the primary tumour leads to enlarged LNs and, in turn, a larger yield. To the authors' knowledge, however, a thorough evaluation of tumour–host immune landscape, such

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Received: 8 March 2023 Revised: 5 July 2023 Accepted: 18 July 2023
Published online: 28 July 2023

Table 1. Clinicopathologic and molecular characteristics according to LN yield.

	LN high	LN low	*P* value
Age (years)	67.4 ± 14.9	70.7 ± 12.3	0.009
Gender (male)	381/804 (47.4%)	64/122 (52.5%)	0.296
BMI (kg/m^2)	27.4 ± 6.1	27.8 ± 6.4	0.443
Smoking	88/804 (10.9%)	19/122 (15.6%)	0.136
Leucocyte at diagnosis (10^9/L)	8.1 ± 3.2	7.9 ± 2.8	0.511
Period (diagnosed after 2010)	329/780 (42.2%)	13/120 (10.8%)	<0.001
Laparoscopic surgery	286/804 (35.6%)	28/121 (23.1%)	0.007
LN positivity	369/803 (46.0%)	52/122 (42.6%)	0.704
Resection length	26.2 ± 16.4	22.6 ± 10.7	0.023
Tumour size (cm)	5.1 ± 2.7	4.0 ± 2.5	<0.001
AJCC stage III–IV disease	379/804 (47.1%)	58/122 (47.5%)	0.934
T stage 3–4 disease	632/804 (78.6%)	88/122 (72.1%)	0.109
Right-sided tumour	409/788 (51.9%)	38/118 (32.2%)	<0.001
High grade	126/790 (15.9%)	21/118 (17.8%)	0.611
EMVI present	271/804 (33.7%)	41/122 (33.6%)	0.983
PNI present	243/803 (30.3%)	41/122 (33.6%)	0.455
Distant metastasis	74/751 (9.9%)	27/120 (22.5%)	<0.001
dMMR	120/399 (30.1%)	4/33 (12.1%)	0.028
BRAF V600E	66/146 (45.2%)	0/4 (0%)	0.131

BMI body mass index, *AJCC* American Joint Committee on Cancer, *EMVI* extramural venous invasion, *PNI* perineural invasion.

as immune cells, to include CD8+ cells, histiocytes, and regulatory FoxP3+ lymphocytes, the expression of tumour and immune and tumour regulatory proteins, such as programmed death ligand-1 (PD-L1), and the expression of tumour antigen-presenting proteins such as human leucocyte antigen (HLA) class I and II and beta-2-microglobulin (B2MG) has not been evaluated.

We seek to further expound on the above-mentioned studies by scrutinising the primary tumour and its association with LN yield to understand better the survival benefit of tumours with greater LN harvests and the influence of the tumour microenvironment on LN yield.

MATERIAL AND METHODS

Patient populations

A total of 953 consecutive patients with treatment naïve colorectal cancer resected at Massachusetts General Hospital between August 2001 and October 2015 were evaluated. Among them, patients with inflammatory bowel diseases (IBDs) ($N = 27$) were excluded since these patients showed more LN yield compared to patients without IBDs (mean LN yield, 35 ± 30 vs. 23 ± 11, $P = 0.047$). The study was approved by the hospital institutional review board (IRB; MGB no. 2017P61) and informed consent was obtained from all patients.

LN harvest was performed with a comparable grossing technique in a single institution, harvesting as many identifiable lymph nodes as possible without using lymph node revealing solution or instruments.

We evaluated clinicopathologic parameters, including surgical approaches, AJCC stage, histologic grade based on WHO guidelines, presence of perineural invasion (PNI) and extramural venous invasion (EMVI), number of examined LNs and positive LNs, and others. We evaluated immunohistochemistry (IHC) for mismatch repair (MMR) proteins including MLH1, MSH2, MSH6, and PMS2 and BRAF V600E. IHC staining for four MMR proteins was classified as intact with retained nuclear expression or absent with loss of nuclear expression. Deficient MMR (dMMR) is defined when one or more MMR proteins are lost.

IHC was performed on tissue microarrays (TMA) for immune cell markers, including CD8, CD163, PD-L1, FoxP3, lymphocyte activation gene-3 (LAG3), and tumour cell markers, including PD-L1, HLA class I, HLA class II, and B2MG. The central portion of each tumour was used for the TMA. Detailed information about the clone, dilution, type of antibody, methods, and company are provided in the previously described method [24].

The clinicopathologic parameters and immunologic profiles were compared between patients with colorectal adenocarcinoma who have more than 12 LN (LN-high) yields and patients who have 12 or less than 12 LN yields (LN-low).

Automated quantification

The stained TMA slides were scanned and automatically quantitated for immune and tumour cell markers [24]. The number of positive cells for these immune markers in the entire available tissue was calculated and expressed per mm^2.

Statistical analysis

Follow-up duration was calculated from the time of operation to the time of death or last follow-up. Survival curves were plotted using the Kaplan–Meier method and the difference of disease-specific survival (DSS) between groups was analysed by the log-rank test. Non-parametric data were tested using the Chi-square test or Fisher's exact test for two independent groups. Parametric data were tested using the Student's *t* test for two-group analysis. Multivariate analyses were performed using linear regression model and Cox proportional-hazards model for analysing variables associated with LN yield and variables associated with prognosis, respectively. All analyses were performed using SPSS version 26 and Prism v6. *P* value of <0.05 was considered statistically significant.

RESULTS

Patient characteristics according to the LN yield

We evaluated 926 consecutive resections from patients with colorectal adenocarcinoma. The mean age was 67.8 ± 14.7 years-old, and the mean number of LN identified was 23 ± 11. Patients were classified as (1) LN-high: those with >12 LNs, and (2) LN-low: ≤12 LNs. Patients in the LN-high group were younger, diagnosed after 2010, more likely to undergo laparoscopic surgery, right-sided and larger tumours, and longer resection length (Table 1); however, AJCC stage, depth of invasion (T stage), EMVI, and PNI were not different between the two groups, implying that biologically aggressive tumours did not yield higher numbers of

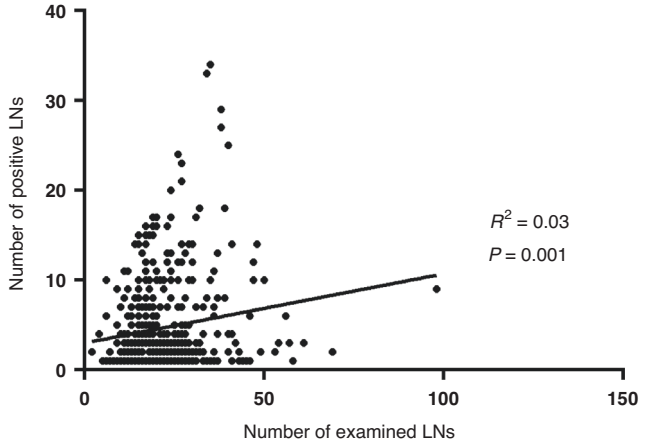

Fig. 1 Correlation between number of positive LN and LN yield. Scatter plot by linear regression analysis shows a significant correlation, albeit weak, between the number of positive LNs and LN yield in patients with positive LNs.

LNs (Table 1). Higher LN yields were more commonly associated with dMMR ($P = 0.028$). LN yield was not associated with BRAF V600E IHC expression. In multivariate analysis for LN yield, year of diagnosis ($P < 0.001$), tumour size ($P = 0.001$), and tumour location ($P = 0.001$) were the independent factors associated with higher LN yield.

The absolute number of positive LNs was higher in LN-high group than LN-low group (mean number of positive LNs, 4.9 ± 5.2 vs. 3.5 ± 3.0, $P = 0.005$) among patients with positive LNs. There was a weak but significant linear correlation between the number of LNs harvested and the number of positive LNs among patients with positive LNs (Fig. 1). Interestingly, the number of patients with distant metastasis at presentation (stage IV) was higher in LN-low group than in LN-high group (Table 1; $P < 0.001$) and in the same context, patients with higher stage tumours showed significantly lower LN yield than the early-stage disease (mean number of LN yield in Stage III–IV vs. stage I–II, 22.0 ± 9.9 vs. 23.5 ± 12.6, $P = 0.041$). The mean LN yield was 22.2 ± 11.7 in open surgery and 23.8 ± 10.8 in laparoscopic surgery, respectively ($P = 0.042$). Regarding the year of diagnosis, the mean LN yield was 20.4 ± 10.4 in patients diagnosed 2001–2010 and 26.2 ± 11.1 in those diagnosed in 2011–2015, respectively ($P < 0.001$). The laparoscopic surgery was performed in 50.1% in 2011–2015, while it was performed in 23.3% in 2001–2010 ($P < 0.001$).

Tumour immune response

Patients in the LN-high group showed significantly higher numbers of CD8+ immune cell infiltration ($P = 0.024$) and B2MG ($P = 0.009$) expression on tumour cells (Fig. 2). The number of other immune cells, including FoxP3, CD163, LAG3, PD-L1+ cells, and the expression of immune markers on tumour cells, including HLA class II, HC10, and PD-L1, were not different between two the groups (Table 2).

We hypothesised that tumour–host immune interaction might be associated with disease progression. In this context, we compared the immune profile to the AJCC stage. We confirmed that early stages disease showed a higher number of CD8, CD163, PD-L1+ cell infiltration and higher expression of B2MG on tumour cells than advanced disease (Table 3).

Disease-specific survival according to LN yield

The mean DSS for patients in the LN-high group was significantly higher than those in the LN-low group, with a mean estimated DSS of 131.7 months (range, 126.3–137.2) in LN-high group compared to a mean estimated DSS of 114.9 months (range, 100.1–129.7) in LN-low group ($P = 0.016$) (Fig. 3a). Since most of patients in LN-low group were diagnosed before 2011 ($N = 107$), survival analysis from 2011 was performed to avoid the influence of time period on prognosis. Among the patients diagnosed from 2011, the mean DSS for patients in the LN-high group was significantly higher than those in the LN-low group, with mean estimated DSS of 71.6 months (range, 68.0–75.3) in the LN-high group compared to a mean estimated DSS of 29.3 months (range, 6.4–16.7) in the LN-low group ($P = 0.003$).

Survival according to the LN positivity

When the survival analysis was performed in patients with negative LNs ($N = 484$), higher LN yields were associated with improved survival, with a mean estimated DSS of 155.8 months (range, 150.7–160.8) in LN-high group compared to a mean estimated DSS of 126.1 months (range, 108.8–143.5) in LN-low group ($P < 0.001$) in patients with negative LNs (Fig. 3b). However, in the patients with positive LNs ($N = 387$, 314 stage III and 73 stage IV), there was no significant difference in the estimated DSS between patients in LN-high group (104.6 months, range, 95.8–113.5) and those in LN-low group (93.7 months, range, 69.4–118.0, $P = 0.320$) (Fig. 3c).

Survival according to the stage

When the analysis was separately performed in patients with stage I–II disease ($N = 461$), the estimated DSS was longer in LN-high group with a mean estimated DSS of 158.6 months (range, 153.8–163.4) compared to LN-low group with a mean estimated DSS of 141.4 months (range, 125.7–157.1) ($P = 0.015$) (Fig. 4a). In patients with stage III–IV disease ($N = 413$), patients in the LN-high group also showed better survival than those in LN-low group (103.4 months (range, 94.6 –112.2) vs. 82.9 months (range, 60.3–105.5), $P = 0.039$) (Fig. 4b).

Survival according to the immune profile

The cutoff value of CD8+ cells and B2MG expression for comparison of survival was decided based on the mean value of each in LN-low group. Patients with higher numbers of CD8+ cells (>1000/mm^2) showed better survival compared to the patients with lower CD8+ cells (≤1000/mm^2) (the estimated DSS, 139.9 months (range, 131.7–148.0) vs. 119.7 (range, 112.2–127.3), $P < 0.001$, Fig. 4c). The estimated DSS according to the level of B2MG expression showed similar results with CD8+ cells with the mean estimated DSS of 140.8 months (range, 131.7–149.9) in patients with higher B2MG expression and the mean estimated DSS of 124.1 months in patients with lower B2MG expression (range, 117.2–131.0) ($P = 0.007$) (Fig. 4d).

Multivariate analysis of prognostic factors

LN yield and CD8+ cell infiltration were independent prognostic factors (LN yield, HR 0.4, 95% CI 0.2–0.9, $P = 0.038$ and CD8+ cells, HR 0.4, 95% CI 0.2–0.9, $P = 0.021$) along with EMVI (HR 3.7, 95% CI 2.0–7.0, $P < 0.001$) and PNI (HR 2.3, 95% CI 1.3–4.3, $P = 0.007$), and AJCC stage (HR 2.9, 95% CI 1.3–6.5, $P = 0.012$).

DISCUSSION

LN harvesting is essential for adequate tumour staging and is a valuable biomarker in predicting outcomes in colon cancer. This study corroborates an evolving consensus, which acknowledges that a greater LN harvest is associated with improved survival, ultimately endorsing a 'more the better' approach for the pathologist. Our data suggest that LN-high tumours (>12 nodes) are more often right-sided and larger. Patients with higher stage tumours showed significantly lower LN yield than the early-stage disease. Additionally, LN-high tumours are more commonly dMMR, associated with higher numbers of CD8+ cells and higher

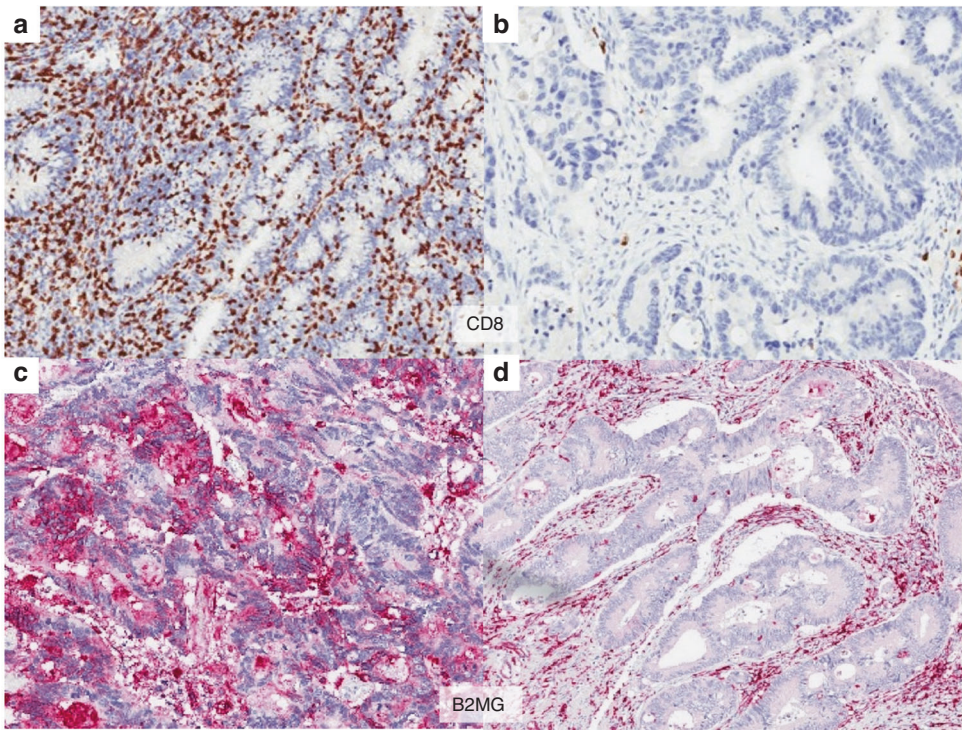

Fig. 2 Comparison between immune-high and immune-low tumours. a Immunohistochemical stains for higher CD8+ cell infiltration, and **b** lower CD8+ cell infiltration. **c** Higher expression of beta-2-microglobulin (B2MG) on tumour cells, and **d** lower expression of B2MG on tumour cells.

Table 2. Immunologic profile according to LN yield.

Markers	LN high	LN low	*P* value
Tumour microenvironment (mean number of cells per mm^2)			
CD8	1290.1 ± 1696.4	1030.9 ± 999.9	0.024
FoxP3	635.4 ± 1635.6	748.6 ± 2237.3	0.544
CD163	1566.5 ± 1496.7	1642.5 ± 1291.8	0.627
LAG3	34.3 ± 163.9	20.6 ± 46.5	0.436
PD-L1	131.7 ± 264.8	103.5 ± 252.9	0.326
Markers on tumour cells (mean percentage of expression on tumour cells)			
B2MG	22.9 ± 34.4	14.7 ± 27.8	0.009
HLA class II	65.6 ± 36.2	63.9 ± 33.7	0.657
HC10	71.9 ± 34.5	70.9 ± 31.9	0.797
PD-L1	1.2 ± 6.9	1.2 ± 10.3	0.975

B2MG beta-2-microglobulin.

Table 3. Immunologic profile according to AJCC stage.

Markers	Stage I–II	Stage III–IV	*P* value
Tumour microenvironment (mean number of cells per mm^2)			
CD8	1491.6 ± 1781.6	982.1 ± 1362.9	<0.001
FoxP3	732.4 ± 2060.8	555.2 ± 1219.8	0.149
CD163	1689.7 ± 1669.9	1449.9 ± 1196.3	0.021
LAG3	36.7 ± 132.1	28.3 ± 177.4	0.455
PD-L1	157.6 ± 301.0	94.3 ± 206.9	0.001
Markers on tumour cells (mean percentage of expression on tumour cells)			
B2MG	28.6 ± 37.1	14.2 ± 27.6	<0.001
HLA class II	65.6 ± 36.6	65.2 ± 35.2	0.859
HC10	72.6 ± 34.5	70.7 ± 33.8	0.423
PD-L1	1.5 ± 8.9	0.8 ± 5.1	0.677

B2MG beta-2-microglobulin.

tumour cell B2MG expression, arguing that tumour antigenicity and immune response may impact LN yield. Collectively, the data argue that the intrinsic properties of the tumour significantly influence LN yield.

The association of right-sided colon cancers with LN-high tumours may be due to anatomy; right-sided resections are commonly longer and associated with more mesentery, lympho-vascular trunks, and vascular pedicles [13, 16, 17, 25–29]. Right colectomies tend to occur in younger patients, where more extensive lymphadenectomies are likely performed due to less concern for co-morbidities in a younger cohort. Additionally, younger patients are also purported to have less LN 'involution' with age, which would also be expected to contribute to a larger yield [12, 14, 16]. Lastly, as right-sided tumours tend to more commonly demonstrate a dMMR phenotype, it is possible that ample LN yields seen in this cohort, are in part due to a beneficial tumour–host interaction (see below discussion) [22, 30].

Multiple studies have demonstrated a linear relationship between tumour size, which is the maximum horizontal diameter of the tumour, and LN yield [15, 17, 19]. An explanation for this phenomenon may be because larger tumours have more access to more lymphatics and drain al wider LN basin [31–33]. Others have argued that larger tumours tend to be associated with a greater mutational burden, and the increased antigenicity of these tumours elicits a greater immunogenic host response and, in turn, a greater LN yield and size [21, 31]. However, other biologically aggressive/infiltrative features, such as AJCC stage, EMVI, and PNI, did not demonstrate a significant association with LN yield,

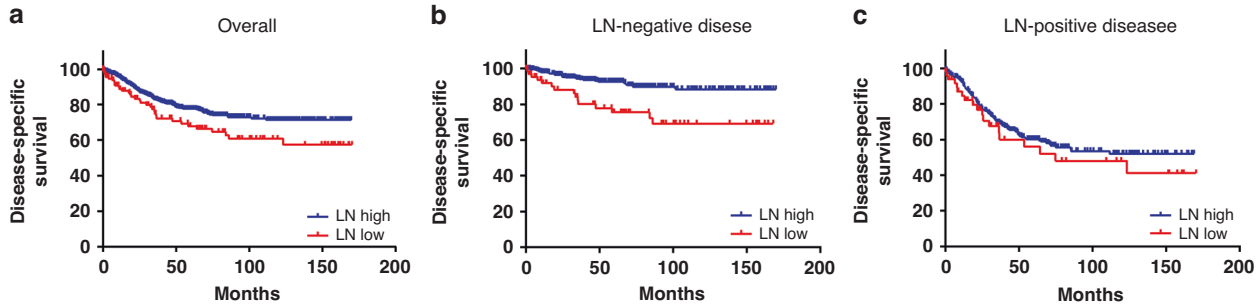

Fig. 3 The disease-specific survival according to the LN positivity (Kaplan–Meier method). a The estimated disease-specific survival in entire patients ($P = 0.016$), **b** in patients with LN-negative disease ($P < 0.001$), **c** in patients with LN-positive disease ($P = 0.320$), respectively. LN lymph node, DSS disease-specific survival.

arguing, at least in part, against these proposed mechanisms. We believe this point is likely multifactorial and requires further elaboration that may include additional factors (and confounders) beyond anatomy and tumour–host immune response.

Our data indicates that year of diagnosis is associated with the LN yield, which corroborates prior studies [17, 34]. Among factors influencing this result, both the realisation and education of the importance of LN yield as well as the effort of surgeons and pathologists likely contributed to a more robust LN harvest. It is also interesting to note that laparoscopic surgery revealed higher LN yield compared to open surgery. This result, however, could have been affected by year of diagnosis considering that half of the current study patients underwent laparoscopic surgery after 2010 and surgical approach lost the significance in the multivariate analysis. Nonetheless, this study can corroborate that surgical approach does not compromise LN yield [35–37].

Our data suggest that the tumour immune milieu significantly influences LN yield and that MMR status may be a factor that affects this relationship. The current study is in line with prior studies demonstrating dMMR to be associated with LN-high tumours [22, 30, 38]. The implication of this finding is two-fold: LN yield may be a surrogate marker of a highly antigenic tumour that leads to secondary LN activation, and the survival benefit seen in LN-high tumours may be at least partially due to the survival benefit commonly associated with dMMR tumours as well as the accompanying CD8+ cells [39, 40].

The evaluation of the tumour immune microenvironment associated with LN yield has been limited. Kim et al. assessed a small cohort ($N = 63$) of stage II and III colon cancers and found, utilising a limited panel of CD3, CD8, and CD45RO, that LN-high tumours are associated with a greater number of CD8+ cells and inflammatory cell infiltration [20]. More recently, Lal and colleagues evaluated The Cancer Genome Atlas (TCGA) for colorectal adenocarcinoma ($N = 377$) and found that LN-high tumours are associated with hallmark gene sets of immune response on transcriptomic analysis (regardless of LN metastasis), and associated with more prominent adaptive and dendritic cell immune response [23]. Using IHC, we sought to investigate biomarkers for adaptive and dendritic immunity, immune regulatory/checkpoint proteins, and proteins involved in tumour and immune cell antigen presentation. To the authors, this study is the largest ($N = 926$) to evaluate the association of the immune milieu and LN yield. The current study corroborates that CD8+ immune-high tumours are associated with LN-high tumours. This is in keeping with the idea that CD8+ cells are valuable biomarkers associated with favourable outcome [41, 42]. Indeed, CD8 is commonly seen in dMMR tumours [41–46], and collectively supports a robust immune-host interaction that leads to a prominent LN harvest. Tumour B2MG expression, which is a component of HLA class I, and essential for neoantigen presentation, is upregulated in LN-high tumours, arguing that an intact HLA machinery may also elicit an immune hot environment that may help enhance LN hyperplasia and enlargement sufficient for gross identification [47, 48]. However, there was no significance identified with immune and tumour regulatory proteins, including PD-L1, LAG3, histiocytes, FoxP3+ regulatory T-cells, peritumoural lymphocytic response, and additional antigen-presenting proteins, including HLA class I and HLA class II, highlighting that the LN yield associated with the overall immune response is not concerted but rather more complex and nuanced.

Corroborating multiple studies [8–10, 28, 29, 49], the current study demonstrated improved survival that was associated with LN count, and was significant not only in stage I and II disease but also, albeit to a lesser degree, in stage III and IV disease. Additionally, LN-high carcinoma showed less distant metastasis, while tumours that presented with distant metastasis (stage IV neoplasia) were more commonly associated with the LN-low group. We hypothesised that the underpinnings of these associations might be rooted in tumour–host immune environment interaction such that a robust immune response may partly play a role in preventing distant metastasis while yielding a greater LN harvest. Our data supports this belief as we found a lower density of CD8, CD163, PD-L1+ tumour microenvironment immune cells and less expression of B2MG in tumours cells for metastatic disease (AJCC stage III–IV) than in non-metastatic disease (AJCC stage I–II) (Table 3). Correspondingly, high stage tumours were associated with lower LN yield (stage I–II mean LN yield, 24 vs. stage III–IV mean LN yield, 22, $P = 0.041$). As a caveat, we note that survival was not different when the analysis was separately performed in stage III and stage IV, but showed a trend of better survival in patients having only haematogenous metastasis with no nodal metastasis (stage IV) for the LN-high group compared to those in the LN-low group (data not shown). This finding might suggest different tumour host immune interactions in haematogenous metastasis vs. lymphatic metastasis. However, due to the number of limited cases in this cohort, it is challenging to draw definitive conclusions and currently represents a future direction of investigation.

Resectable colon cancer may benefit from adjuvant chemotherapy with a suboptimal LN yield, as poor LN yield has been shown to be a negative prognosticator of outcome [50]. The current study offers a viewpoint that highlights the association of LN yield and the tumour host immune microenvironment and suggests that the favourable outcomes associated with LN-high tumour are in part due to a beneficial tumour host immune response. This understanding may potentially have a role in triaging treatment, in that it not only supports the benefit of adjuvant chemotherapy in LN-low tumours, but also offers an additional wrinkle in how to treat recurrent or metastatic LN-high tumours. LN-high tumours, which are more characteristically dMMR and CD8+ cells high, may benefit from immunomodulatory therapy in refractory cases [51, 52]. Indeed, the density of CD8+ cells in the tumour

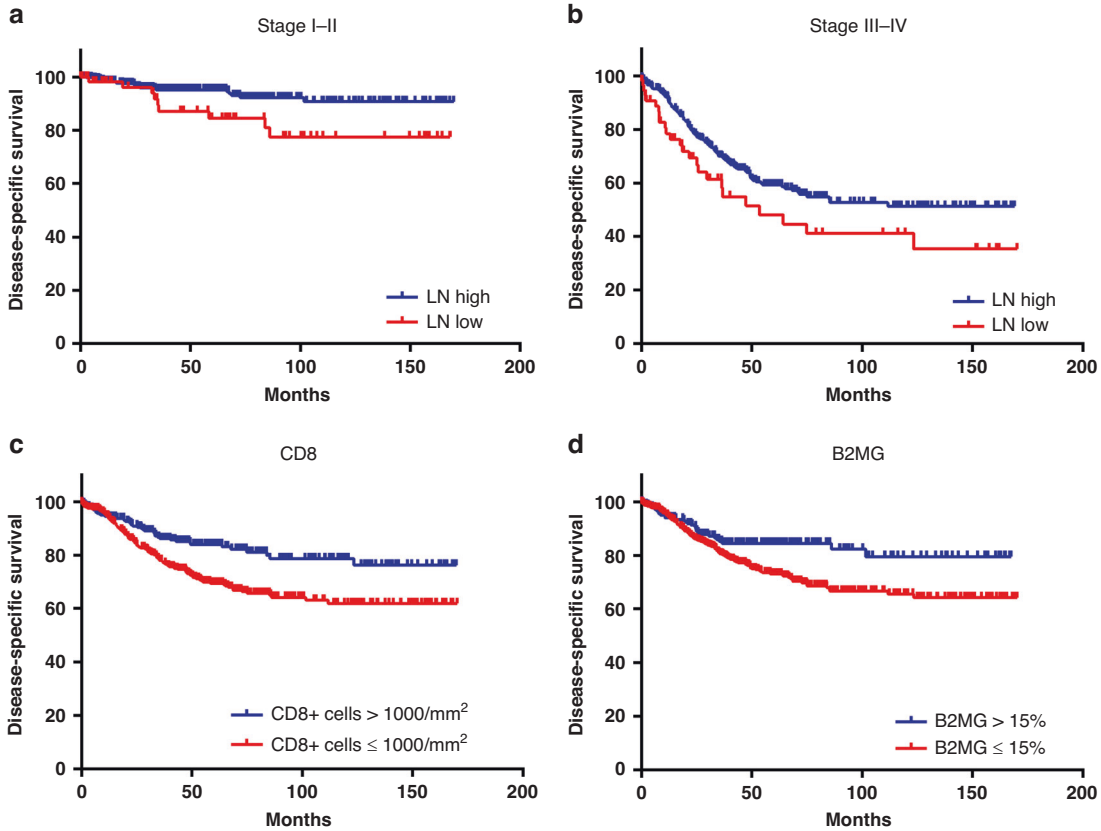

Fig. 4 The disease-specific survival according to the stage and immune profile (Kaplan–Meier method). a The estimated disease-specific survival in patients with stage I–II ($P = 0.015$), **b** in patients with stage III–IV ($P = 0.039$), **c** in patients with higher CD8+ cell infiltration ($P < 0.001$), and **d** in patients with a higher beta-2-microglobulin expression on tumour cells ($P = 0.007$), respectively.

environment is an important factor in predicting the efficacy of anti-PD-L1 therapy [53] and, more importantly, in dMMR disease [51, 52]. Therefore, high LN yield might be a surrogate marker for the tumour immune environment and potentially a predictive factor for immunotherapy. Future studies are required to further investigate this association.

Theoretically, a larger LN harvest has a greater chance to identify LNs positive for metastatic disease. The current study, however, is partly in line with an evolving consensus [54, 55] that fails to demonstrate a strong association between increased LN yield and LN positivity. Indeed, we found that larger LN yield did not affect LN positivity at the designated cut-off value of 12 LNs and was only weakly associated with the number of positive LNs in LN positive disease ($r^2 = 0.03$ in Fig. 2). We believe that the benefit seen with a robust LN harvest is because it is in response to a beneficial tumour–host immune microenvironment, and this is why finding more lymph nodes does not identify more metastatic disease and why LN-high tumours have better disease-specific survival. Indeed, comparable to LN yield, the level of CD8+ cells in the primary tumour is an independent variable in predicting DSS, arguing that the beneficial outcomes in LN-high tumours may be due to LN activation from a beneficial tumour–host immune response [39, 56–58]. We do reiterate, however, a weak association with LN yield in LN positive cases and acknowledge that a larger yield may still be a variable in identifying LN positive disease.

A limitation of this study is that it did not evaluate additional biomarkers, such as other immune biomarkers, stimulatory and regulatory proteins, such as cytotoxic T-lymphocyte-associated-protein-4 (CTLA-4) that may shed additional light on the tumour–immune crosstalk associated with LN yield. Nonetheless, this is the most comprehensive evaluation of the immune milieu in relation to LN yield ever performed. Additionally, as the study was performed on tissue microarrays, we do not account for the heterogeneity typically associated with immune markers. However, the tissue microarray approach allows for evaluating multiple immunohistochemical markers in one of the largest cohorts systematically evaluated for immune markers. We acknowledge that while a weakness of this analysis is that the LN cut-off is designated at 12, this value is not arbitrary as it is the endorsed threshold to evaluate LN metastasis by many societies and is more than 90% accurate in identifying a positive LN [1–4]. Lastly, the data does not account for the variation of LN yield related to pathologists and surgical technique; however, given that all the specimens involved a single large tertiary institution with standardised grossing and surgical approach, it is less likely to impact the outcome.

In conclusion, several key factors that influence LN yield are related to the immune milieu of the tumour. Patients with dMMR tumours, CD8+ immune high microenvironment, and greater tumour B2MG expression are more likely to yield a higher number of LN. Thus, the association between LN yield and favourable outcome may be related to the tumour immune milieu. Therefore, we again ask the question: how many LNs are adequate? This has practical implications in that oncologists, per American Society of Clinical Oncology and NCCN guidelines, consider <12 LNs as a high-risk factor along with other high-risk factors such as tumour perforation and perineural or lymphovascular invasion, will commonly treat N0 tumours as N1 if less than 12 LNs are found [50]. At a practical level, while we endorse the 12 LN cutoff, we argue for a multifactorial approach. Our findings indicated that certain tumours, including dMMR and CD8+ high tumours, were intrinsically likely to reveal more LNs. A nuanced view that may include variables such as length of the specimen, size of the

tumour, MMR status, and possibly a CD8+ cell count, may have practical value in determining whether a 12 LN threshold is required for adequate staging and whether it should impact decision making regarding adjuvant therapy in cases with <12 LNs.

DATA AVAILABILITY

The data that support the findings of this study are not openly available to maintain patient confidentiality, but de-identified data are available from the corresponding author upon reasonable request.

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ACKNOWLEDGEMENTS

VD is partially funded by NIH grant.

AUTHOR CONTRIBUTIONS

OY and VD performed study concept and design; AP, AN, YO, and SR performed development of methodology; CF, DT, DP, OY, and DB provided acquisition of data and revision of paper; SL, OY, and VD analysed and interpreted data; SL, OY, and VD performed writing the paper. All authors reviewed and approved the final paper.

COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The analyses of the cohort were conducted in adherence of the Declaration of Helsinki. The study was approved by the hospital institutional review board (IRB; MGB no. 2017P61).

ADDITIONAL INFORMATION

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