UNIVERSITAT DE BARCELONA

Caracterización de la estadificación molecular en carcinoma de colon. Correlación clínico-histológica

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TRABAJOS REALIZADOS, MÉTODOS Y RESULTADOS

La descripción de los pacientes, la metodología utilizada así como los resultados encontrados en las diferentes investigaciones realizadas, se encuentran detalladamente explicadas en las secciones de "*Materials and methods*" y *"Results"* de cada uno de los dos artículos que constituyen el cuerpo de la presente tesis doctoral.

Los dos artículos que se incluyen a continuación tal y como se encuentran en la literatura científica.

Estudio 1

Molecularly determined total tumour load in lymph nodes of stage I–II colon cancer patients correlates with high-risk factors. A multicentre prospective study

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Resumen

Los pacientes con carcinoma colorrectal (CCR) en estadios I-II (pN0) son tratados quirúrgicamente, aunque hasta el 25% de los mismos fallecen por recidiva de la enfermedad. El estado de los ganglios linfáticos (GLs) es un factor pronóstico independiente en el CCR, y la detección molecular de neoplasia en GLs de pacientes con CCRs en estadios iniciales se asocia a un mayor riesgo de recidiva de la enfermedad y peor supervivencia. El objetivo del presente estudio multicéntrico prospectivo es determinar la relación entre la carga tumoral molecular en GLs y los factores de riesgo convencionales en pacientes con cáncer de colon en estadios I-II.

Se obtuvieron 1940 GLs de 149 pacientes con cáncer de colon con estadio histológico pN0. Se cuantificó la cantidad de ARNm mensajero (ARNm) de citoqueratina 19 (*CK19*) en los GLs mediante la técnica *Reverse Transcription Loop-Mediated Isothermal Amplification* denominada *One-Step Nucleic Acid Amplification*. Se definió la carga tumoral total (CTT) de cada paciente como la suma de todas las copias de ARNm de *CK19*/µL de cada GL positivo por colectomía.

Se obtuvo una mediana de 15 GLs por caso (RIC 12;20). La positividad molecular se correlacionó con la presencia de áreas de alto grado (p <0,01), histología mucinosa/anillo de sello (p = 0,017), el sexo masculino (p = 0,02), el número GLs aislados (p = 0,012) y el peso total de GLs por caso (p < 0,01). La CTT se relacionó con estadio pT (p = 0,01) y tamaño tumoral (p <0,01) en los tumores de bajo grado. El estudio de regresión logística multivariante mostró una correlación independiente de positividad molecular con el género, grado tumoral y número de GLs en fresco [(IC del 95% = 0,62 a 0,79) AUC = 0,71].

Nuestros resultados muestran que la detección de ARNm de *CK19* en ganglios linfáticos se correlaciona con factores de alto riesgo clásicos en pacientes con cáncer de colon en estadio I-II. La CTT es una medida cuantitativa y objetiva que puede contribuir a una mejor estadificación de pacientes con cáncer de colon precoz.

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ORIGINAL ARTICLE

Molecularly determined total tumour load in lymph nodes of stage I–II colon cancer patients correlates with high-risk factors. A multicentre prospective study

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Abstract Stage I–II (pN0) colorectal cancer patients are surgically treated although up to 25 % will eventually die from disease recurrence. Lymph node (LN) status is an independent prognostic factor in colorectal cancer (CRC), and molecular tumour detection in LN of early-stage CRC patients is associated with an increased risk of disease recurrence and poor survival. This prospective multicentre study aimed to determine the relationship between LN molecular tumour burden and conventional high-risk factors in stage I–II colon cancer patients. A total of 1940 LN from 149 pathologically assessed pN0 colon cancer patients were analysed for the amount of tumour cytokeratin 19 (CK19) messenger RNA (mRNA) with the quantitative reverse transcription loop-mediated isothermal amplification molecular assay One-Step Nucleic Acid Amplification. Patient's total tumour load (TTL) resulted from the sum of all CK19 mRNA tumour copies/μL of each positive LN from the colectomy specimen. A median of 15 LN were procured per case (IQR 12;20). Molecular positivity correlated with high-grade ($p < 0.01$), mucinous/signet ring type ($p = 0.017$), male gender ($p = 0.02$), number of collected LN ($p = 0.012$) and total LN weight per case ($p < 0.01$). The TTL was related to pT stage ($p = 0.01$) and tumour size ($p < 0.01$) in low-grade tumours. Multivariate logistic regression showed independent correlation of molecular positivity with gender,

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tumour grade and number of fresh LN [AUC = 0.71 (95 % CI = 0.62–0.79)]. Our results show that lymph node CK19 mRNA detection correlates with classical high-risk factors in stage I–II colon cancer patients. Total tumour load is a quantitative and objective measure that may help to better stage early colon cancer patients.

Keywords Colorectal neoplasms · Neoplasm staging · Molecular pathology · Lymph nodes · Cytokeratin 19

Introduction

Surgical resection with no adjuvant therapy is recommended for most stage I–II colorectal cancer (CRC) patients, except for selected high-risk stage II patients given the significant impact of chemotherapy on stage III disease [1, 2]. Although there is evidence that pathological nodal staging is far from being optimal, current NCCN guidelines are based on haematoxylin and eosin (HE) lymph node (LN) staging, [3–7]. Its major weakness is the limited scope of histological LN analysis, based on a small sample provided by 2–5 μm LN sections, which comprise less than 0.5 % of the entire LN, and it may lead to false negative diagnoses [3, 7–9]. This may partly explain why up to 25 % of CRC patients with histologically negative LN die from recurrent disease after a potentially curative surgical resection. Some of these patients may have had undetected LN metastases [3, 10, 11].

The use of additional techniques, i.e. immunohistochemistry (IHC) or reverse transcriptase polymerase chain reaction, makes it possible to find LN tumour burden not detected with conventional HE analysis in 25 to 50 % of CRC patients, due to both increased sensitivity and the more extensive study than usually permitted by histological sections [3, 6, 7, 10–17].

Although the prognostic value of LN molecular tumour detection in early-stage CRC is controversial [14–18], there are enough data to support the use of more sensitive (i.e. molecular) methods of LN staging. As stated in three meta-analyses, the molecular detection of tumour cells in regional LN of stage I–II CRC patients is associated with an increased risk of disease recurrence and poor survival [3, 10, 11].

Most studies have focused in dichotomic (positive-negative) or semi-quantitative scales (isolated tumour cells (ITC), micro- and macrometastases) to assess molecular results [8, 10, 11, 14, 15, 17, 19, 20]. The molecular assay One-Step Nucleic Acid Amplification (OSNA; Sysmex Corporation, Kobe, Japan) is a quantitative method which analyses the entire LN. It amplifies cytokeratin 19 (CK19) mRNA from LN tissue lysates using the reverse transcription loop-mediated isothermal amplification (RT-LAMP) method [21]. CK19 mRNA was selected among other CRC markers showing the highest diagnostic performance and reproducibility, with 94.9–95.2 % sensitivity and 97.7–97.9 % specificity [22]. The system has been validated for breast and colon cancer LN assessment, providing results comparable to extensive histological and IHC LN analysis [8, 9, 21, 23–28]. The amount of CK19 mRNA detected correlates with the size of the metastatic foci [8, 9, 21, 27, 29]. It also makes it possible to calculate the total tumour load (TTL) present in a given patient, by adding all CK19 mRNA copies from each positive LN of a colectomy specimen [27, 28].

In this multicentre prospective study, we tried to correlate the TTL, as determined by OSNA, with classical clinical and pathologic high-risk factors, in an effort to determine whether the TTL could be used as an additional factor to better select stage I–II patients at risk of recurrence. Such an approach is now widely used in the treatment of breast cancer [27, 30, 31].

Materials and methods

Study sample

This is a prospective observational study including 10 institutions. Inclusion criteria were patients over 18 years old, with primary histologically confirmed colon cancer, cN0 preoperative diagnosis and positive CK19 IHC of the primary tumour. Exclusion criteria included rectal tumours, non-invasive pTis and pT0 tumours, positive LN on HE, synchronous tumours or other malignancies, cN1, gross adipose tissue involvement by the tumour, metastatic cancer, neo-adjuvant chemotherapy, familial adenomatous polyposis, carcinomas on inflammatory bowel disease and presence of stent-type intraluminal devices.

Study procedure

Sample processing and fresh lymph node procuring

Fresh LN procurement from the mesocolon fat was performed within 50 min after surgical excision. When immediate LN dissection was not possible, the surgical specimen was kept up to 3 h in the refrigerator at 4 °C until LN dissection was done. During the LN harvesting process, the dissection area was kept cold by putting a thick layer of chopped ice under an elevated metallic surface and covered with a clean filter paper for LN dissection (Figure available at Online Resource 1a). Microcentrifuge tubes were also kept cold by punching them in chopped ice (Figure 2 available at Online Resource 1b). We first detached the mesocolon fat from the colon wall with a surgical blade. pT4 tumours corresponded to antimesenteric serous tumour infiltration, and pT3 tumours corresponded only to specimens with minimal tumour infiltration of the mesocolon. We then dissected one by one all LN from the mesocolon fat using different clean areas of the surgical blade for small LN, or changing it after each LN dissection. When a LN was grossly suspicious of being positive, a cytology

touch-prep was performed to confirm or discard metastasis. Positive cases were discarded. All freshly dissected LN were analysed by both methods, HE and OSNA, using a modified protocol from previous studies [8, 9, 21]. Each LN was numbered and cut along the long axis. A central 1-mm slice was submitted for conventional formalin-fixation paraffin-embedding (FFPE) and HE analysis. The rest of the LN was stored at −80 °C in microcentrifuge tubes for 1 to 7 days until deferred OSNA analysis was performed. Lymph nodes with weight ≤0.07 g (average 5.5 mm) were defined as small.

After fresh LN harvesting, the specimen was fixed overnight in 10 % neutral buffered formalin. Then, the mesocolon fat was re-examined for remaining LNs, which were submitted only for conventional histopathology analysis.

CK19 immunohistochemistry

CK19 IHC was performed on representative sections of all the primary colon carcinomas to ensure reliable negative molecular CK19 mRNA results. A 2-μm section of each primary tumour was mounted on FLEX IHC microscope slides and pre-treated in PT-LINK (Dako, Glostrup, Denmark). Incubation for 20 min with the primary CK19 antibody (CK19 mouse monoclonal, clone RCK108; IR615 pre-diluted. Dako) was performed in the AutostainerLink 48 (Dako). Membranous staining with or without cytoplasm staining of ≥10 % of the tumour cells was defined as positive IHC in colon carcinomas (Fig. 1a, b).

Pathology report and LN staging

LN staging and pathology report were performed from the analysis of HE stains according to the AJCC/UICC TNM, 7th edition [32, 33]. Tumours ≥4 cm were defined as large. Pathologists and clinicians were both blinded to the OSNA results.

OSNA procedure

The OSNA method was performed at each institution following the manufacturer's instructions, using the protocol described by Tsujimoto et al. [21]. Briefly, LNs were weighed, homogenized with the lysis buffer Lynorhag (Sysmex) for mRNA stabilization and genomic DNA precipitation. After centrifugation for 1 min at 10,000 × *g* at room temperature; a 2-μL sample of the intermediate phase was mixed with the reagent LynoampBC (Sysmex). Analysis was performed using the RT-LAMP isothermal amplification method with the RD-100i automated gene-amplification system (Sysmex). The amount of CK19 mRNA amplified was detected by a change in turbidity upon precipitation of magnesium pyrophosphate (Fig. 1c). The result was correlated to CK19 mRNA copy number/μL of the original lysate through calibrated standard curves containing different CK19 mRNA concentrations. In every assay, a standard positive control sample containing 5×10^3 copies/μL of CK19 mRNA, and a negative control sample without CK19 mRNA were used for validation. The results were based on the number of CK19 mRNA copies/μL obtained for each LN. The cutoff value was 250 copies/μL, based on a previous study [9].

Statistical analysis

The Fisher exact test and Pearson's correlation coefficient were used for testing the association between categorical or numerical variables, respectively. The Mann-Whitney-Wilcoxon and Kruskal-Wallis test were applied to compare groups' distributions. A $p < 0.05$ was considered statistically significant.

Logistic regression analysis was used to predict the OSNA outcome. The backward stepwise algorithm was used to determine the best-fitting model. The classification avidity of the model was assessed by the ROC curve and a 10-fold cross-validation technique was applied for the model validation. The Cohen's kappa was used to assess the degree of agreement between OSNA outcome and model prediction. All analyses were performed using *R* statistical environment (V.3.1.0) [34].

Results

Sample size and characteristics

We analysed 3512 LN from 211 colon cancer patients recruited in 10 hospitals between June 2012 and December 2013. We excluded 27 non-invasive tumours (9 pT0 and 18 pTis), 34 cases with HE-positive LN, and one patient with synchronous tumours. Finally, 149 stage I–II colon cancer patients met the study selection criteria. All primary tumours showed positivity for CK19 IHC. Demographic, clinical, pathological, and lymph node characteristics of the study sample and correlation with CK19 mRNA results are summarized in Table 1 and the study flow diagram (Fig. 2).

Lymph node features and CK19 mRNA results

Among the 2483 LN procured from 149 cases, slightly over 78 % of them were freshly isolated and submitted for OSNA analysis (1940 fresh LN vs 543 FFPE LN). Hence, we obtained a median of 15 LN per case, of which 12 LN were analysed by OSNA and HE.

CK19 mRNA positivity was observed in 76/149 patients (51 %). Most of those positive cases; i.e. 80 %, had only 1 to 3 positive LN. Thus, among all OSNA LN analysed, 9,8 % (190/1940) were positive. Association analysis showed that OSNA-positive cases harboured more freshly procured LN

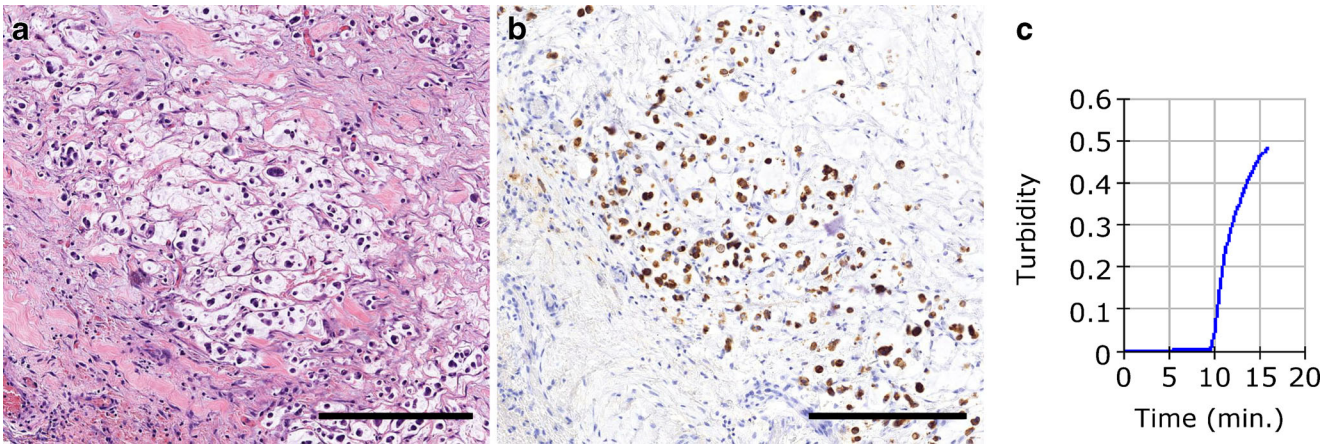

Fig. 1 **a** A primary high-grade mucinous carcinoma with signet ring cells. **b** Positive CK19 immunohistochemistry from the same case. **c** Amplification curve of *CK19* mRNA from a positive LN, assessed through turbidity variation (*Y*-axis) over time (*X*-axis, minutes). (Fig. 1a, b, scale bar 250 μm)

(p = 0.012) and had a higher total LN weight per case ($p < 0.01$) than negative ones (Fig. 3). Regarding the size of the freshly dissected LN, CK19 mRNA-negative LN were significantly smaller (i.e. weight ≤0.07 g) than positive ones (29.3 vs 15.6 %, $p < 0.01$).

In conclusion, fresh LN dissection did not impede an effective LN dissection and was an efficient way to obtain the minimum number of LN required for pN assessment. Although a substantial part of cases harboured tumour burden, only a few LN were positive among them.

High-risk factors and CK19 mRNA detection

The pathological high-risk features of CRC that were related to the presence of tumour CK19 mRNA in the LN were as follows: mucinous/signet ring histological types, high histological grade and tumour size. Among demographic variables, male gender was significantly related to OSNA positivity. No relationship was found with pT stage, vascular invasion, macroscopic tumour configuration, or other features.

High-grade tumours were significantly larger than their low-grade counterparts ($p < 0.01$), with a trend between histological type and tumour size ($p = 0.11$). The logistic regression model including OSNA results as dependent variable and tumour size as independent variable adjusted by histological type or grade showed a no significant relation between tumour size and OSNA response ($p = 0.26$ and $p = 0.15$, respectively).

The multivariate logistic regression analysis gave a reduced predictive model for OSNA molecular output including the independent variables gender, grade, and the number of fresh lymph nodes (Table 2). The receiver operating characteristic (ROC) curve was performed to test the avidity of the generated model to predict CK19 mRNA outcome, giving an AUC = 0.71 (95 % CI = 0.62–0.79). With a decision boundary of 0.5, the sensitivity, specificity, and accuracy were 61, 68, and 64 %, respectively. A 10-fold cross-validation was done for the model assessment, the resulting AUC was 0.67 (95 % CI = 0.59–0.76) (Fig. 4). Therefore, male gender, high histological grade and higher yields of LN were independent predictors of CK19 mRNA positivity.

Total tumour load

The median TTL of CK19 mRNA copies/μL among positive cases was 2015 (IQR: 940–6222.5). Cumulative TTL showed that 29 % of patients (22/76) had more than 5000 CK19 mRNA copies/μL. Although not significant (p = 0.18), there was a positive trend between CK19 mRNA copies/μL and pT stage; while TTL in pT1 cases was 1280 CK19 mRNA copies/μL and 1790 CK19 mRNA copies/μL in pT2, it rose to 3080 CK19 mRNA copies/μL in pT3 tumours (Fig. 3e). Surprisingly, the median TTL among pT4a tumours was 540 CK19 mRNA copies/μL. This could be explained by the inclusion of antimesenteric pT4 tumours rather than bulky tumours with marked adipose tissue infiltration, to the scarce amount of positive cases (5 among pT4a tumours), and by the fact than in 3 of them less than 65 % of the LNs were analysed by OSNA. Distribution of the cases regarding their TTLs and divided by pT stage is provided as Supplementary Table 1 in Online Resource 2. Albeit also not significant, all OSNA positive cases with vascular invasion had >2000 CK19 mRNA copies/μL.

In order to evaluate the distribution of the TTL, it was categorized by quartiles and compared with classical high-risk factors (data not shown). When stratified by tumour grade, it showed that among low-grade tumours, TTL increased with tumour stage (pT) (p = 0.01) and tumour size ($p < 0.01$; rho = 0.27) (Table 3). This trend was not observed in high-grade tumours. Taken together,

Table 1 Patient's demographics and specimen characteristics and correlation with CK19 mRNA results

Clinical parameter	*n* (%)	CK19 mRNA negative n (%)	CK19 mRNA positive n (%)	*p* value
Cases	149 (100)	73 (49.0)	76 (51.0)	–
Gender				0.02
Male	97 (65.1)	41 (42.3)	56 (57.7)	
Female	52 (34.9)	32 (61.5)	20 (38.5)	
Age (years)—median (IQR)	67 (61–75)	68 (61–74)	66 (61–75)	0.89
Surgical specimen characteristics				
Specimen size (cm)—median (IQR)	20 (15–25)	20 (15–25)	19.5 (15–25)	0.81
Tumour size (cm)[a]—median (IQR)	3 (2–5)	3 (2–4)	4 (2–5.5)	0.045
Large tumours (>4 cm)	39 (26.7)	14 (38.9)	25 (64.1)	0.09
Small tumours (≤4 cm)	107 (73.3)	57 (53.3)	50 (46.7)	
Tumour location				0.33
Right colon and caecum (incl. hepatic flexure)	67 (45)	37 (55.2)	30 (44.8)	
Transverse colon	14 (9.4)	5 (35.7)	9 (64.3)	
Left colon and sigmoid colon (incl. splenic flexure)	68 (45.6)	31 (45.6)	37 (54.4)	
Macroscopic tumour configuration				0.57
Annular	8 (5.4)	5 (62.5)	3 (37.5)	
Ulcerated	65 (43.6)	33 (50.8)	32 (49.2)	
Polypoid	75 (50.3)	34 (45.3)	41 (54.7)	
Other	1 (0.7)	1 (100)	0 (0)	
Vascular invasion				0.45
No	137 (91.9)	65 (47.4)	72 (52.6)	
Yes	12 (8.1)	8 (66.7)	4 (33.3)	
Histological type				0.017
Adenocarcinoma	136 (91.3)	71 (52.2)	65 (47.8)	
Mucinous/signet ring cell AC	13 (8.7)	2 (15.4)	11 (84.6)	
Grade[b]				<0.01
High grade	22 (15.0)	4 (18.2)	18 (81.8)	
Low grade	125 (85.0)	69 (55.2)	56 (44.8)	
pT				0.19
pT1	40 (26.8)	17 (42.5)	23 (57.5)	
pT2	31 (20.8)	20 (64.5)	11 (35.5)	
pT3	66 (44.3)	29 (43.9)	37 (56.1)	
pT4a	12 (8.1)	7 (58.3)	5 (41.7)	

IQR interquartile range, *AC* adenocarcinoma

[a] In three patients, tumour size could not be assessed

[b] In two patients, tumour grade could not be assessed

our results show that most patients had low TTLs. These values increased with tumour stage and followed different distributions among low- and high-grade tumours.

Patient's follow-up

At the time of writing of this manuscript, all patients had a follow-up of 2 years (median 33 months, IQR 25–32.5); 8.7 % (13/149) patients recurred (3 died and 10 were alive with metastatic disease). Four of the cases were OSNA positive, with a median TTL of 4375 CK19 mRNA copies/μL (range 360–47,600); 2 of the 3 dead patients were OSNA positive.

Discussion

This study quantifies the amount of total tumour load within the lymph nodes of stage I–II colon cancer patients, and suggests that LN molecular detection of CK19 mRNA could

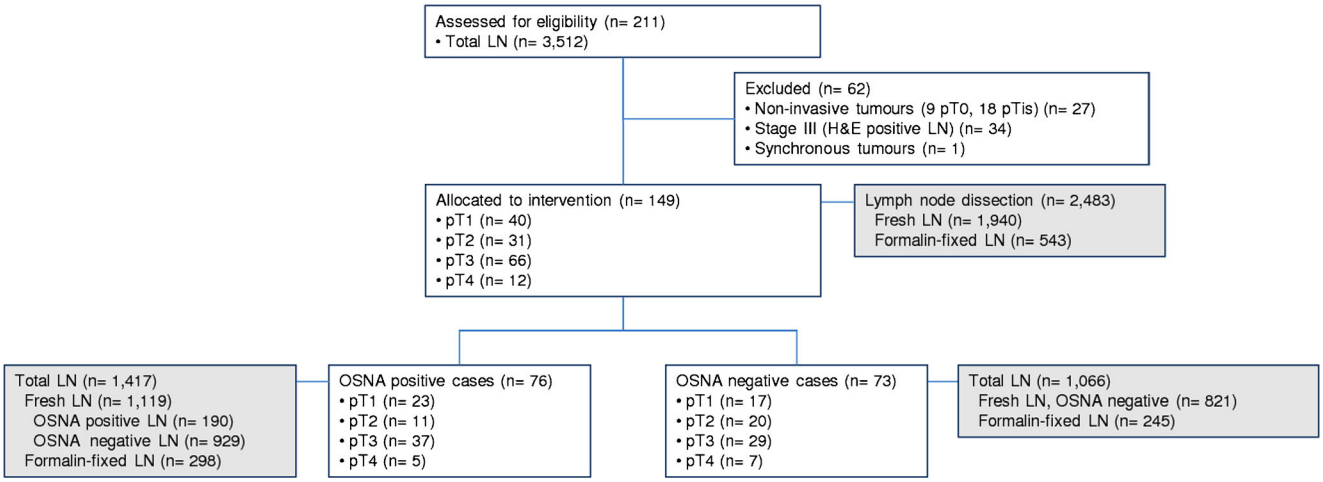

Fig. 2 Study flow diagram

become an objective indicator of risk in such individuals [11, 20, 28, 35]. Stage I–II CRC is amenable to complete surgical resection, but patients at risk of tumour recurrence are clinically and pathologically difficult to identify [36, 37]. In addition, cancer dissemination to LN may occur at early stages of tumour development [6, 38], potentially implying a poor

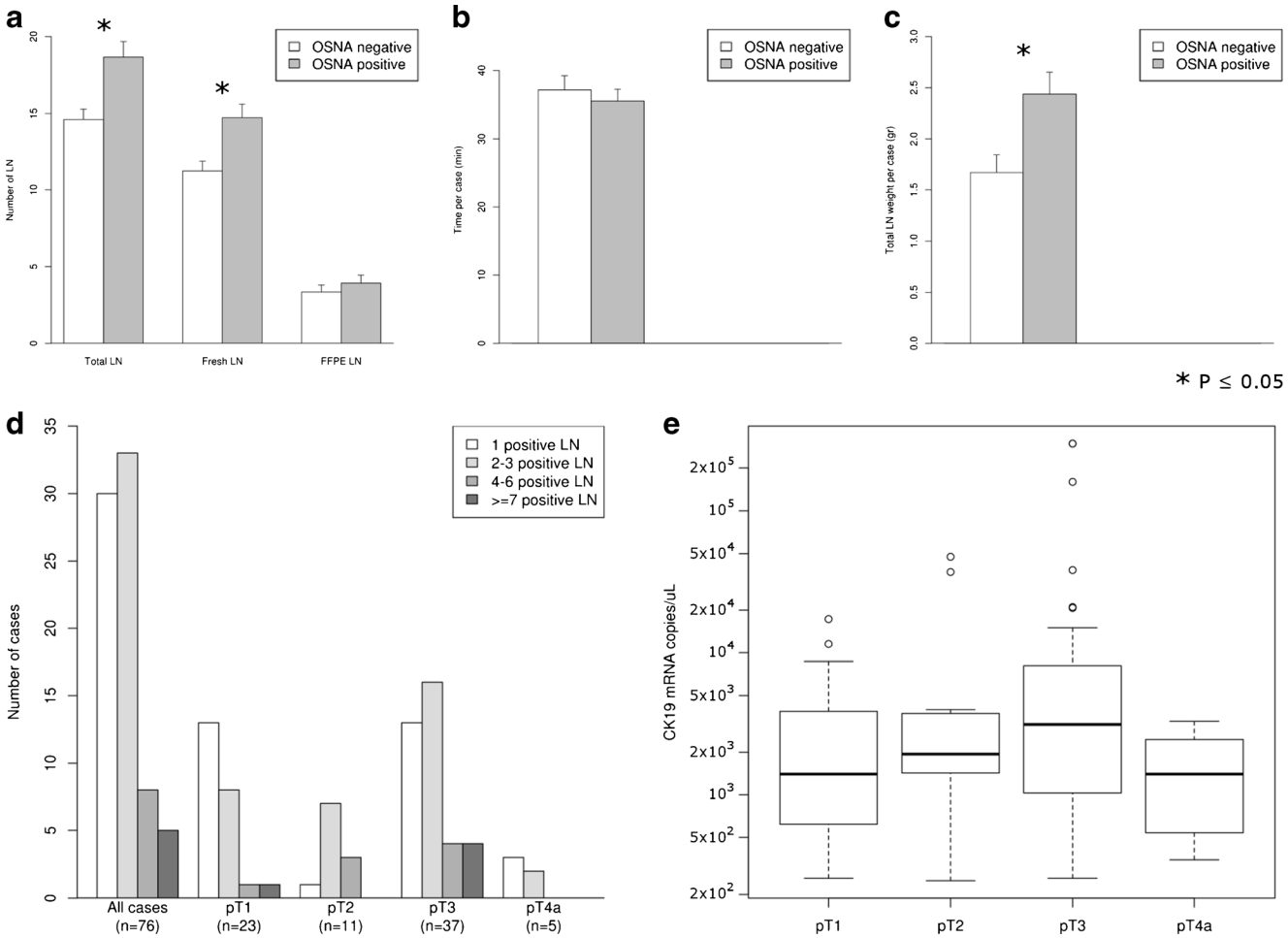

Fig. 3 OSNA results and correlation with LN retrieval and pT stage, regarding number of LN retrieved (**a**), time spent on fresh LN search in minutes (**b**) and total weight of fresh LN per case (gr) (**c**). OSNA positive cases held significantly more LN due to a higher fresh LN yields. **d** Shows that most cases in every pT stage held up to 3 OSNA positive LN. **e** Although not significant, a trend was observed between pT stage and TTL

Table 2 Logistic regression of clinical and histological variables related to CK19 mRNA positivity

Variables	Univariate model		Multivariate model	
	OR (95 % CI)	Wald test *p*	OR (95 % CI)	Wald test *p*
Gender (male vs female)	2.2 (1.1–4.4)	0.026	3.1 (1.4–7.0)	0.006
Age (years)	0.99 (0.96–1.03)	0.74	–	–
Tumour size	1.2 (1.0–1.4)	0.06	–	–
Tumour location		0.31	–	–
Transverse colon vs right colon and caecum (including hepatic flexure)	2.2 (0.7–7.9)			
Left colon and sigmoid colon (including splenic flexure) vs Right colon and caecum (including hepatic flexure)	1.4 (0.7–2.9)			
Macroscopic tumour configuration		0.59	–	–
Ulcerated vs annular	1.6 (0.4–8.4)			
Polypoid vs annular	2.0 (0.5–10.4)			
Histological type (mucinous / signet ring cell AC vs adenocarcinoma)		0.02	–	–
	6.0 (1.5–39.8)			
Vascular invasion (yes vs no)	0.5 (0.1–1.5)	0.21	–	–
Grade (high vs low)	5.4 (1.9–19.5)	<0.01	4.8 (1.5–18.9)	0.013
pT		0.20	–	–
pT2 vs pT1	0.4 (0.2–1.1)			
pT3 vs pT1	0.9 (0.4–2.1)			
pT4a vs pT1	0.5 (0.1–1.9)			
Fresh LN per case weight (gr)	1.1 (1.0–1.1)	<0.01	1.1 (1.0–1.2)	0.017

AC adenocarcinoma, *gr* grammes

prognostic impact [3, 7, 11, 15]. Thus, the assessment of the nodal staging arises as a key factor for CRC therapeutic management [3, 10, 11].

Our findings confirm the presence of undetected nodal tumour burden in early-stage colon cancer using the OSNA method. Compared to previous studies, we have the highest percentage of cases with CK19 mRNA detection in LN (i.e. 51 %) [23–25, 28]. Discrepancies may be due to differences in the LN collection and analysis, such as the fact that we analysed a larger amount of LN, including all evaluable LN by OSNA regardless of its size. Current guidelines recommend evaluating at least 12 LN to achieve a reliable histological staging [16, 32, 33, 39–42]. In compliance with them, we obtained a median of 15 LN per patient, of which 12 were freshly obtained and analysed by OSNA.

CRC lymph nodes are usually small, especially in early stages of the disease, but may still contain tumour burden, as size is not a good preoperative indicator of LN staging, or a predictor of the presence of tumour [43–45]. Although we identified a significant association between CK19 mRNA detection and larger LN size, we found CK19 mRNA in 15.6 % of small LN. Furthermore, the multivariate analysis of OSNA results showed that the number of collected LN, the gender and the histologic grade were independent predictors of OSNA results. Our data highlights the importance of procuring any identifiable LN irrespective of its size [40–42, 45–47].

Our results show that the presence of CK19 mRNA in regional LN was associated with other classical high-risk factors such as mucinous/signet ring types, histologic high-grade, tumour size and male gender. Moreover, it is noteworthy the trend observed between TTL and pT stage, as pT3 had the highest TTL (3080 CK19 mRNA copies/μL) and rate of CK19 mRNA detection (37/66).

We also observed a different behaviour between low and high-grade tumours, with a significant orderly increase of TTL in regional LN with the increase of pT stage and tumour size in low-grade tumours. This phenomenon may be accounted for by the fact that low-grade tumours tend to follow an orderly sequence of accumulation of molecular alterations as they grow and acquire infiltrative proprieties [48]. In contrast, no association was found with any assessed variables in high-grade tumours. This may be explained by the different molecular pathways involved in high-grade tumours and their aggressive behaviour from the beginning [48]. Reinforcing the importance of tumour grade in neoplastic LN spread, tumour size did not correlate with CK19 mRNA positivity when stratified by grade or histological type.

The biologic significance of LN molecular tumour detection is of leading importance. Although the sole presence of small amounts of tumour cells in regional LN does not necessarily imply poor prognosis, it may indicate a yet undefined risk of disease recurrence. This would explain why, with

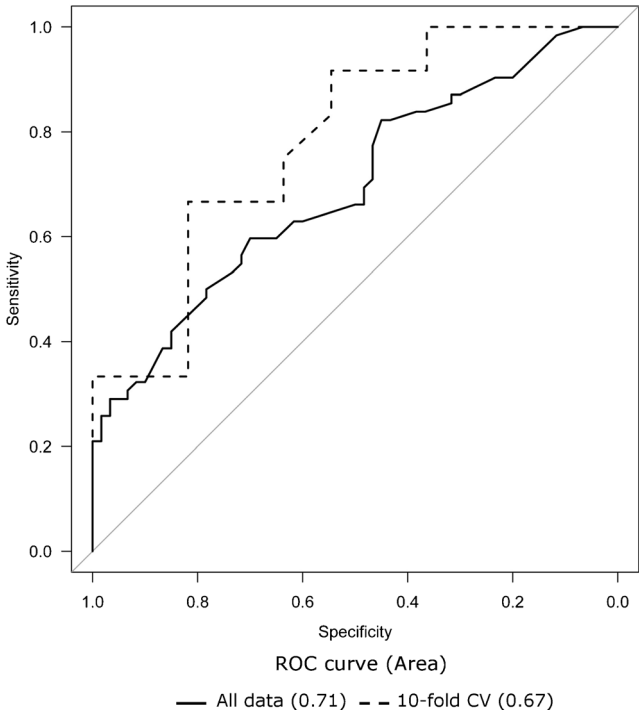

Fig. 4 ROC curve of the multivariate logistic regression model for OSNA results including gender, grade and the number of fresh lymph nodes as variables predicting molecular positivity. *Continuous line*: All data (0.71 (95 % CI = 0.62–0.79); *Dashed line*: 10-fold CV (AUC = 0.67 [95 % CI = 0.59–0.76])

historical recurrence records of 2.5 % in pT1 N0 tumours, we have found that almost 60 % had some CK19 mRNA tumour burden in their LN [11, 49]. In this context, low TTLs may have no further biologic consequences, but large amounts of tumour burden within the LN may imply higher odds of recurrence [7, 27]. A study using RT-qPCR detection of Guanylyl cyclase C (GUCY2C) by RT-qPCR found 87.5 % patients with positive LN, although only 20.9 % developed recurrent disease [7].

Quantifying the amount of tumour burden, not just the presence or absence of tumour, in regional lymph nodes is therefore important in prognosticating from molecular results, as has been stated in CRC [3, 7, 11, 20, 35] and breast cancer [27, 31]. It should be highlighted that whereas the median TTL of our cohort was 2015 CK19 mRNA copies/μL, values up to 15,000 CK19 mRNA copies/μL have been recently set in breast cancer sentinel LN as clinically relevant to predict additional axillary LN metastases [27]. Some authors have also demonstrated the relationship between the amount of metastatic tumour and prognosis, stressing the importance of distinguishing ITC from micrometastases [7, 11, 23, 24, 50]. Two novel Japanese multicentre studies stress the need to stratify molecular outcomes in LN assessment. The first one addresses that TTL significantly increases with pN stage [28] and suggests it as a potential staging technique. The second one has shown the correlation of LN micrometastasis volume, measured by qRT-PCR for CEA mRNA, as a predictor of

Table 3 Total tumour load distribution in high and low-grade tumours

pT	CK19 mRNA copies/μL per case *n* (%)				Total *n* (%)	*p* value
	<1000	<2000	<6000	≥6000		
All cases						0.246
pT1	8 (34.8)[a]	6 (26.1)	6 (26.1)	3 (13.0)	23 (100)	
pT2	1 (9.1)	5 (45.4)	3 (27.3)	2 (18.2)	11 (100)	
pT3	9 (24.3)	6 (16.2)	8 (21.6)[a]	14 (37.8)	37 (100)	
pT4a	2 (40.0)	1 (20.0)	2 (40.0)	0 (0.0)	5 (100)	
Total	20 (26.3)	18 (23.7)	19 (25)	19 (25)	76 (100)	
High grade						0.61
pT1	1 (20.0)	1 (20.0)	2 (40.0)	1 (20.0)	5 (100)	
pT2	1 (50.0)	0 (0.0)	0 (0.0)	1 (50.0)	2 (100)	
pT3	1 (12.5)	4 (50.0)	1 (12.5)	2 (25.0)	8 (100)	
pT4a	1 (33.3)	0 (0.0)	2 (66.7)	0 (0.0)	3 (100)	
Total	4 (22.2)	5 (27.8)	5 (27.8)	4 (22.2)	18 (100)	
Low grade						0.01
pT1	6 (35.3)	5 (29.4)	4 (23.5)	2 (11.8)	17 (100)	
pT2	0 (0.0)	5 (55.6)	3 (33.3)	1 (11.1)	9 (100)	
pT3	8 (28.6)	2 (7.1)	6 (21.4)	12 (42.9)	28 (100)	
pT4a	1 (50.0)	1 (50.0)	0 (0.0)	0 (0.0)	2 (100)	
Total	15 (26.8)	13 (23.2)	13 (23.2)	15 (26.8)	56 (100)	

[a] In two patients, tumour grade could not be assessed

recurrence in stage II patients [51]. In addition to these findings, we have found that TTL unveils distinct progression patterns in low- and high-grade neoplasms.

Our study has some drawbacks; firstly, a follow-up period of 2 years is a limited time to establish the amount of CK19 mRNA copies that would have clinical significance; thus, the OSNA results could not be correlated with prognosis. Nevertheless, this study was not designed to determine the role of the molecular results as a prognostic factor, but to find out whether the OSNA results correlated with other classical CRC high-risk factors. Secondly, in order to evaluate the histological high-risk factors, we focused on histologically pN0 specimens. To assess the potential predictive value of the TTL, colectomy specimens should be studied regardless of their LN status, thus comparing histological and molecular evaluation methods and their ability to predict disease recurrence.

In conclusion, this study identifies the presence of undetected tumour burden in LN of early colon cancer patients. TTL correlates with other CRC classical high-risk factors and may be placed among them. We thereby support CK19 mRNA TTL as a fast and reliable approach to help better stage early colon cancer. Long-term follow-up and validation studies are needed to obtain a predictive prognostic scale for stage I–II colon cancer patients based on the patient's TTL.

AC, adenocarcinoma; AUC, area under the curve; CK19, cytokeratin 19; CRC, colorectal cancer; FFPE, formalin-fixation paraffin-embedding; HE, Haematoxylin and Eosin; IHC, immunohistochemistry; IQR, interquartile range; ITC, isolated tumour cells; LN, lymph node; OSNA, one-step nucleic acid amplification; pT, tumour stage; ROC, receiver-operator characteristic; RT-LAMP, reverse transcription loop-mediated isothermal amplification; TTL, total tumour load

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Compliance with ethical standards The study protocol was approved by the Ethics and Scientific committee of each participating institution. All patients signed a written informed consent document for participation in the study. The study was performed in compliance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

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Conflict of interest The authors declare that they have no competing interests.

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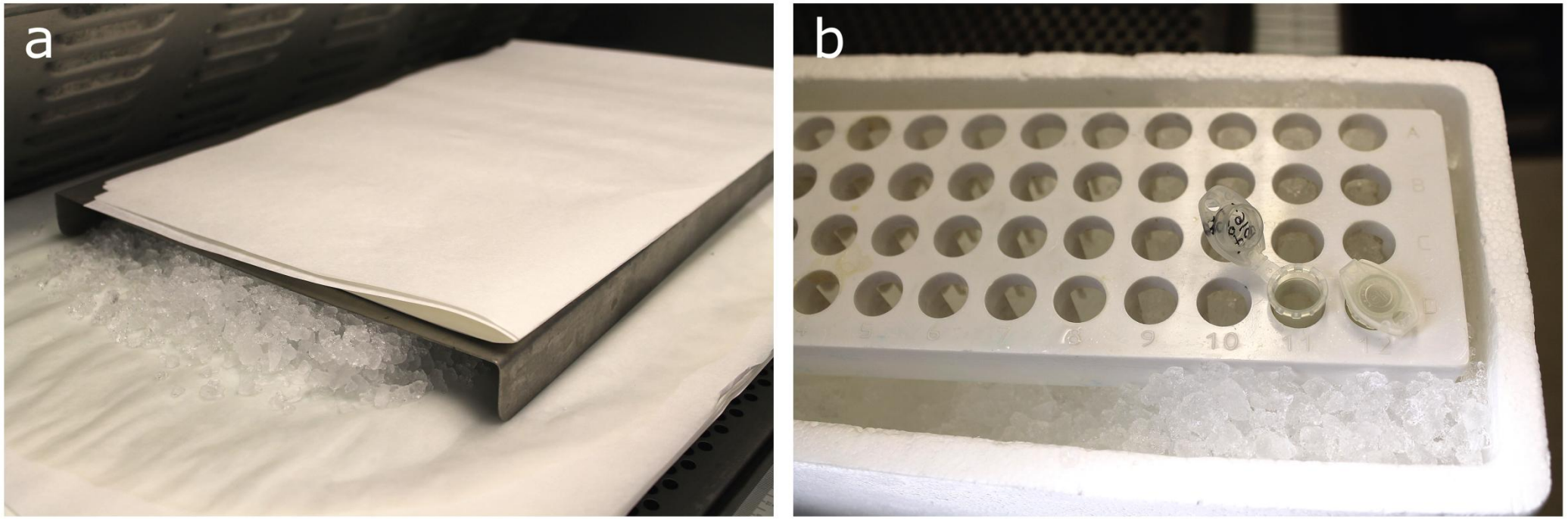
Electronic Supplementary Material nº1

Material used for fresh lymph node retrieval



(**a**) Image of the dissection area, with a thick layer of chopped ice beneath an elevated metallic surface. By placing filter paper above, the fresh LN dissection could be performed on a clean surface. (**b**) Microcentrifuge tubes were also kept cold by holding them in chopped ice

Electronic Supplementary Material nº2

Case distribution regarding TTL values and pT stage

	Total		pT1		pT2		pT3		pT4a	
Copies	**n**	**Cum (%)**	**n**	**Cum (%)**	**n**	**Cum (%)**	**n**	**Cum (%)**	**n**	**Cum (%)**
250	1	1,32	0	0,00	1	9,09	0	0,00	0	0,00
260	2	3,95	1	4,35	0	9,09	1	2,70	0	0,00
270	3	7,89	0	4,35	0	9,09	3	10,81	0	0,00
320	1	9,21	0	4,35	0	9,09	1	13,51	0	0,00
350	1	10,53	0	4,35	0	9,09	0	13,51	1	20,00
360	1	11,84	0	4,35	0	9,09	1	16,22	0	20,00
400	1	13,16	1	8,70	0	9,09	0	16,22	0	20,00
470	2	15,79	2	17,39	0	9,09	0	16,22	0	20,00
540	2	18,42	0	17,39	0	9,09	1	18,92	1	40,00
620	4	23,68	3	30,43	0	9,09	1	21,62	0	40,00
880	1	25,00	0	30,43	0	9,09	1	24,32	0	40,00
960	1	26,32	1	34,78	0	9,09	0	24,32	0	40,00
1030	1	27,63	0	34,78	0	9,09	1	27,03	0	40,00
1180	3	31,58	1	39,13	2	27,27	0	27,03	0	40,00
1270	1	32,89	1	43,48	0	27,27	0	27,03	0	40,00
1280	1	34,21	1	47,83	0	27,27	0	27,03	0	40,00
1320	1	35,53	0	47,83	0	27,27	1	29,73	0	40,00
1400	2	38,16	1	52,17	0	27,27	0	29,73	1	60,00
1500	1	39,47	1	56,52	0	27,27	0	29,73	0	60,00
1540	1	40,79	0	56,52	0	27,27	1	32,43	0	60,00
1650	1	42,11	0	56,52	0	27,27	1	35,14	0	60,00
1670	1	43,42	0	56,52	1	36,36	0	35,14	0	60,00
1790	1	44,74	0	56,52	1	45,45	0	35,14	0	60,00
1900	1	46,05	1	60,87	0	45,45	0	35,14	0	60,00
1940	1	47,37	0	60,87	1	54,55	0	35,14	0	60,00
1980	1	48,68	0	60,87	0	54,55	1	37,84	0	60,00
2000	1	50,00	0	60,87	0	54,55	1	40,54	0	60,00
2030	1	51,32	0	60,87	1	63,64	0	40,54	0	60,00
2200	1	52,63	1	65,22	0	63,64	0	40,54	0	60,00
2260	1	53,95	1	69,57	0	63,64	0	40,54	0	60,00
2420	1	55,26	0	69,57	0	63,64	1	43,24	0	60,00
2460	1	56,58	0	69,57	0	63,64	0	43,24	1	80,00
2560	1	57,89	0	69,57	0	63,64	1	45,95	0	80,00
3080	1	59,21	0	69,57	0	63,64	1	48,65	0	80,00
3140	1	60,53	0	69,57	0	63,64	1	51,35	0	80,00
3300	2	63,16	0	69,57	0	63,64	1	54,05	1	100,00
3510	1	64,47	0	69,57	1	72,73	0	54,05	0	100,00
3600	1	65,79	1	73,91	0	72,73	0	54,05	0	100,00
4000	1	67,11	0	73,91	1	81,82	0	54,05	0	100,00
4170	1	68,42	1	78,26	0	81,82	0	54,05	0	100,00

4250	1	69,74	1	82,61	0	81,82	0	54,05	0	100,00
4500	1	71,05	0	82,61	0	81,82	1	56,76	0	100,00
5220	1	72,37	1	86,96	0	81,82	0	56,76	0	100,00
5600	1	73,68	0	86,96	0	81,82	1	59,46	0	100,00
5940	1	75,00	0	86,96	0	81,82	1	62,16	0	100,00
7070	1	76,32	0	86,96	0	81,82	1	64,86	0	100,00
7120	1	77,63	0	86,96	0	81,82	1	67,57	0	100,00
7400	1	78,95	0	86,96	0	81,82	1	70,27	0	100,00
7520	1	80,26	0	86,96	0	81,82	1	72,97	0	100,00
8110	1	81,58	0	86,96	0	81,82	1	75,68	0	100,00
8210	1	82,89	0	86,96	0	81,82	1	78,38	0	100,00
8700	1	84,21	1	91,30	0	81,82	0	78,38	0	100,00
11530	1	85,53	1	95,65	0	81,82	0	78,38	0	100,00
13100	1	86,84	0	95,65	0	81,82	1	81,08	0	100,00
15000	1	88,16	0	95,65	0	81,82	1	83,78	0	100,00
17280	1	89,47	1	100,00	0	81,82	0	83,78	0	100,00
20700	1	90,79	0	100,00	0	81,82	1	86,49	0	100,00
21000	1	92,11	0	100,00	0	81,82	1	89,19	0	100,00
37180	1	93,42	0	100,00	1	90,91	0	89,19	0	100,00
38370	1	94,74	0	100,00	0	90,91	1	91,89	0	100,00
47600	1	96,05	0	100,00	1	100,00	0	91,89	0	100,00
160130	1	97,37	0	100,00	0	100,00	1	94,59	0	100,00
297090	1	98,68	0	100,00	0	100,00	1	97,30	0	100,00
297420	1	100,00	0	100,00	0	100,00	1	100,00	0	100,00

The distribution of TTL (CK19 mRNA copies/µL) shows different median values for each pT stage. Copies: CK19 mRNA copies/µl; Cum (%): cumulative percentage; n: number of cases

Estudio 2

Endoscopic tattooing of early colon carcinoma enhances detection of lymph nodes most prone to harbor tumor burden

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Resumen

Antecedentes: Los programas de cribado del cáncer colorrectal (CCR) han derivado en una mayor detección de carcinomas asintomáticos en etapas iniciales, potencialmente curables con manejo quirúrgico. Los ganglios linfáticos (GLs) de los CCRs en estadios iniciales suelen ser pequeños y pueden ser difíciles de identificar macroscópicamente. Aún así, al menos 12 GLs deben ser evaluados por espécimen para poder garantizar un estadio pN0 fiable. El tatuaje endoscópico parece mejorar las tasas de GLs aislados. De manera adicional, la detección molecular de carga tumoral en GLs de pacientes con CCR histológicamente pN0 se asocia con una menor tasa de supervivencia.

El objetivo del presente estudio es evaluar el impacto del tatuaje endoscópico prequirúrgico en la detección molecular de carga tumoral en GLs de neoplasias de colon en estadios iniciales.

Métodos: Se trata de un estudio de cohorte prospectivo basado en una población de cribado de CCR en un hospital universitario terciario. Se evaluaron los GLs de colectomías con y sin tatuaje endoscópico preoperatorio mediante dos métodos, HE y RT-LAMP, detectando este último ARNm tumoral de citoqueratina 19 (*CK19*). Se comparó la cantidad de la carga tumoral y los valores de GLs obtenidos entre especímenes tatuados y no tatuados.

Resultados: Se evaluaron mediante HE y RT-LAMP 936 GLs obtenidos de 71 colectomías que contenían carcinomas precoces y adenomas endoscópicamente irresecables (8 pT0, 17 PTI, 27 pT1, 19 pT2); 47 de 71 (66,2%) estaban tatuados. La positividad molecular en GLs se correlacionó con la presencia de tatuaje ganglionar [p <0,001; odds ratio 3.1 (95% IC 1.7 a 5.5)]. Se obtuvo un número significativamente mayor de GLs en especímenes tatuados en comparación con los no tatuados (mediana 17 GLs vs. 14,5 GLs; p = 0,019).

Conclusiones: el tatuaje endoscópico permite la detección de los ganglios linfáticos más proclives a albergar células tumorales y aumenta el número de ganglios linfáticos aislados.

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Endoscopic tattooing of early colon carcinoma enhances detection of lymph nodes most prone to harbor tumor burden

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Abstract

Background Colorectal cancer (CRC) screening programs result in the detection of early-stage asymptomatic carcinomas suitable to be surgically cured. Lymph nodes (LN) from early CRC are usually small and may be difficult to collect. Still, at least 12 LNs should be analyzed from colectomies, to ensure a reliable pN0 stage. Presurgical endoscopic tattooing improves LN procurement. In addition, molecular detection of occult LN tumor burden in histologically pN0 CRC patients is associated with a decreased survival rate. We aimed to study the impact of presurgical endoscopic tattooing on the molecular detection of LN tumor burden in early colon neoplasms.

Methods A prospective cohort study from a CRC screening-based population was performed at a tertiary academic hospital. LNs from colectomies with and without preoperative endoscopic tattooing were assessed by two methods, hematoxylin and eosin (HE), and RT-LAMP, to detect tumor cytokeratin 19 (CK19) mRNA. We compared the amount of tumor burden and LN yields from tattooed and non-tattooed specimens.

Results HE and RT-LAMP analyses of 936 LNs were performed from 71 colectomies containing early carcinomas and endoscopically unresectable adenomas (8 pT0, 17 pTis, 27 pT1, 19 pT2); 47 out of 71 (66.2 %) were tattooed. Molecular positivity correlated with the presence of tattoo in LN [$p < 0.001$; OR 3.1 (95 % CI 1.7–5.5)]. A significantly higher number of LNs were obtained in tattooed specimens (median 17 LN vs. 14.5 LN; $p = 0.019$).

Conclusions Endoscopic tattooing enables the analysis of those LNs most prone to harbor tumor cells and improves the number of LN harvested.

Keywords Colorectal cancer · Lymph nodes · Endoscopic tattooing · India ink · OSNA

Endoscopic tattooing was originally introduced as a reliable and accurate method to localize colonic lesions on follow-up colonoscopies, i.e., incompletely resected polyps or scars from prior polypectomies [1]. Its use has been extended with the introduction of laparoscopic and robotic surgery to locate lesions and determine the extent of colonic resection [1–4]. In addition, colorectal cancer (CRC) population screening programs have increased the detection of early-stage asymptomatic CRC and malignant polyps [5]. This has led to new challenging diagnostic and management issues, including the need to adequately

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define the underlying malignant potential of a certain polyp [6], the decision of endoscopic control or surgical treatment, and the indication for postoperative adjuvant chemotherapy.

Surgically removed early colorectal tumors face the challenge of assessing the lymph node (LN) metastatic potential of a single case, based on the information included in the histopathology report [7]. Considering that LN staging is an accurate prognostic factor, there is a broad consensus on the need to achieve the highest number of LNs [7–9]. In order to increase histological diagnostic sensitivity, gross LN enhancement with nodal revealing solutions [10] and molecular LN staging assessment have been proposed [11–18]. Nevertheless, despite the evidences of impaired survival rates associated with the molecular detection of tumor cells in regional LN of stage I–II CRC patients [12, 14, 19], LN molecular analysis is not performed on a daily basis, mainly due to its high costs and time-consuming nature.

Moreover, the impact of presurgical submucosal tattooing on the LN yield obtained is controversial [9, 20–22]. Methodological differences in the type and number of LN studied, as well as the different aims of the studies, may account for the discrepancies, i.e., some authors have used India ink to identify sentinel lymph nodes (SLN), advocating that it may help to identify metastatic LN, while others have used it for LN mapping and increasing the number of LN [20, 23–25].

The aim of this study was to assess the impact of endoscopic tattooing on the molecular detection of LN tumor burden in early colon neoplasia. We used the molecular assay one-step nucleic acid amplification (OSNA, Sysmex Corporation, Kobe, Japan), which is based on the quantitative detection of cytokeratin 19 (CK19) mRNA. This technique is widely used in the evaluation of breast cancer SLN [26] and is being introduced to assess regional LN in colon [27, 28], lung [29], endometrial [30], and thyroid cancer [31]. In addition, we also aimed to assess the enhancement of LN procurement in tattooed colectomies.

Materials and methods

Patients

From May 2012 to December 2013, we included all patients submitted to surgery for colon carcinomas or endoscopically unresectable adenomas diagnosed at our institution. The study was performed in the context of a population-based CRC screening program using fecal immunochemical test (FIT) (OC-Sensor®, Eiken, Japan; cutoff, ≥20 μg of hemoglobin/g of feces). FIT was offered every 2 years to asymptomatic individuals between 50 and 69 years old.

This study is focused on the molecular identification of LN metastasis in early colon neoplasms (pT0–2) and the influence of tattooing in this setting. We included individuals over 18 years old with endoscopically unresectable adenomas, malignant polyps with confirmed pT1 carcinoma containing adverse prognostic factors associated with LN metastasis (i.e., the presence of at least one of the following features: poor differentiation, lymphovascular invasion, high-grade tumor budding, tumor margin ≤1 mm, and submucosal invasion ≥2 mm), and pT1–2 stage colon carcinomas. pT3–4 carcinomas were excluded since they are seldom tattooed at our institution as they are regarded as large infiltrating masses easy to be surgically localized. Other exclusion criteria were tumor infiltration of the mesocolon fat on gross examination, rectal tumors, synchronous colorectal carcinomas, appendicular carcinomas, the presence of colonic stent, inflammatory bowel disease or other malignancies, and reception of surgical specimen immersed in formalin.

Ethical considerations

The study was approved by the Ethics Committee of Hospital Clinic of Barcelona, Spain. All patients signed and kept a copy of the informed consent document for participation in the study. Another copy was kept with the patient's clinical files.

Study procedures

Colonoscopy and tattooing

Colonoscopies were performed on patients with a positive FIT result. Our institution has a high-quality endoscopy unit dedicated to screening and follow-up colonoscopy, with highly skilled, experienced endoscopists (performing more than 200 endoscopies per year), and quality assurance controls according to the European guidelines on quality in screening colonoscopy [32]. The standard practice of colonoscopy tattooing to provide accurate localization of lesions for later identification is performed at the endoscopist criteria, following the clinical protocols of the Gastroenterology Department at Hospital Clínic, Barcelona, Spain, performed in compliance with the European guidelines [32]. Thus, colonoscopy tattooing is performed after removal of 2-cm polyps or larger, and on all suspicious lesions at colonoscopy situated outside of the cecum or rectum: namely non-resectable polyps, lesions suspected of submucosal invasive carcinoma, or endoscopically partially resected advanced adenomas (>10 mm, villous, or after a pathology diagnosis of high-grade dysplasia).

For tattooing, 10 % of diluted and sterilized commercial India ink was used (Pelikan drawing ink color black (A17), Pelikan Vertriebsgesellschaft mbH & Co. KG, Hannover, Germany), which was crafted at the Pharmacy Department of Hospital Clinic by mixing 6 ml of ink and 54 ml of bi-distilled water, divided in 2-mL glass vials, closed, and sterilized by autoclaving at 120 °C for 20 min. India ink tattooing was performed by injection of 1 mL of ink in the submucosa adjacent to the lesion after previous saline submucosal injection, to verify its correct location and to avoid ink injection into the peritoneum (Fig. 1a). For lesions located at the sigmoid or ascending colon, tattoos were placed 1 to 2 cm distally to the lesion and on two opposite sides of the colonic lumen. For lesions located at the transverse colon, tattoos were placed both distally and proximally to the lesion and on both sides of the lumen (i.e., at 3 and 9 o'clock).

Surgery

Laparoscopy-assisted colectomy under general anesthesia was performed in all patients by a single gastrointestinal surgical team with wide experience in laparoscopic procedures [4]. Throughout the operation, a pneumoperitoneum with intra-abdominal pressure between 10 and 14 mmHg was maintained. In all patients, colon resection was carried out following oncological criteria, consisting in proximal vessel ligation, en bloc lymphadenectomy, and broad macroscopic resection margins. Routine measures used to prevent port-site metastasis included initial vascular ligation, the use of a wound edge protector, reduction in intra-abdominal pressure before tumor extraction, and exhaustive cleansing with 5 % iodopovidone solution.

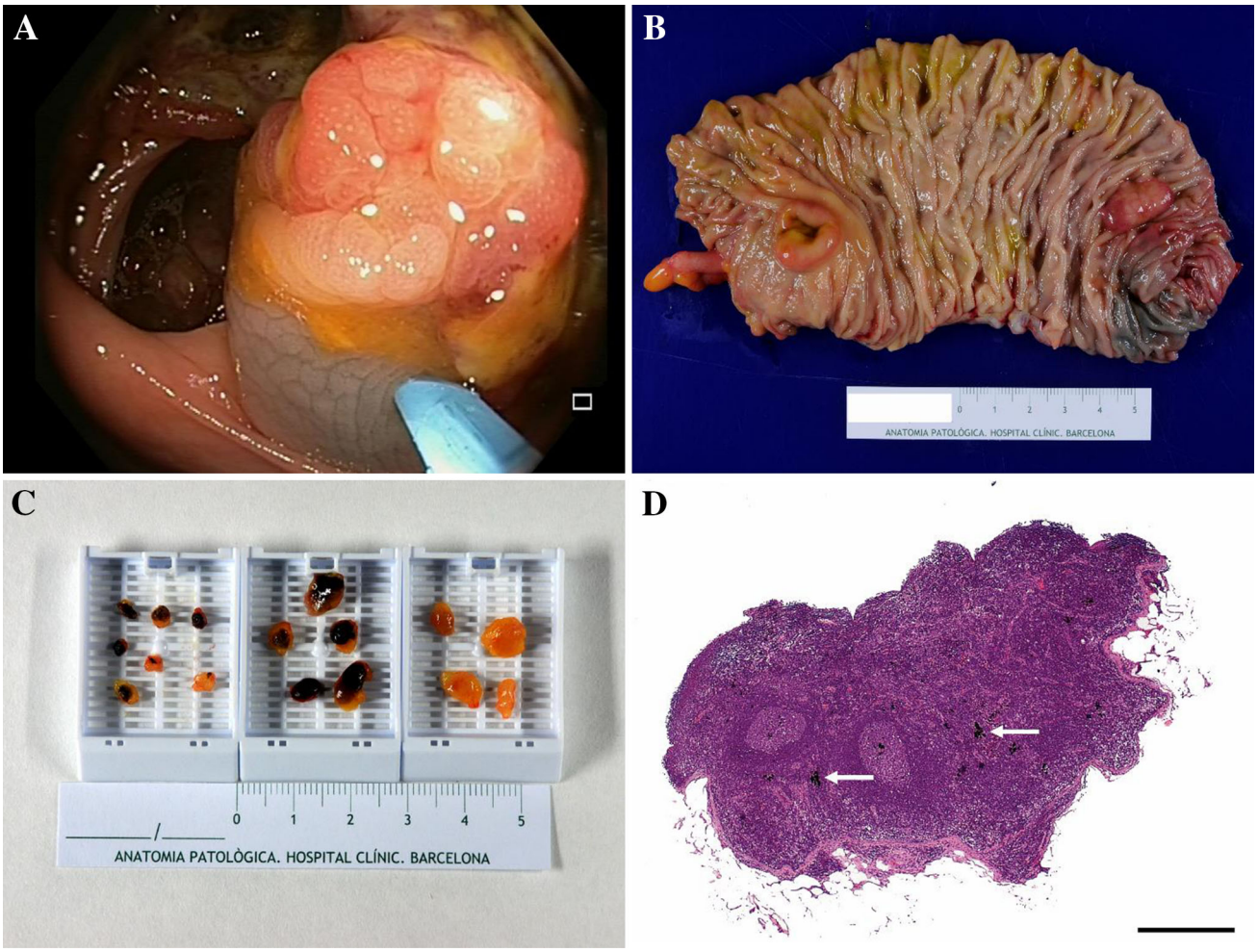

Fig. 1 **A** Endoscopic tattooing of a colon carcinoma. Notice submucosal ink injection adjacent to the lesion; **B** gross surgical specimen of a right-side hemicolectomy with tattooed area near an advanced adenoma. Ruler in centimeters; **C** lymph nodes harvested from one surgical specimen. The darker tonalities of tattooed lymph nodes eased their procurement. Notice the left cassette with some small tattooed lymph nodes. A few lymph nodes placed on the right cassette had no ink. Ruler in centimeters; **D** histological HE section of a LN with presence of carbon particles inside (*arrows*) from previous tattooing (*scale bar* 500 μm)

Surgical specimens

The study included colectomies containing adenocarcinomas, partially resected advanced adenomas, non-resectable polyps, adenomas with suspicion of submucosal invasive carcinoma, and endoscopically resected malignant polyps that contained adverse prognostic factors listed above, which required complete colectomy (Fig. 1B). Pathological data from both endoscopically and surgically resected neoplasms were collected and recorded (Table 1). Patient's staging resulted from the pathological evaluation of both endoscopic and surgical specimens. Gross processing and pathology reports were performed according to the protocols of the pathology department, adopted from the latest version of the colorectal protocol of the AJCC [33]. All cases were diagnosed and reviewed by two senior gastrointestinal pathologists (JAB, MC).

Lymph node harvest

Lymph nodes from all surgical specimens were identically procured and assessed (Fig. 1C). LNs were also dissected and analyzed from colectomies with no residual tumor performed after an endoscopic polypectomy containing a pT1 carcinoma with adverse prognostic factors. Fresh LN procurement from the mesocolon fat was performed at the pathology department within 50 min after surgical resection by four pathologists, IA, CM, NR, and MC. Fresh LNs were sectioned and submitted for both conventional histology with HE and the OSNA molecular assay, for the detection and amplification of tumor CK19 mRNA [28]. A second-look LN search was performed after 18–30 h of formalin fixation. LNs revealing solutions (i.e., alcohol) were not used. All formalin-fixed paraffin-embedded (FFPE) LNs were submitted only for HE analysis. The presence of India ink in the LN was recorded after evaluation of the HE slides from all LNs using a conventional Olympus BX41 microscope (Olympus, Tokyo, Japan) (Fig. 1D).

OSNA lymph node molecular analysis for CK19 mRNA quantification

The OSNA method was performed following the manufacturer's instructions, using a modified protocol from Tsujimoto et al. [28, 34, 35]. In this assay, the amount of CK19 mRNA/μL copies correlates with the size of the metastasis [34]. The results were based on the number of CK19 mRNA copies/μL obtained for each LN, with a cutoff of 100 CK19 mRNA copies/μL. Values from 100 to 250 CK19 mRNA copies/μL corresponded to isolated tumor cells (ITC). The total tumor load (TTL) of a given specimen resulted from the sum of all CK19 mRNA copies/μL from each positive LN. Evaluation of the molecular results was performed blindly with respect to both clinical and pathological assessments.

India ink and OSNA assay interference test

In order to determine whether the carbon particles from India ink interfered with the RT-LAMP reaction, we tested the RT-LAMP CK19 mRNA reaction with and without India ink at 1:100 dilution, which represents a surplus of carbon particles compared to the real traces of carbon particles present in the LN of a tattooed surgical specimen. From a 2000 μL mix containing 20 μL of the positive control containing human CK19 mRNA sample and 1980 μL of lysis buffer Lynorhag (Sysmex Corp. Kobe, Japan), we transferred 200 μL of the mix into 10 OSNA vials and performed the analysis using the Lynoamp BC gene amplification reagent (Sysmex Corp. Kobe, Japan). The same process was done with 20 μL of India ink, 20 μL of positive control CK19 mRNA sample, and 1960 μL of Lynorhag (Sysmex). The amplification product was detected by measuring the rise time required to exceed a predetermined threshold turbidity caused by the by-product magnesium pyrophosphate. The values obtained among samples with high concentration of India ink and without India ink were not significant, showing the absence of India ink interference in the RT-LAMP reaction (Supplementary figure).

Outcomes

The main outcome was to compare the presence of CK19 mRNA tumor burden in LN among tattooed and non-tattooed specimens.

The secondary outcome was to determine the differences in the yield of LN procured in tattooed and non-tattooed specimens.

Statistical analysis

Continuous variables were described as median and interquartile ranges (IQR), and categorical variables as absolute frequencies and percentages. Spearman's rank correlation coefficient was applied to assess correlation between continuous variables. The association of categorical variables was assessed by Fisher's exact test. The Mann–Whitney–Wilcoxon test was performed to analyze the statistical significance of differences in continuous parameters. Cohen's kappa was used to assess the degree of agreement. A mixed-effects logistic model with a random patient effect was used to assess the prediction of OSNA outcome in tattooed cases. A p value of <0.05 was considered statistically significant. All analyses were performed using R statistical environment (V.3.0.2) [36].

Table 1 Patient demographics and specimen characteristics

Variables	Total	Tattooed specimens	Non-tattooed specimens	*p* value
Cases	71	47	24	
Gender				0.61
Male	43 (60.6)	27 (57.4)	16 (66.7)	
Female	28 (39.4)	20 (42.6)	8 (33.3)	
Age (years)	64 (59–70)	63 (59–68)	66 (62–74)	0.15
Surgical specimen characteristics				
Specimen size (cm)	14 (11–18)	14 (12–17.3)	13.3 (10.9–19.3)	0.93
Adenocarcinoma size (cm)	1.5 (0.9–3)	1.5 (0.9–2.5)	1.8 (0.8–3.4)	0.62
Tumor location				0.12
Cecum	12 (16.9)	4 (8.5)	8 (33.3)	
Ascending colon	16 (22.5)	9 (19.1)	7 (29.2)	
Hepatic flexure	3 (4.2)	3 (6.4)	0 (0.0)	
Transverse colon	6 (8.5)	6 (12.8)	0 (0.0)	
Splenic flexure	5 (7.0)	3 (6.4)	2 (8.3)	
Descending colon	3 (4.2)	3 (6.4)	0 (0.0)	
Sigmoid colon	26 (36.6)	19 (40.4)	7 (29.2)	
Surgical specimen type[a]				0.17
Completely resected	18 (25.3)	15 (31.9)	3 (12.5)	
Partially resected	6 (8.5)	3 (6.4)	3 (12.5)	
Non-resected	47 (66.2)	29 (61.7)	18 (75.0)	
Lymphovascular invasion[b]				0.09
No	65 (91.5)	41 (87.2)	24 (100)	
Yes	6 (8.5)	6 (12.8)	0 (0.0)	
Grade				0.15
High grade	9 (12.7)	8 (17.0)	1 (4.2)	
Low grade	62 (87.3)	39 (83.0)	23 (95.8)	
MS instability	5 (7.0)	2 (4.3)	3 (12.5)	0.33
Tumor budding ($n = 43$)[c]				1.00
High grade	30 (69.8)	21 (70.0)	9 (69.2)	
Low grade	13 (30.2)	9 (30.0)	4 (30.8)	
pTMN				0.53
pT0	8 (11.3)	4 (8.5)	4 (16.7)	
pTis	17 (23.9)	10 (21.3)	7 (29.2)	
pT1	27 (38.0)	20 (42.6)	7 (29.2)	
pT2	19 (26.8)	13 (27.7)	6 (25.0)	

Categorical variables are shown as absolute frequencies and percentages. Numerical variables are described as median and interquartile range (IQR)

[a] With respect to the endoscopic resection

[b] In one case, lymphatic invasion could not be assessed

[c] Tumor budding was assessed in 43 infiltrating carcinomas

Results

Sample characteristics

The flowchart of the study is detailed in Fig. 2. A total of 2980 colonoscopies were performed on patients with a positive FIT result. We excluded 1820 patients who had a normal colonoscopy or non-advanced adenomas which were endoscopically treated. We found 140 CRC and 1020 advanced adenomas. Most of the latter were endoscopically treated. A total of 103 surgically treated cases were included for LN analysis with OSNA and HE. Of them, 32 pT3–4 carcinomas were excluded. Finally, 71 patients met the study selection criteria. These individuals comprised of (a) 18 patients with endoscopically resected malignant polyps with adverse prognostic factors submitted to

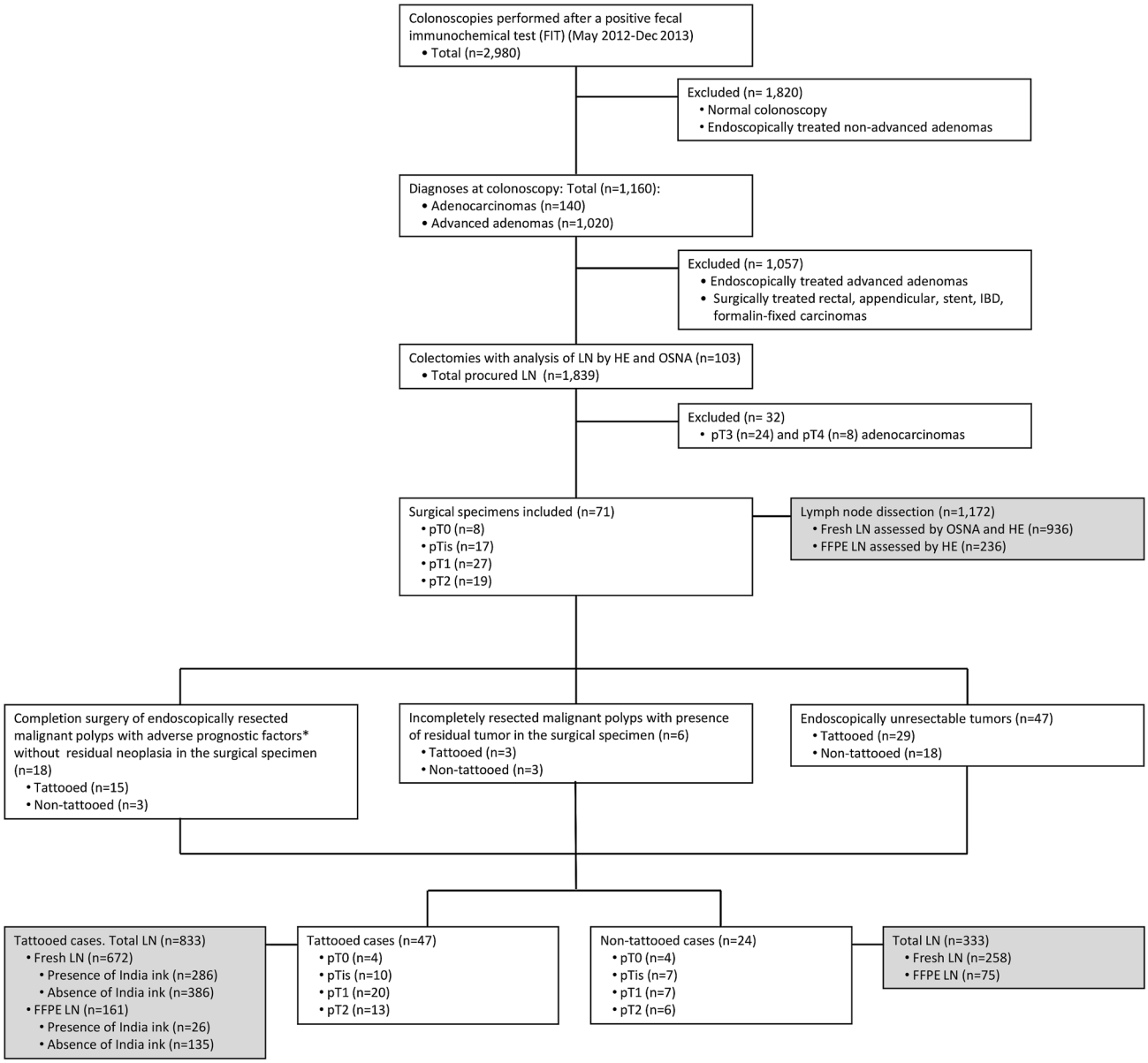

Fig. 2 Study flow diagram. Selection and classification of patients according to endoscopic tattooing and pathological findings. *The presence of at least one of the following features: poor differentiation, lymphovascular invasion, high-grade tumor budding, tumor margin ≤1 mm, submucosal invasion >2 mm

surgery. No residual tumor was found in the colectomy specimen; (b) 6 patients with partially resected malignant polyps at colonoscopy, with the presence of residual tumor in the surgical specimen; and c) 47 patients with endoscopically unresectable tumors.

Colectomy specimens were 14 cm (IQR 11–18 cm) in average size. Forty-seven (66.2 %) cases were tattooed and 24 (33.8 %) non-tattooed. The median period of time between endoscopic tattooing and surgery was 63 days (IQR 38–92 days). No differences were observed between both groups regarding demographic and pathologic specimen characteristics (Table 1). The median adenocarcinoma size was 1.5 cm (IQR 0.9–3.0 cm). Two cases had LN metastases on HE analysis. Regarding classical high-risk factors, 9 carcinomas contained high-grade areas and 6 presented angiovascular invasion. Perineural invasion was not observed in any case.

Lymph node assessment

The number of LN assessed in this study is detailed at the bottom of the flowchart (Fig. 2) and in Table 2. From the 71 surgical specimens included, 1172 LNs were procured; 936 (79.9 %) were freshly dissected and analyzed for both

OSNA and HE. After formalin fixation, 236 (20.1 %) LNs were obtained and analyzed with HE. A median of 15 lymph nodes was obtained per patient, 12 of them freshly harvested. The number of total LN procured per case was significantly higher in tattooed cases (median, 17 LNs in tattooed specimens vs. 14.5 LNs in non-tattooed specimens; $p = 0.019$) (Table 2).

Fresh LN procurement was performed within a median of 30 min (IQR 20.0–38.5 min). Although no differences were found in time expended on LN harvesting among tattooed and non-tattooed cases, a significant reduction in the harvesting time was observed in the former, when LN search time was corrected by the number of LN collected ($p = 0.014$, Table 2).

Analysis of the presence of India ink and tumor CK19 mRNA in LN among tattooed cases

We assessed with the optical microscope the presence of traces of India ink in the form of carbon particles among the 833 LNs obtained from the 47 tattooed cases; 672 LNs were freshly collected. India ink was present in a total of 312 LNs (286; 42.6 % fresh LNs, and 26; 16 % FFPE), See flowchart in Fig. 2. Carbon particles were present in a median of 7 LNs (IQR 4–8) per case.

Twenty-nine tattooed patients were positive for CK19 mRNA (61.7 %). We analyzed the association between the presence of India ink in LN and the detection of tumor CK19 mRNA (Table 2). Of the 672 freshly harvested LNs, 72 (10.7 %) contained tumor CK19 mRNA (44 LNs with India ink and 28 without). Importantly, 15.3 % (44/286) of LNs with carbon particles contained tumor CK19 mRNA, while less than 7.3 % (28/386) of LNs without India ink were positive for CK19 mRNA (Table 3). The logistic model with a random patient effect gave a significant effect of ink ($p < 0.001$) in the CK19 mRNA detection with an odds ratio of 3.1 (95 % CI 1.7–5.5).

Analysis of CK19 mRNA in the whole cohort

CK19 mRNA assessment revealed the presence of traces of tumor CK19 mRNA in LN of 42 out of 71 cases (59.2 %). The median TTL was 1350 (IQR 640–2938) CK19 mRNA copies/μL. Two tattooed cases with LN metastases on HE had a significantly higher TTL of 560,000 and 41,160 CK19 mRNA copies/μL, respectively. The median TTL of patients with histologically negative LN was 1275 CK19 mRNA copies/μL (IQR 620–2262). Additional analysis between OSNA results and classical high-risk factors showed an association with tumor size (positive cases showed larger tumors ($p = 0.02$)) and higher tumor grade ($p < 0.01$) (data not shown).

Treatment and clinical follow-up

All patients were surgically treated by a high-volume practice surgical team specialized in laparoscopic CRC surgery. The two patients with positive LN on HE received adjuvant chemotherapy. Median follow-up was 984 days (IQR 801.5–1118.5 days). Two patients developed distant

Table 2 Lymph node characteristics per case

Variables	Total ($n = 71$)	Tattooed specimens ($n = 47$)	Non-tattooed specimens ($n = 24$)	p value
Total lymph nodes	15 (12–20)	17 (13–21)	14.5 (10–17)	0.019
Fresh lymph nodes	12 (9–16.5)	13 (10–18)	10.5 (7.7–13.2)	0.02
FFPE lymph nodes	2 (1–5)	2 (1–5)	3 (1.7–4.2)	0.96
Lymph node harvest time (min)[a]	30 (20–38.5)	30 (20–38.5)	27.5 (20–36.2)	0.91
Lymph node harvest time (min) adjusted per LN	2.2 (1.8–3.0)	2.1 (1.8–2.5)	3.2 (1.9–3.8)	0.014
CK19 mRNA detection				0.61
CK19 mRNA detected	42 (59.2)	29 (61.7)	13 (54.2)	
CK19 mRNA not-detected	29 (40.8)	18 (38.3)	11 (45.8)	
TTL[b]	1350 (640–2938)	1420 (700–4270)	1270 (620–2200)	0.76

Categorical variables are shown as absolute frequencies and percentages. Numerical variables are described as median and interquartile range (IQR)

FFPE formalin-fixed paraffin-embedded

[a] Time spent on fresh lymph node harvesting per case

[b] Total tumor load (TTL) was calculated as the sum of CK19 mRNA copies/μL from all positive lymph nodes in one given case. The median and IQR shown was obtained from the cohort of positive CK19 mRNA cases ($n = 42$)

Table 3 CK19 mRNA detection in tattooed and non-tattooed lymph nodes

	Total LN no. (%)	Tattooed LN no. (%)	Non-tattooed LN no. (%)	
CK19 mRNA detected	72 (100)	44 (61.1)	28 (38.9)	$p < 0.001$
CK19 mRNA not-detected	600 (100)	242 (40.3)	358 (59.7)	
Total LN	672	286	386	

LN lymph node

metastases at 4 and 21 months after surgery, respectively. Both patients presented pT2N0 right-sided tumors with no high-grade features and comparable tumor sizes (30 and 25 mm). The first patient was not tattooed, with 14 out of 18 LNs analyzed by OSNA, and a high TTL of 47,760 CK19 mRNA copies/μL. The second patient was a tattooed case, negative for CK19 mRNA detection, but had only 10 LNs analyzed by OSNA out of 20 procured LNs.

Discussion

Colonoscopic submucosal tattooing was originally developed to aid in the localization of colon tumors by the endoscopist and surgeon. Although it has been a useful clinically oriented method [1–4], its impact on and benefit in relation to LN retrieval and CRC staging is controversial [9, 20–25]. While some studies find that preoperative tattooing improves LN harvest [9, 20, 21, 24], other publications with divergent results have recently appeared [22]. It is widely accepted that the number of pathologically assessed LN is a critical issue, as it has been demonstrated that it correlates with survival, particularly for node-negative colon cancer patients. In fact, the greater the number of LN examined, the greater the chance to detect metastases [7–9]. Current guidelines recommend that at least 12 LNs should be pathologically assessed to ensure an adequate specimen evaluation and a reliable pathologic staging [33, 37].

Although the number of LN obtained depends on patient, surgical and pathological factors, the latter are decisive and may include revealing solutions that ease LN procuring, i.e., ether and alcohol. [7, 9, 38]. It seems that placement of tattoo by injection of carbon-based dyes causes a permanent deposit of carbon particles in LN, resulting in a dark stain of the LN which helps gross detection and dissection, especially of small-sized LN. Of notice, the median time period between endoscopic tattooing and surgical resection in our study was of 63 days, with a correct microscopic analysis of the presence of carbon particles, being in other series from intraoperative injection to 30 days between both events [20–22, 24].

In agreement with previous studies [9, 20, 21, 23–25], we have evaluated the usefulness of presurgical colonoscopic tattooing to obtain higher LN retrievals. We obtained a median of 17 LNs in tattooed colectomies compared to 14.5 LNs in non-tattooed ones ($p = 0.02$). We obtained at least 12 LNs in 87.3 % of tattooed cases, in compliance with international guidelines [33], which represented an increase of 20.6 % with respect to non-tattooed cases.

Nodal metastases in early CRC are often present in small, difficult to identify, LN of <5 mm in greatest diameter [17, 18, 33]. Presurgical colonoscopic tattooing enabled us to easily detect those LNs. The presence of tattooing may also enhance the likelihood of harvesting the first level of nodal drainage, as well as other LNs in the drainage basin that can also shelter metastatic disease [9, 20, 23, 25]. Spatz et al. [23] analyzed 311 LNs from 21 specimens concluding that colonoscopic tattooing is potentially beneficial for appropriate colon cancer staging.

Our results reinforce previous findings, having found a significantly higher amount of tumor CK19 mRNA among tattooed LNs, with an odds ratio of 3.1 ($p < 0.001$). In our study, we tried to evaluate the practical benefit of a tattooed specimen. As Bartels did, we considered cases to be tattooed only when they had traces of India ink either at gross or at LN microscopic analysis [20]. Differences in methodology and limitations of previous studies may account for controversial results. Feo et al. [22] retrospectively studied 250 colorectal specimens divided into two cohorts, with and without preoperative colonoscopic tattooing. They concluded that preoperative tattooing did not improve the LN yield. Nevertheless, histologic assessment seeking for LN carbon deposits was performed in only 30 out of 107 tattooed cases. They found LN carbon particles in only 2 of the 30 cases [22]. In our prospective cohort, all 47 tattooed cases had gross and histologic traces of carbon particles.

The prognostic significance of LN tumor burden detection using molecular techniques has already been established for breast and CRC [12–16, 26, 39]. Several meta-analyses have found an independent significant association between molecular LN tumor detection and an increased

risk of disease recurrence and poor survival in CRC patients [12–15, 19, 40]. Nevertheless, tumor burden detected using molecular techniques may not always be clinically relevant. A study using RT-qPCR detection of guanylyl cyclase C in colon cancer patients found positive LN in 87.5 % of them, although only 20.9 % developed recurrent disease [13]. Other highly sensitive molecular techniques, such as liquid biopsy, have also demonstrated the presence of circulating cell-free tumor DNA in patients with precursor lesions and in situ carcinomas [41, 42].

The aim of our study was not to prove the prognostic significance of LN tumor burden detection using molecular techniques, but to demonstrate the usefulness of tattooing in the detection of those LN most prone to hold tumor burden. For that reason, we included in our analysis those LN holding isolated tumor cells, or small traces of tumor CK19 mRNA, which are known to date to have no clinical significance. In other studies using OSNA, the presence of small amounts of TTL is overlooked. In fact, TTL over 15,000 CK19 mRNA copies/μL has been settled for breast and thyroid cancer sentinel LN studies to determine the likelihood of additional nodal metastases [26, 31]. In our study, the TTL obtained was much lower, with a median of 1350 CK19 mRNA copies/μL per patient. Interestingly, patients with high TTL had either disease recurrence or LN metastases, except for one patient with disease recurrence that had only 50 % of the retrieved LN assessed by OSNA. Forthcoming studies are needed in CRC to determine the amount of clinically significant nodal total tumor load.

Our study has some drawbacks. Firstly, it was performed in a single institution and has a small number of cases analyzed, although it was performed in a screening-based program which allowed us to obtain early-stage carcinomas. Secondly, the distance of the tattoo from the neoplastic lesion was not recorded and, therefore, the correlation between this variable and the CK19 mRNA values could not be ascertained.

Our study highlights colonoscopic tattooing as a highly efficient LN procurement. Tattooing helps the endoscopist and the surgeon to localize the tumor. In addition, it can be used as an extra pathology tool to harvest a higher amount of LN, but more importantly, it makes it possible to find those LNs which might shelter tumor. In a CRC screening-based population, an expanded use of presurgical endoscopic tattooing could benefit patient's diagnosis and therapeutic management.

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Author contributions M.C., M.P., A.C., and I.A. were involved in study conception and design; I.A., C.M., G.F., A.G., S.D., D.M., L.M., and G.M. were responsible for data acquisition; M.C., I.A., and N.P. were involved in statistical analysis and data interpretation; M.C., I.A., and M.P. drafted the manuscript; M.P., F.B., and A.C. critically revised the article for important intellectual content; M.C. and A.C. finally approved the article.

Compliance with ethical standards

Disclosures None of the authors of this manuscript, Iban Aldecoa M.D.; Carla Montironi M.D.; Nuria Planell B.Sc; Maria Pellise M.D., PhD; Gloria Fernandez-Esparrach M.D., PhD; Angels Gines M.D., PhD; Salvadora Delgado M.D., PhD; Dulce Momblan M.D.; Leticia Moreira M.D., PhD; Maria Lopez-Ceron M.D., PhD; Natalia Rakislova M.D.; Graciela Martínez-Palli M.D., PhD; Jaume Balust M.D.; Josep Antoni Bombi M.D., PhD; Antonio de Lacy M.D., PhD; Antoni Castells M.D., PhD; Francesc Balaguer M.D., PhD; and Miriam Cuatrecasas M.D., PhD, have any conflicts of interest or financial ties to disclose.

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Electronic Supplementary Material nº1

India ink and OSNA assay interference test

India ink RT-LAMP interference test

Vial number	CK 19 mRNA Rise time	
	0 (Positive control without India ink)	1/100 (Positive control with India ink)
1	10,7	10,7
2	10,8	10,8
3	10,8	10,8
4	10,8	10,9
5	10,9	10,9
6	11	10,9
7	11,1	10,9
8	11,1	10,9
9	11,1	11
10	11,1	11
11	11,2	11,1
12	11,3	11,1
Average	11,0	10,9
StDEv	0,19	0,12
St Error	0,05	0,03
Conc.[copies/uL]	3.0E+02	4.6E+02
Qualitative	(+)	(+)