# Molecular Staging of Patients with Colon Cancer. The C-Closer-II Study: A Multicentre Study in Portugal

# Estadiamento Molecular de Doentes com Carcinoma do Colon. O Estudo C-Closer-II: Um Estudo Multicêntrico em Portugal

ARTIGO ORIGINAL

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## ABSTRACT

**Introduction:** Approximately 20% - 30% of histological lymph node-negative patients with colorectal cancer relapse at five years after surgical treatment. This recurrence is likely due to occult nodal disease undetected by standard histopathological practice which has implications in terms of the clinical management of patients.

**Material and Methods:** Lymph nodes were collected from colectomy specimens. A central section from each lymph node was histologically examined following haematoxylin-eosin staining and the remaining tissue was subjected to OSNA - one step nucleic acid amplification analysis.

**Results:** A total of 1046 lymph nodes from 59 pN0 patients were assessed. Of these, 753 lymph nodes were examined by both methods. The median number of lymph nodes assessed with OSNA - one step nucleic acid amplification was 12 (IQR: 7;16). Among pN0 patients, 17 had OSNA - one step nucleic acid amplification-positive lymph nodes, resulting in a positive molecular staging rate of 28.8% (95% CI: 17.8 - 42.1). Among these patients, 12 (70.59%) were molecular-staged as pN1 and 5 (29.41%) were molecular staged as pN2. The tumour burden of lymph nodes assessed with OSNA - one step nucleic acid amplification ranged from 270 to 17 000 cytokeratin 19 mRNA copies/μL. Most of these patients (88.2%) were found to have lymph nodes with micrometastases only (250 - 4999 copies/μL).

**Discussion:** We provide the results from the first study of the use of the OSNA - one step nucleic acid amplification assay in colorectal cancer patients in Portugal. Our results are in-line with other international studies, showing the improvement on patients' staging by molecular examination of lymph nodes.

**Conclusion:** In our study, 28.8% of patients with histologically negative lymph nodes were found to have metastatic lymph nodes using OSNA - one step nucleic acid molecular assessment. OSNA - one step nucleic acid assay allows a more accurate staging of patients with colorectal cancer and standardizes lymph node assessment.

**Keywords:** Colorectal Neoplasms; Lymph Nodes; Lymphatic Metastasis; Neoplasm Staging; Nucleic Acid Amplification Techniques

## RESUMO

**Introdução:** Cerca de 20% - 30% dos doentes com cancro colo-rectal, com gânglios linfáticos regionais negativos por histologia têm recidiva do carcinoma colo-rectal, após cinco anos do tratamento cirúrgico. Esta recorrência é provavelmente devida à presença de metástases ganglionares ocultas, não detetadas no exame anatomo-patológico *standard*.

**Material e Métodos:** Os gânglios linfáticos foram obtidos a partir de espécimes de colectomia. Uma secção central de cada gânglio linfático foi analisada histologicamente com a coloração de hematoxilina-eosina e o tecido restante foi sujeito a análise de OSNA - *one step nucleic acid amplification*.

**Resultados:** Um total de 1046 gânglios linfáticos de 59 doentes pN0 foram avaliados. Foram examinados 753 gânglios linfáticos por ambos os métodos. A mediana de gânglios linfáticos avaliados com OSNA - *one step nucleic acid amplification* foi de 12 (IQR:7; 16). Entre os doentes pN0, 17 tinham gânglios linfáticos positivos para OSNA - *one step nucleic acid amplification*, resultando numa taxa de estadiamento molecular positiva de 28,8% (95% CI: 17,8 - 42,1). De entre esses doentes, 12 (70,59%) apresentaram-se molecularmente pN1 e cinco (29,41%) pN2. A carga tumoral dos gânglios linfáticos avaliada com OSNA - *one step nucleic acid amplification* variou de 270 a 17 000 cópias/μL de ARNm de citoqueratina 19. A maioria desses doentes (88,2%) apresentou gânglios linfáticos com micrometástases (250 - 4999 cópias/μL).

**Discussão:** Apresentamos os resultados do primeiro estudo do ensaio OSNA - *one step nucleic acid amplification* levado a cabo, em pacientes com cancro colo-rectal, em Portugal. Os nossos resultados estão em linha com outros estudos internacionais, demostrando uma melhoria no estadiamento dos doentes, pelo exame molecular dos gânglios linfáticos.

**Conclusão:** Verificou-se que 28,8% dos doentes com gânglios linfáticos histologicamente negativos apresentavam doença ganglionar metastática, usando a avaliação molecular de OSNA - *one step nucleic acid amplification*. O ensaio OSNA - *one step nucleic acid amplification* permite um estadiamento mais preciso de doentes com carcinoma do colon e padroniza a avaliação dos gânglios linfáticos.

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**Palavras-chave:** Estadiamento de Neoplasias; Gânglios Linfáticos; Metástase Linfática; Neoplasias Colo-rectais; Técnicas de Amplificação de Ácido Nucleico

## INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancers worldwide with 1.36 million cases and 694 000 deaths reported annually.[1] According to recent estimates, CRC is the most common cancer and the second most common cause of cancer-related deaths in Europe. In Portugal, the number of deaths from CRC increased by 3% per year between 2000 and 2005, and the overall survival rates at 1, 3 and 5 years are estimated at 73%, 55% and 46%, respectively.[2]

After potentially curative surgery for CRC, nodal involvement is the major determinant for prognosis and represents the main indicator for adjuvant chemotherapy. Nonetheless, 20% to 30% of node-negative patients (stages I and II according to AJCC classification) develop loco-regional or distant disease recurrence after surgery within 5 years of follow-up.[3,4] The recurrence in these patients, designated as pN0 and excluded from adjuvant therapy, is most likely due to occult nodal disease which remains undetected and hence untreated, since only about 1% of each whole node is examined in conventional histopathological examination with haematoxylin-eosin (H&E). Therefore, strategies that improve accuracy in diagnosis and the staging of patients with CRC should help to detect patients that would benefit from adjuvant therapy.

Large numbers of lymph nodes (LN) are frequently harvested from the colorectal resection, which makes in-depth analysis using immunohistochemical (IHC) staining a very time-consuming and impractical procedure.[5] The feasibility of a molecular approach to nodal staging has been investigated in several studies.[6–8] The OSNA (one step nucleic acid amplification) assay (Sysmex Corp., Japan) uses the RT-LAMP (reverse transcription loop-mediated isothermal amplification) technique to rapidly amplify the CK19 mRNA tumour marker. OSNA provides an indirect quantification of CK19 mRNA amounts based on the turbidity of the reaction and analyses up to four nodes in a single run. The OSNA assay allows for the standardization of the diagnosis of LN metastasis, and is suitable for intraoperative LN evaluation, particularly in breast cancer patients.[6,7] Additionally, the OSNA assay discriminates between macrometastasis and micrometastasis in LN based on the number of CK19 mRNA copies per microliter.[6,8]

The diagnostic advantage of OSNA in comparison with histological examination has been proved in CRC, providing values of 94.9% - 95.2% sensitivity and 97.7% - 97.9% specificity.[8–11] Several studies have reported nodal molecular staging of CRC patients using OSNA.[11–14] A European multicentre study detected over 25% positive results in molecular staging of patients with initially histopathological-negative lymph nodes.[12] Among them, 16.2% patients were molecular-staged to UICC-I and 30.3% to UICC-II. The multicentre study conducted in Japan with 204 CRC patients (pN0 and pN1) found 11.3% were molecularly upstaged.[14] In this study most pN0 patients (71%) had a single LN affected. Moreover, although sentinel lymph nodes (SLN) in CRC are still unclear,[15,16] a Dutch study has shown that the molecular staging of SLN using OSNA is a promising tool for predicting the prognosis of CRC patients.[13] Thus, the molecular diagnosis of LN status using OSNA improves the ability to identify patients that may benefit from an additional systemic treatment.

In this multicentre prospective study, we aim to assess the capacity of OSNA to identify the undetected tumour burden in cases with histologically negative lymph nodes.

## MATERIAL AND METHODS

### Study design

This collaborative prospective study was carried out in four Portuguese hospitals during a 2-year accrual from 2013 to 2015. Eligible patients were over the age of 18 years, with primary histologically confirmed colon cancer with preoperative clinically and radiologically negative nodes, non-infiltration of the mesocolon, and CK19 immunopositivity of the primary tumour. The exclusion criteria included non-invasive tumours, positive LN on H&E examination, synchronous tumours or other malignancies, cN1, metastatic cancer, neoadjuvant chemotherapy, familial adenomatous polyposis, carcinomas in inflammatory bowel disease, and the presence of stent-type intraluminal devices. The study was approved by the Ethics Committee of each hospital and all enrolled patients provided the signed informed consent form.

### Lymph node collection and assessment

After surgical extraction, the colectomy specimen was immediately taken to the pathology department. The mesocolon was separated from the colon wall, and fresh LN were harvested from the mesocolic fat within 60 minutes after surgical resection. A correlative number was assigned to each freshly dissected LN to compare H&E and OSNA results. The LN were cut along the long axis. A central 1-mm slice was subjected to conventional formalin-fixation and paraffin-embedded (FFPE) and H&E staining (Fig. 1). The remaining node tissue was collected in a microcentrifuge tube and stored at -80 ºC for subsequent OSNA analysis. For small LN (< 5 mm of diameter), one half was used for OSNA and the other for light microscopy. After LN collection, the specimen was fixed overnight in 10% neutral buffered formalin and the mesocolon fat was re-examined. The FFPE lymph nodes were histologically examined according to the standard protocols of the Pathology Department. LN staging and the pathological report were performed based on histological examination according to the AJCC/UICC TNM, 7$^{th}$ edition.[17] The pathologist and the physician were blind to the OSNA results.

Expression of cytokeratin 19 was confirmed in all

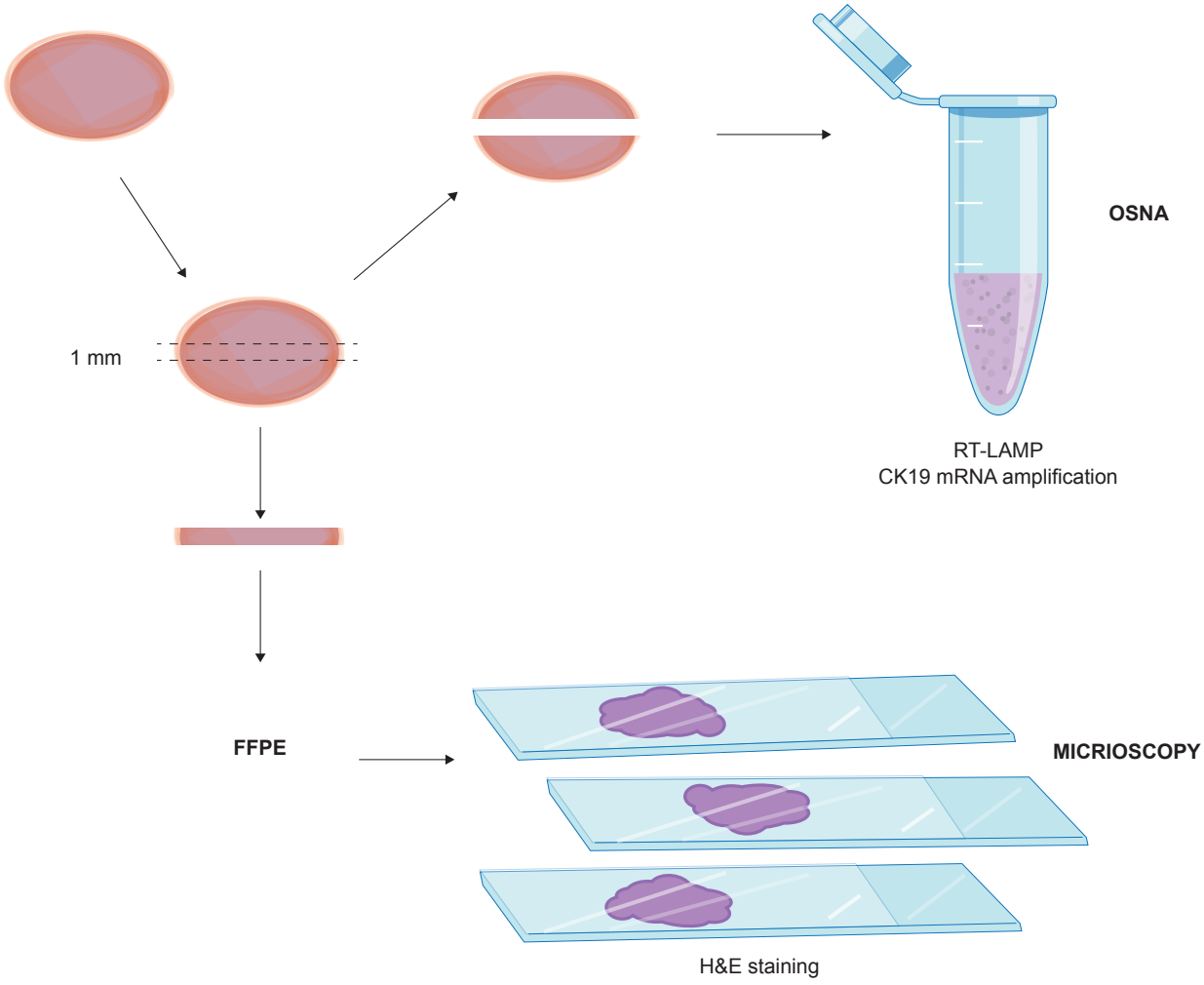


Figure 1 – Lymph node procedure and assessment

CK19: cytokeratin 19; FFPE: formalin-fixation and paraffin-embedded; H&E: haematoxylin-eosin; OSNA: one step nucleic acid amplification; RT-LAMP: reverse transcription loop-mediated isothermal amplification

primary tumour specimens by immunohistochemistry (IHC) to ensure reliable negative results on OSNA assay.

### OSNA procedure

The OSNA method has been previously described in detail.[6,10,11] The LN were analysed with the RD-100i system and OSNA assay (Sysmex Corp., Japan) following the manufacturer's instructions. Briefly, the tissue samples were homogenized with 4 mL of Lynorhag® buffer and the CK19 mRNA amplification was performed using the Lynoamp® CC kit reagents. The entire analysis of 3 - 4 LN, from the sampling process to OSNA results, lasted approximately 40 - 45 minutes. The OSNA results were interpreted as reported previously,[6,10,11] as negative results for < 250 copies/μL, micrometastasis from 250 - 4999 copies/μL, and macrometastasis with ≥ 5000 copies/μL.

### Statistical analysis

The analyses were carried out using Stata version 14.1. Continuous variables are shown using the median and quartiles, and ordinal/categorical variables are reported as absolute and relative frequencies. The 95% exact binomial confidence interval was calculated. Group comparisons in categorical variables were performed using the chi-squared test. The Fisher test was applied for expected absolute frequencies under 5. The non-absolute parametric Mann-Whitney U-test was used to compare groups of continuous variables when normality could not be assumed. A two-sided significance level ($\alpha = 0.05$) was used in all hypothesis tests.

## RESULTS

### Patient characteristics

A total of 64 patients with colon carcinoma were considered for the study (Fig. 2). Five of them were excluded due to LN involvement (pN1 or pN2) by pathologic assessment. A total of 59 pN0 patients were enrolled in the study. The patients were mostly women (69.5%) and the median age was 71 years (Table 1). In 44.1% of the cases a right hemicolectomy was performed, sigmoidectomy in 23%, and left hemicolectomy and rectosigmoidectomy in 10%. All patients underwent a complete tumour resection

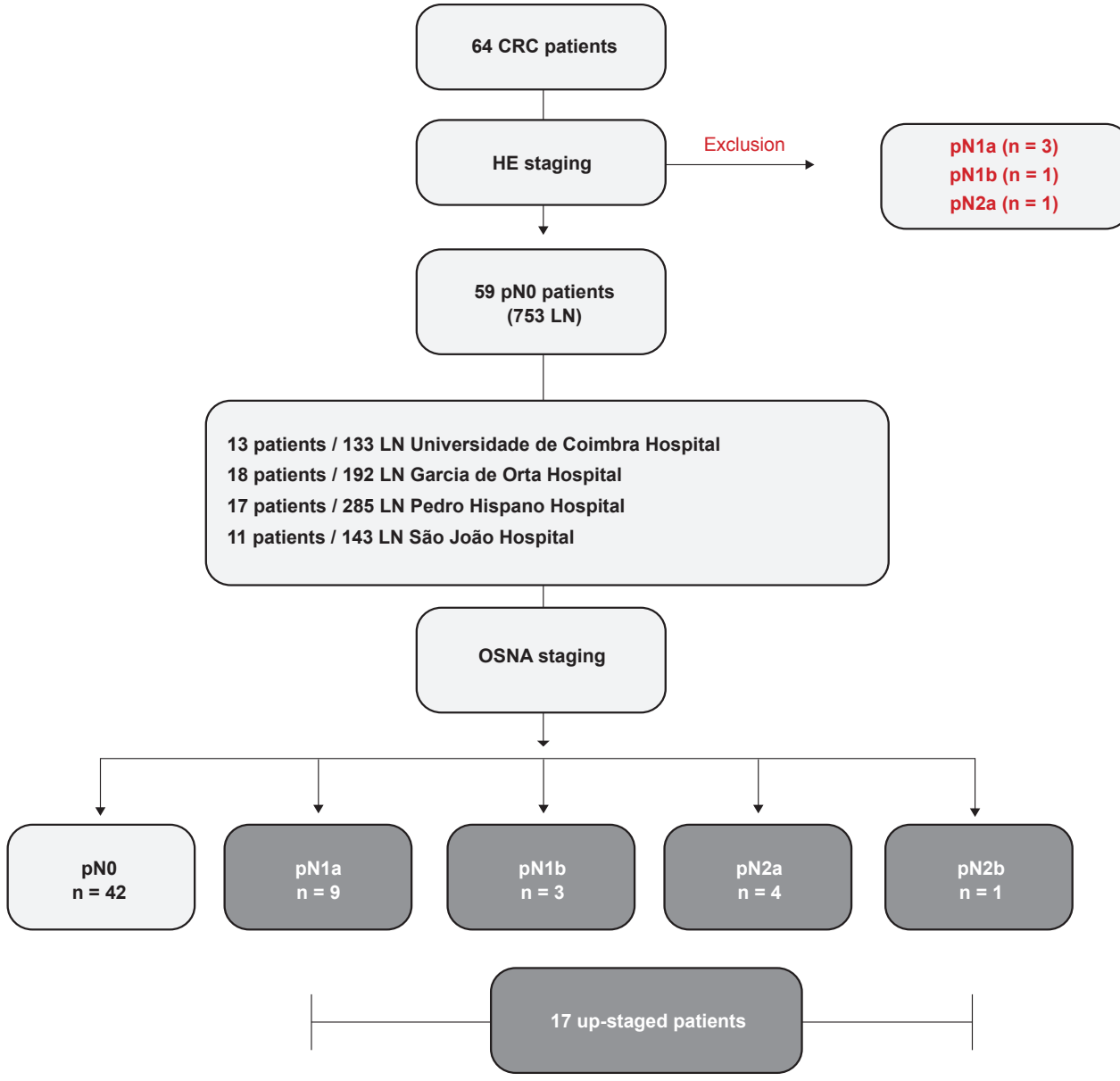


Figure 2 – Study flow-chart

CRC: colorectal cancer; HE: haematoxylin-eosin; LN: lymph nodes; OSNA: one step nucleic acid amplification; pN: pathological stage of lymph nodes according to AJCC/UICC TNM, 7th edition

(R0). Most patients (91.5%) had an adenocarcinoma, and were staged as pT1 (30.5%), pT2 (25.4%) and pT3 (30.5%) tumours. In 94.9% of cases, patients had low-grade tumours and most of them (91.5%) did not show lymphatic invasion. Out of total, three patients received adjuvant chemotherapy; none of them exhibited OSNA-positive results. CK19 protein expression was confirmed in all primary tumours by IHC, thus eliminating the false negative results in OSNA assessment.

### Lymph nodes collection

The molecular assessment of LN status with OSNA entails fresh nodal dissection from the surgical specimen. The outcomes of fresh LN dissection procedure are summarized in Table 2. Dissection took a median of 45 min (IQR: 30; 60). The subsequent examination of the mesocolic fat demonstrated the failure to isolate the totality of the LN during fresh dissection in 10 of 59 cases. Among these cases, a median of 1 (0; 4) LN were detected following the fixation of the specimen. In twelve patients, less than 12 (range 6 - 11) LN were harvested, including fresh and post-fixation procedures. A total of 1046 LN were collected (814 freshly dissected and 232 post-fixation). The median number of LN examined per patient was 13 (9; 19). Small LN were only examined by conventional histology. A total of 753 LN were examined using both OSNA and H&E. The median number of LN analysed with OSNA was 12 (7; 16).

### Molecular staging of pN0 patients

From the 753 LN assessed with both methods, 40 LN were OSNA-positive (≥ 250 copies/μL). Thus, OSNA assay demonstrated 17 patients [28.8% (95% CI: 17.8 - 42.1)] with

Table 1 – Demographic and pathological characteristics of colorectal cancer patients

| | Total (n = 59)<br>n (%) | OSNA-positive (n = 17)<br>n (%) |
|---|---|---|
| **Sex** | | |
| Male | 18 (30.5) | 7 (41.2) |
| Female | 41 (69.5) | 10 (58.8) |
| **Age*** | **71 (62;77)** | **75 (71;77)** |
| **Surgery** | | |
| Right hemicolectomy | 26 (44.1) | 7 (41.2) |
| Left hemicolectomy | 8 (13.5) | 1 (5.9) |
| Rectum-sigmoidectomy | 2 (3.4) | 2 (11.8) |
| Sigmoidectomy | 23 (39.0) | 7 (41.2) |
| **Tumour localization** | | |
| Cecum | 10 (17.0) | 2 (11.8) |
| Right colon (ascending) | 11 (18.6) | 2 (11.8) |
| Left colon (descending) | 3 (5.1) | 0 (0.0) |
| Sigmoid colon | 23 (39.0) | 8 (47.0) |
| Transverse colon | 3 (5.1) | 2 (11.8) |
| Rectum-sigma | 2 (3.4) | 1 (5.9) |
| Splenic flexure | 3 (5.1) | 0 (0.0) |
| Hepatic angle | 4 (6.8) | 2 (11.8) |
| **Resection completed** | | |
| No | 0 (0.0) | 0 (0.0) |
| Yes | 59 (100.0) | 17 (100.0) |
| **Tumour deposits** | | |
| Not detected | 58 (98.3) | 17 (100.0) |
| Unknown | 1 (1.7) | 0 (0.0) |
| **Round border** | | |
| Negative | 59 (100.0) | 17 (100.0) |
| Positive | 0 (0.0) | 0 (0.0) |
| **Lymphatic invasion** | | |
| Not detected | 54 (91.5) | 16 (94.1) |
| Angiolymphatic | 3 (5.1) | 1 (5.9) |
| Perineural | 1 (1.7) | 0 (0.0) |
| Venous | 1 (1.7) | 0 (0.0) |
| **Histological type** | | |
| Adenocarcinoma | 54 (91.5) | 16 (94.1) |
| Mucinous adenocarcinoma (50%) | 2 (3.4) | 0 (0.0) |
| Others | 3 (5.1) | 1 (5.9) |
| **Histological grade** | | |
| Low grade | 56 (94.9) | 16 (94.1) |
| Not determined | 1 (1.7) | 0 (0.0) |
| Others | 2 (3.4) | 1 (5.9) |
| **T stage** | | |
| pTis | 3 (5.1) | 1 (5.9) |
| pT1 | 18 (30.5) | 5 (29.4) |
| pT2 | 15 (25.4) | 6 (35.3) |
| pT3 | 18 (30.5) | 4 (23.5) |
| pT4a | 4 (6.8) | 1 (5.9) |
| pT4b | 1 (1.7) | 0 (0.0) |
| **Lymph node examination*** | | |
| H&E | 13 (10;20) | 14 (13;18) |
| OSNA | 12 (7;16) | 14 (12;18) |
| **Chemotherapy** | | |
| Yes | 6 (10.2) | 0 (0.0) |
| No | 53 (89.8) | 17 (100.0) |

H&E: haematoxylin-eosin; LN: lymph node; OSNA: one step nucleic acid amplification; pT: pathological stage of primary tumour according to AJCC/UICC TNM, 7th edition; (*) Median; percentile 25, percentile 75.
Data are shown with absolute and relative frequencies. Specific data (*) are described as median and interquartile.

Table 2 – Lymph node dissection procedure

| | Total (n = 59) | | OSNA-positive (n = 17) | |
|---|---|---|---|---|
| Number of fresh nodes for OSNA | 12 | **(7;16)** | 14 | (12;18) |
| Number of nodes (paraffin-embedded) | 13 | (10;20) | 18 | (13;27) |
| Dissection time (min) | 45 | (30;60) | 60 | (35;60) |
| Number of nodes (post-fixation) | 1 | (0;4) | 0 | (0;4) |
| Total number of nodes dissected | 17 | (11;21) | 16 | (14;27) |
| Fresh to formalin-fixed node ratio* | 2.0 | (1.3;8.0) | 4.1 | (1.4; 9.4) |
| Fresh to total node ratio | 0.9 | (0.6;1.0) | 1 | (0.9;1) |

(*) This figure cannot be calculated for cases without the nodes retrieved after post-fixation (n = 36 and n = 8 for total and OSNA positive group, respectively).
Data are shown as median and interquartile.

metastatic involvement of LN (Fig. 2). The improvement of patient staging by molecular analysis was mainly observed in two centres, Pedro Hispano Hospital and García de Orta Hospital, with 47.1% (8/17) and 38.9% (7/18) respectively, whereas the University of Coimbra Hospital had 15.4% (2/17) and São João Hospital had no OSNA-positive cases. Among the OSNA-positive patients, 11 were histologically classified as stage I (64.7%), 4 as stage IIA (23.5%), 1 as stage IIB (5.9%) and 1 as stage 0 (5.9%), according to AJCC guidelines. Molecular staging showed 9 patients with pN1a (52.9%), 3 patients with pN1b (17.7%), 4 patients with pN2a (23.5%) and 1 patient with pN2b (5.9%). The characteristics of OSNA-positive patients are described in Table 1. The median of OSNA-positive LN per patient was 1 (1:4). Over half of the patients (9/17) had only one OSNA-positive LN while one had 6 molecular-positive nodes (Table 3). Most patients (88.2%) had nodes with only micrometastases (OSNA +; 250 - 4999 copies/μL). In one case (5.9%) only a macrometastasis (OSNA ++; ≥ 5000 copies/μL) was retrieved and one case (5.9%) had nodes with both micro- and macrometastasis. The OSNA results ranged from 270 to 17 000 CK19 mRNA copies/μL. Among patients with only micrometastases, 53.3% (8/15) had only one node affected, with OSNA results ranging from 270 to 2100 copies/μL. The patient who only had macrometastases had just one affected node with 17 000 copies/μL. Non-significant differences of clinical-pathological characteristics were found between OSNA-positive patients and patients with negative results both by OSNA and H&E assessments (Table 4).

After one year of follow-up, four patients enrolled in the study relapsed with metastatic disease; two of them had OSNA-positive LN. Five patients died and three of them had OSNA-positive LN. Only one death was tumour-related.

Table 3 – OSNA-positive lymph nodes per patient according to molecular tumour burden

| | Exclusively micrometastasis (n = 15) | Exclusively macrometastasis (n = 1) | Micrometastasis + macrometastasis (n = 1) |
|---|---|---|---|
| 1 | 8 | 1 | 0 |
| 2 | 1 | 0 | 0 |
| 3 | 2 | 0 | 0 |
| 4 | 2 | 0 | 1 |
| 5 | 1 | 0 | 0 |
| 6 | 1 | 0 | 0 |

LN: lymph node; micrometastasis, 250 - 4999 copies/μL; macrometastasis ≥ 5000 copies/μL

## DISCUSSION

This is the first study conducted in Portugal focusing on molecular LN assessment in colon cancer. We show that the staging of 28.8% of CRC patients with histologically negative nodes has been improved using the OSNA assay. These results are in accordance with proportions reported in other studies that ranged between 15% – 25%.[11–14] Using the same methodological approach based on the histological examination of the central slide, Croner et al. found an upstaging rate of 25.2% that correlated with the number of LN analysed and the tumour stage.[12] Most patients (73.1%) had only one OSNA-positive LN. Güller *et al* reported a lower rate of 15.3%.[11] They analysed half of each node with each method which results in a higher allocation bias and would explain a decrease in molecular staging. Similarly, the multicentre Japanese study of pN0 and pN1 patients with colorectal cancer, which used half node assessment, provided a 11.3% of molecular positive rate and most of the pN0 patients (71%) had a single LN affected.[14] They observed differences in tumour size and lymphatic invasion between OSNA-positive and OSNA-negative patients, and most OSNA-positive cases were above the T3 tumour stage. Vogelaar *et al*, who focused on SLN performance in CRC, reported a diagnostic value of OSNA of 82.1% in comparison with routine pathological examination (single H&E-slide examination) and a molecular staging rate of 20.2%.[13]

Retrospective surveys of CRC patient survival have expressed the need for CRC screening programmes in Portugal.[18] The Portuguese Directorate-General of Health recommends the annual prescription of faecal occult blood tests (FOBT) in all asymptomatic individuals aged between 50 and 74 years which would increase opportunistic screening[19] A pilot screening programme started in 2009 in the central region of the country, but by 2012, only two regions in Portugal had implemented an organised screening program.[19,20] The implementation of CRC screening programmes worldwide increases numbers of patients suspected of CRC at early stages. In these patients, surgical management by tumour resection is the current favoured clinical procedure, without systemic therapy. Relapse occurs within five years of surgery in 20% – 30% of these patients without apparent lymph node involvement which is thought to be due to occult lymph node metastases.[3,21] Hence, better and more accurate

Table 4 – Patients' characteristics according to lymph node status assessed with OSNA

| | OSNA-positive LN | | |
|---|---|---|---|
| | No (n = 42) | Yes (n = 17) | |
| | n (%) | n (%) | *p*-value |
| **Histological type** | | | |
| Adenocarcinoma | 38 (90.4) | 16 (94.1) | 1.000† |
| Mucinous adenocarcinoma (50%) | 2 (4.8) | 0 (0.0) | |
| Others | 2 (4.8) | 1 (5.9) | |
| **Histological grade** | | | |
| Low grade | 40 (95.2) | 16 (94.1) | 0.647† |
| Not determined | 1 (2.4) | 0 (0.0) | |
| Others | 1 (2.4) | 1 (5.9) | |
| **Lymphatic invasion** | | | |
| Not detected | 38 (90.5) | 16 (94.1) | 1.000† |
| Angiolymphatic | 2 (4.8) | 1 (5.9) | |
| Perineural | 1 (2.4) | 0 (0.0) | |
| Venous | 1 (2.4) | 0 (0.0) | |
| **Tumour location** | | | |
| Right | 20 (47.6) | 8 (47.1) | 0.969‡ |
| Left | 22 (52.4) | 9 (52.9) | |
| **Tumour size (cm)*** | 3 (2.5;4.3) | 2.2 (1.7;3.5) | 0.192§ |
| **pTNM stage** | | | |
| pTis | 2 (4.8) | 1 (5.9) | |
| pT1 | 13 (30.9) | 5 (29.4) | |
| pT2 | 9 (21.4) | 6 (35.3) | |
| pT3 | 14 (33.3) | 4 (23.5) | |
| pT4a | 3 (7.1) | 1 (5.9) | |
| pT4b | 1 (2.4) | 0 (0.0) | |
| **Number of lymph node assessed*** | | | |
| H&E | 12 (7;19) | 14 (13;18) | 0.028§ |
| OSNA | 11 (6;15) | 14 (12;18) | 0.013§ |
| **Chemotherapy** | | | |
| No | 39 (92.9) | 17 (100.0) | 0.550† |
| Yes | 3 (7.1) | 0 (0.0) | |

H&E: haematoxylin-eosin; LN: lymph node; OSNA: one step nucleic acid amplification; pTNM: pathological stage according to AJCC/UICC TNM, 7th edition; (*) Median; percentile 25, percentile 75. (†) Fisher's exact test; (‡) Pearson chi-square test; (§) U Mann-Whitney test.
Data are shown with absolute and relative frequencies. Specific data (*) is described as median and interquartile.

staging of these patients is essential so that they might also benefit from adjuvant chemotherapy. However, extensive histological assessment of the large number of LN that is required for an accurate diagnosis in accordance with clinical guidelines is time-consuming and unfeasible in most pathology laboratories. Molecular assessment of LN status is a response to these issues. The meta-analysis conducted by Iddings *et al* reported a statistically significant difference of 3-year overall survival of patients with molecular positive LN (pN0mol+) in comparison with conventional histological staging.[3] More recently, a multicentre Spanish study demonstrated that OSNA positivity correlates with high-risk factors in stage I-II CRC patients such as gender, the number of LN collected and histologic grade, which were independent predictors of the OSNA results.[22] They propose the molecular tumour burden assessed with OSNA (TTL, total tumour load) as a prognostic factor in early staged CRC, although long-term surveillance and validation studies are needed. Furthermore, Yamamoto et al. have shown that TTL increased significantly as the pathological status increased.[14]

OSNA assessment of SLN in breast cancer has recently been recommended in the UK pathology guidelines.[23] Although several studies in Europe and Japan have reported the contribution of OSNA to better staging of CRC patients and a satisfactory diagnostic accuracy of LN status,[8–14,24] the value of OSNA for pathological assessment is still hampered due to the absence of up-to-date surveillance data. At the time of preparing this manuscript, only one-year survival outcomes were available. Further information on patient follow-up would provide insights about whether they would have benefited from treatment according to the molecular staging with OSNA.

The study has some drawbacks. Firstly, the small sample size limits the strength of the results and detailed inferential statistical analysis. Although four reference hospitals participated in the study, most CRC patients are diagnosed at an advanced stage, and the screening programmes in Portugal only cover 9.3% of the population at risk.[19] Secondly, there are the unavoidable methodological biases, due to the assessment of different sections of LN in the histological and molecular procedures. In addition, we cannot rule out an improper staging in some cases because of low LN collection. Molecular assessment entails

fresh LN dissection in contrast with the standard practice of dissection after fixation. Certainly, increased expertise in fresh dissection would improve the number of LN collected, which would be more in line with the clinical guideline consensus of 12 LN.[17,25,26] Finally, the 5-year follow-up period necessary to evaluate the disease-free status of the OSNA-positive cases is currently unfinished.

## CONCLUSION

Our results have shown that the OSNA assay is a feasible tool to evaluate LN status in patients with early CRC and allows more accurate staging in comparison with the standard histological method. According to the current practice at the hospitals involved in this study, most patients were not subjected to adjuvant treatment. However, they could have benefited from systemic therapy based on molecular staging. Hence, further surveillance data are needed to clarify the clinical value of molecular staging in early CRC.

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## PROTECTION OF HUMAN AND ANIMAL SUBJECTS

The authors declare that the research procedures were performed according to the regulations of the institution's ethics committee and the Code of Ethics of the World Medical Association (Declaration of Helsinki).

## CONFIDENTIALITY OF DATA

The authors declare that they have followed the protocols of their work centre regarding the publication of data from patients.

## CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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