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## Postoperative intra-arterial methylene blue injection of colorectal cancer specimens increases the number of lymph nodes recovered

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# Postoperative intra-arterial methylene blue injection of colorectal cancer specimens increases the number of lymph nodes recovered

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**Keywords:** Colorectal cancer, lymph nodes, methylene blue injection.

**Running title:** Methylene improves lymph node recovery.

## Abstract

**Aims:** The aim of this study was to determine the possible advantage of intra-arterial injection of methylene blue with a view to improving lymph node recovery in postoperative examination of colorectal cancer specimens.

**Methods and results:** Thirty-two colorectal cancer specimens were randomly assigned either to dissection with intra-arterial methylene blue injection or to routine dissection (without methylene blue injection). Immediately postoperatively, the specimens in the staining group were injected intra-arterially with methylene blue dye. The two procedures were compared with respect to the number of lymph nodes recovered. The number of recovered lymph nodes was significantly higher in the intra-arterial methylene blue injection group than in the group investigated with routine procedures ( $P < 0.0001$ ).

**Conclusion:** The intra-arterial methylene blue injection method is fairly easy to use postoperatively and significantly increases the number of lymph nodes recovered in colorectal cancer specimens.

## Introduction

Adjuvant chemotherapy significantly reduces mortality and the risk of recurrence in stage III (T1-4, N1-2, M0) colorectal cancer relative to surgery alone.<sup>1</sup> Accurate lymph node staging is therefore crucial in terms of evaluating prognosis and deciding on further treatment. The International Union Against Cancer (UICC) recommends the examination of a minimum of 12 lymph nodes in colorectal cancer specimens for an accurate assessment of lymph node status. However, recommendations in the literature range all the way from 6 examined lymph nodes to as many as possible with no particular minimum number.<sup>2-11</sup> Studies have indicated that both stage II (T3-4, N0, M0) and stage III cancers are in fact very heterogeneous groups regarding prognosis and outcome. Part of this heterogeneity is thought to depend on the number of lymph nodes recovered, which is believed to affect the accuracy of the pathological diagnosis.<sup>2,3,6,8,9,10,13</sup> Swanson et al. examined patients with stage IIA (T3, N0, M0) cancers and found a positive association between the number of lymph nodes recovered and prognosis. Stratifying the patients into groups depending on the number of recovered lymph nodes (1-7, 8-12, or  $\geq 13$  lymph nodes) yielded 5-year relative survival rates of 69.3%, 77.5% and 84.5%, respectively, which all differed significantly from each other<sup>6</sup>. This heterogeneity within TNM stage groups emphasizes the importance of adequate examination of lymph nodes to ensure accurate pathological diagnosis.

Understaging in colorectal cancer is suggested to result from an insufficient number of recovered lymph nodes at pathological examination. This hypothesis is known as the stage migration theory.<sup>12,13</sup> An insufficient assessment of lymph nodes may leave undetected metastases. Patients diagnosed with stage II cancer and with few examined lymph nodes may in fact have stage III cancer. This may cause incorrect oncological treatment postoperatively and give the patient an unfavourable prognosis.

Intra-arterial methylene blue injection was recently introduced by Märkl et al. for improvement of lymph node recovery in colon and rectal cancer specimens.<sup>14</sup> By postoperative injection of methylene blue into the specimen arteries, Märkl et al. significantly improved lymph node recovery compared to a control group: mean  $35 \pm 8$  and  $17 \pm 10$  lymph nodes per specimen, respectively. The technique was fairly simple, efficient and adaptable as a routine procedure for pathological examination of colorectal cancer specimens. The additional lymph nodes recovered in the stained group were mainly small nodes measuring 1-4 mm in diameter.<sup>14</sup> Several studies have shown that smaller lymph nodes (of  $\leq 5$  mm in diameter) contain metastases just as frequently as larger nodes.<sup>15-18</sup>

Accurate specimen preparation and examination is important for detection of lymph nodes and to avoid understaging of colorectal cancer. We assumed that more nodal metastases can be identified and that understaging may be avoided when more lymph nodes are harvested and examined. The aim of this study was to investigate whether postoperative intra-arterial methylene blue injection of colorectal cancer specimens would improve lymph node recovery at pathological examination.

## Material and Methods

Between September 2008 and January 2009, patients undergoing intended curative surgery for colorectal cancer at the Departments of Surgery at Norrköping County Hospital and Linköping University Hospital, Sweden were randomly assigned into two groups, either to

specimen staining with methylene blue or no staining. The exclusion criteria were concomitant inflammatory bowel disease and massively invasive tumours necessitating a surgical resection technique, rendering subsequent specimen staining impossible. The specimens were analysed at the Department of Clinical Pathology and Genetics at Linköping University Hospital, Sweden.

Immediately postoperatively, the specimens in the staining group had their main artery or arteries cannulated with intravenous catheters of appropriate sizes (16–20 G). The proximal ends of the arteries were marked by the surgeon with sutures after the resection. The catheters were inserted using only the plastic tube portion, as there was a risk that the cannula would perforate the artery. The arteries were injected with 10–30 ml of methylene blue dye (50 mg methylene blue in 5 ml distilled water, diluted with 0.9% NaCl solution in the ratio 1:3) until the dye was visible on the surface of the specimen. The amount of dye refers to the amount used for the entire specimen. The number of arteries injected with dye per specimen varied between one and three. The specimens in both the unstained and the stained groups were fixed in formaldehyde for a minimum of 24 hours according to the standard protocol, and were dissected in the same manner and by the same person (AT). After having located the primary tumour and secured representative pieces for histological examination, the mesenterial fatty tissue was sliced and visually examined and palpated (Figure 1). All harvested lymph nodes were examined histopathologically by the same pathologist (HO) according to routine protocol.

All statistical analyses were performed using Statistica software version 7. The significance of differences was calculated using Fisher's exact t-test. P-values  $\leq 0.05$  were assumed to be statistically significant.

*“Figure 1”*

## Results

Of 37 specimens, 20 were randomly assigned to the methylene blue staining group and 17 to the no staining group. Three specimens were excluded in the staining group because of failure to inject them with the methylene blue dye, and one specimen from each group was excluded because later histopathological examination showed adenoma. Sixteen specimens remained in each group for further analysis (Table 1 and 2).

Metastases were found in 6 stained specimens and 9 unstained specimens. The median number of lymph nodes recovered was 61 (range 35–161) and 22 (range 13–51) in the stained and unstained groups, respectively. The median number of harvested lymph nodes was significantly higher in the stained specimen group ( $P < 0.0001$ ) (Figure 2). There was no significant difference regarding the number of lymph node metastases found in the two groups. All specimens in both groups had lymph node recoveries that were better than the UICC-recommended minimum number of 12 nodes.

*“Figure 2  
Table 1-2”*

## Discussion

Our results show that intra-arterial injection of methylene blue into colorectal cancer specimens significantly improves the number of lymph nodes recovered compared to the traditional pathological method. **The difference between the specimen groups is highly**

significant and although the study is fairly small we are confident that this difference would remain even if more specimens were examined.

The number of lymph nodes required for an accurate staging of colorectal cancer specimens is unclear, and the recommendation of at least 12 lymph nodes by the UICC is controversial. Some authors have reported that the benefit of finding additional lymph nodes increases in a linear fashion.<sup>11,19</sup> Much of the controversy is based on studies that have found that the prognosis for patients with metastasis-free (stage II) colorectal cancer is dependent on recovery of lymph nodes, there being a positive correlation between the number of lymph nodes recovered and better prognosis.<sup>6,8,9,13</sup> Based on these data, various techniques for recovering lymph nodes have emerged such as simple fat-clearing techniques, specialised fixatives such as GEWF (glacial acetic acid, ethanol, distilled water and formaldehyde) and lymphatic system dye techniques.<sup>14,20,21</sup>

The methylene blue injection method can provide some procedural difficulties. Difficulties were experienced with arterial catheterization, as the latter proved fragile, and the risk of perforating the arteries therefore proved to be a problem. However, as a failed staining does not appear to have any deleterious effect on specimen dissection, the method can be regarded as safe. **No formal measurements were made regarding time spent on dissection, however in our experience it did not differ between the two specimen groups as the time spent removing the extra lymph nodes in the methylene blue specimens was compensated for by having to spend less time searching for them. The cost effectiveness of the methylene blue technique is completely dependent on whether or not it assists in the detection of lymph node metastases.**

Our results show that it is possible to significantly improve the number of lymph nodes recovered by using a supplementary staining method; however, we cannot make any conclusions about the benefit of finding these additional lymph nodes. The study material was too small for us to examine any correlation between the number of lymph nodes recovered and the frequency of metastases. Richter et al. demonstrated this phenomenon when using an acetone fat clearing method for examining colorectal cancer specimens. They first manually dissected the specimens for lymph nodes and then used their clearing method on the same specimens. Of 188 specimens, 15% were found to have previously undetected metastases and 8% of the specimens had to have their N-stages upgraded. Nine specimens were upgraded from N0 to N1 or N2 (from stage II to stage III).<sup>20</sup>

Lymph node size is another important issue, since the methylene blue technique mainly improves the recovery of very small lymph nodes ( $\leq 4$  mm in diameter).<sup>14</sup> Several authors have reported that even small lymph nodes ( $\leq 5$  mm in diameter) carry metastases.<sup>15-18</sup> However, it is not proven that metastases in small lymph nodes have the same clinical implications as metastases in larger lymph nodes. Care should be taken when referring to lymph node metastases without any reference to the size or nature of those metastases.

There may be different reasons for the great variation in the number of lymph nodes recovered from colorectal cancer specimens. For instance, a strong immune response against the tumour might enlarge lymph nodes, making them easier to find on examination. Surgical technique is a debatable subject. **West et al. compared colon cancer specimens resected using complete mesocolic excision (CME) and central vascular ligation (CVL) with specimens resected using a more conservative and less radical technique. The CME/CVL operations in comparison to the alternative operating technique yielded a significantly larger area of mesentery removed, 19,7 versus 11,8 mm<sup>2</sup>, and a significantly higher specimen lymph node**

recovery, 30 versus 18 lymph nodes, thus demonstrating the important influence of surgical technique on the outcome of the pathologists specimen dissection.<sup>22</sup> The number of lymph nodes recovered is a measure of quality, both from a surgical point of view but also for the pathologists performing the postoperative specimen investigation. Use of the staining method described would eliminate many of the pathologist's difficulties, thus highlighting the role of the surgeon.

In conclusion, intra-arterial methylene blue staining of colorectal cancer specimens significantly improves lymph node recovery. However, a possible correlation between the number of lymph nodes recovered and the frequency of metastases could not be shown in this fairly small study more focusing on pathological laboratory techniques.

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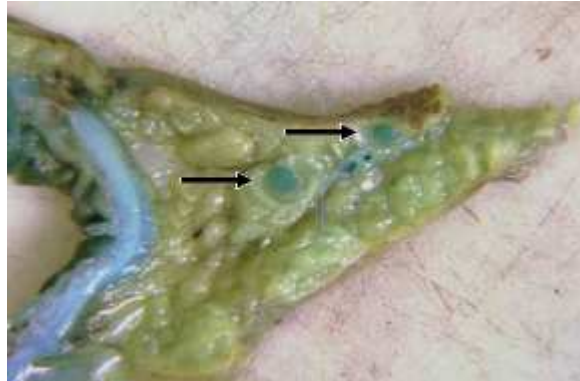
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Table 1. Specimen raw data.

Specimen no.	Staining	Location of tumour	pT stage	Tumour differentiation	Length of specimen (cm)	Pre-operative radiation	No. of lymph nodes recovered	No. of metastases
1	Yes	Colon	3	Moderate	26	No	61	0
2	Yes	Colon	3	Moderate	30	No	101	3
3	Yes	Colon	4	Mucinous	40	No	43	0
4	Yes	Colon	3	Poor	30	No	121	56
5	Yes	Colon	3	Mucinous	20	No	143	0
6	Yes	Colon	3	Moderate	24	No	67	0
7	Yes	Colon	3	Moderate	13	No	35	0
8	Yes	Colon	3	Mucinous	24	No	51	0
9	Yes	Colon	3	Moderate	18	No	75	1
10	Yes	Colon	3	Moderate	13	No	44	0
11	Yes	Rectum	3	Poor	27	Yes	92	10
12	Yes	Rectum	3	Moderate	20	Yes	61	8
13	Yes	Rectum	3	Moderate	26	Yes	80	0
14	Yes	Rectum	3	Moderate	26	Yes	50	1
15	Yes	Rectum	3	Moderate	36	Yes	42	0
16	Yes	Rectum	3	Moderate	24	Yes	54	0
17	No	Colon	3	Moderate	50	No	27	9
18	No	Colon	3	Poor	25	No	51	20
19	No	Colon	3	Mucinous	26	No	13	0
20	No	Colon	3	Moderate	25	No	19	3
21	No	Colon	4	Moderate	28	No	22	0
22	No	Colon	3	Moderate	22	No	20	0
23	No	Colon	3	Poor	19	No	25	0
24	No	Colon	4	Moderate	18	No	13	8
25	No	Colon	3	Poor	14	No	21	11
26	No	Colon	3	Moderate	23	No	40	3
27	No	Rectum	3	Moderate	22	Yes	25	3
28	No	Rectum	3	Moderate	36	Yes	22	0
29	No	Rectum	4	Moderate	29	Yes	18	5
30	No	Rectum	3	Moderate	27	Yes	26	0
31	No	Rectum	3	Moderate	22	Yes	16	3
32	No	Rectum	3	Moderate	31	Yes	29	0

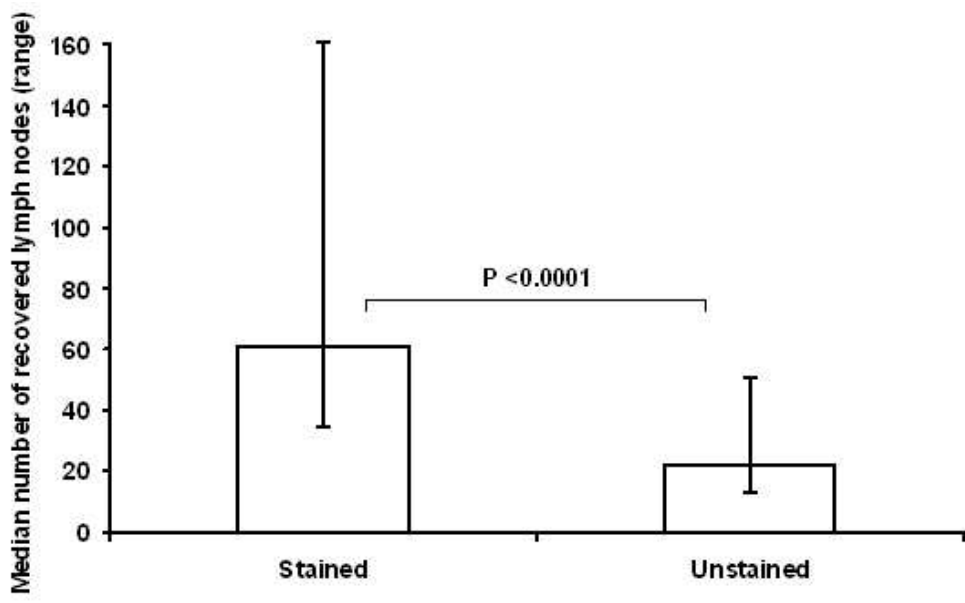
Table 2. Characteristics of patients and specimens.

	Stained group (n = 16)	Unstained group (n = 16)	P- value
Median age (range)	69.5 (23–89)	73.5 (51–92)	Ns
Gender (female/male)	6/10	9/7	Ns
Localization			
Colon	10	10	Ns
Rectum	6	6	Ns
Preoperative radiotherapy	6 (all rectum)	6 (all rectum)	Ns
Median length of specimen in cm (range)	25 (13–40)	25 (14–50)	Ns
TN stage			
T1	0	0	
T2	0	0	
T3	15	13	
T4	1	3	
N0	10	7	
N1	3	4	
N2	3	5	



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Peer Review



145x94mm (96 x 96 DPI)

Review

## Figures

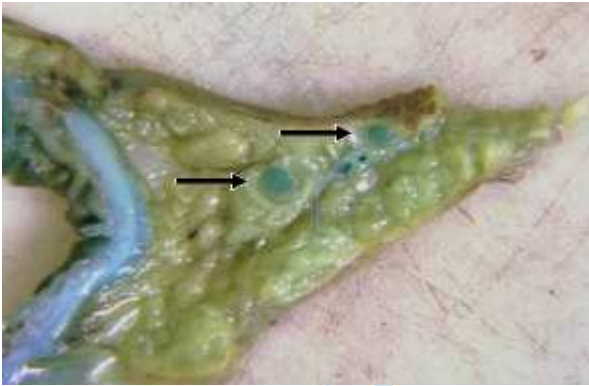


Figure 1

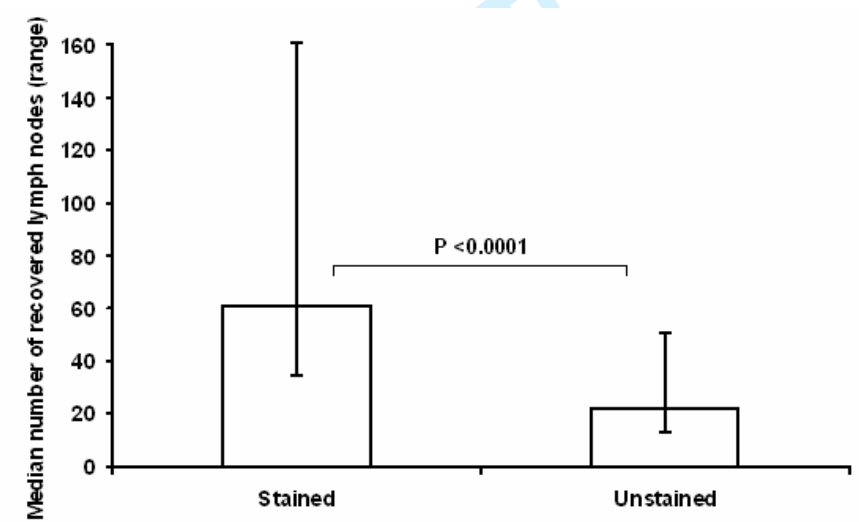


Figure 2

### Figure legends

Figure 1. Stained lymph nodes, marked with arrows.

Figure 2. Median number of lymph nodes recovered (y-axis) in the stained and unstained groups, respectively.