A comparison of formalin and GEWF in fixation of colorectal carcinoma specimens: rates of lymph node retrieval and effect on TNM staging


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ABSTRACT

INTRODUCTION

The Royal College of Pathologists recommend that a median of at least 12 lymph nodes should be harvested during pathological staging of colorectal cancer. It is not always easy to harvest the required number, especially in patients with rectal cancer receiving neoadjuvant therapy.

Lymph node revealing solutions, e.g. GEWF, may improve nodal yield. GEWF is safe, cheap and easy to use.

METHODS

In a controlled trial, lymph node yields were compared after secondary specimen dissection following either 24 hours of further fixation in formalin (n=101) or GEWF immersion (n=99). The number, size and tumour status of additional lymph nodes identified was compared between groups. Twenty-seven cases which received long-course neoadjuvant therapy were also assessed.

RESULTS

Median lymph node yield at primary dissection met national standards overall (19) but also in the long-course neoadjuvant therapy group (13). Lymph nodes were smaller in neoadjuvant cases compared to non-neoadjuvant cases (mean size range 1.3-5.6mm vs 1.5-8.9mm). The use of further fixation and GEWF detected more nodes at secondary dissection. The mean number of additional nodes harvested was greater with formalin (8.3) than GEWF (7.3). There was no significant difference in the mean size of
the additional lymph nodes detected between groups (point estimate 1.02; 95% CI -0.58-2.63; p=0.211). Upstaging triggering adjunct chemotherapy occurred in 1% (2/200) of cases.

CONCLUSIONS
The routine use of adjunct techniques to identify additional lymph nodes is unnecessary with underlying high quality dissection practice. Emphasis should be placed upon education and training, spending appropriate time dissecting, and ensuring specimens are sufficiently fixed beforehand.
INTRODUCTION

Colorectal carcinoma (CRC) is the fourth most common cancer in the UK.[1] Nodal metastasis is inextricably linked to the prognosis of the patient,[2-6] and just one nodal tumour deposit upstages the malignancy from pN0 to pN1 in the TNM system,[5] which has important implications when adjuvant chemotherapy is considered.[7] High quality histopathological assessment includes harvesting an adequate number of lymph nodes. Current recommendations are that a median of at least 12 lymph nodes should be retrieved for adequate staging,[5-6] with all mesentery within the tumour vicinity searched. This is based on evidence demonstrating the prognostic significance of lymph node harvesting with differing numbers of nodes retrieved.[2-5]. Some literature suggests that more lymph nodes should be harvested for adequate staging,[2] but 12 is the current consensus.[5-6]

The recommended number of nodes is not always achieved, even in published studies. Most notably, in patients who have received long-course neoadjuvant chemotherapy for rectal carcinoma, the size of lymph nodes may be reduced making identification more challenging.[8] Other contributory factors may include fixation time, experience of the surgeon and failure by the dissector to appropriately examine or identify all nodes within a specimen, either due to lack of experience or poor technique.[9-14]. In response to this, a number of studies have addressed the issue of lymph node harvesting using a variety of techniques,[9,15-25] including lymph node revealing solutions.[9,19-46] Studies using GEWF, a mixture of glacial acetic acid, ethanol, water and formalin, suggested that its use will identify an increased number of lymph nodes,[9,37-43,45-46] and that this may lead to stage migration from node negative to positive.[19,27,29,34,42-43] GEWF has been shown to facilitate identification of smaller
lymph nodes,[9,38,41,42,46] which may be especially useful for cases where neoadjuvant therapy has been given, as there is often a paucity of lymph nodes which are also smaller in size.[8]

In cases where an inadequate number of nodes have been retrieved, it is common practice to return to the specimen and perform a secondary dissection to look for more nodes. We performed a study to compare the use of GEWF versus further fixation in formalin in terms of the number of nodes retrieved at secondary dissection, their size, and the rate of tumour upstaging.

MATERIALS AND METHODS

We undertook a controlled trial study design using randomisation, pseudoanonymisation and stratification. Two hundred consecutive colorectal specimens received into the diagnostic histopathology department at Southampton General Hospital between June 2012 and June 2014 were entered into the study. A power calculation suggested that 100 cases in each group would be sufficient to detect an extra three nodes with a power of 80%.

Randomisation and anonymisation

Samples were randomly allocated to either further fixation in formalin or GEWF. The allocation was concealed from the chief investigator, as knowledge of allocation could have been a source of sample bias.[47] Blinding was impossible because the intervention chemical was recognisable at secondary dissection via its distinctive smell and appearance. Randomisation was performed using an automated randomisation programme. Cards within sealed envelopes, containing the allocation information for
each case were used. Specimens were assigned a study number and pseudoanonymised, with a link maintained to the patient, in line with the Human Tissue Act, 2004, code of practice 1.[48] Ethical approval was gained from North West Research Ethics Committee.

Recruitment and stratification

Only adenocarcinomas were considered for inclusion in the study. Cases were excluded if there was no informed patient consent for research; the tumour was a recurrence after previous resection; or a timely secondary dissection could not be performed. Samples were stratified into either non-neoadjuvant or neoadjuvant therapy groups, because the use of long-course neoadjuvant therapy is known to be related to retrieval of fewer, smaller lymph nodes.[41,49] Patients receiving short-course neoadjuvant therapy were placed into the non-neoadjuvant group. Within each stratified group, samples were then randomly allocated into one of two intervention groups (Figure 1).

Dissection and interpretation

Specimens were fixed in formalin for 48-96 hours before primary dissection, which was performed by a variety of staff according to routine laboratory protocols. Then, depending on the allocation, the specimen was placed in either fresh formalin or GEWF (glacial acetic acid 80ml, ethanol 500ml, water 150ml, formalin 80ml) for at least 24 hours. A secondary dissection was then performed by the chief investigator with the aim of sampling all residual lymph nodes. The number and sizes of nodes retrieved at primary and secondary dissections were recorded following microscopic examination.
Statistical analysis

As the data were approximately normally distributed (Figure 3), an independent samples t-test was used to compare the difference in number and size of lymph nodes between the two groups. Mean differences are reported alongside 95% CI and p-values. Statistical analysis was performed using IBM SPSS Statistics, version 21.[50]

RESULTS

Of the 200 patients recruited to the study, 119 (59.5%) were male and 81 (40.5%) were female. The mean age for all patients was 70.5 years (range 25 to 97). Pathological data are shown in Table 1. Thirty-nine percent of cancers were right sided whilst 61% were left sided or rectal. Twenty-seven patients (13.5%) received long-course neoadjuvant therapy. The two intervention chemicals were used almost equally (50.5% vs 49.5%).
Table 1. Pathological data. All secondary dissections were performed by the chief investigator.

<table>
<thead>
<tr>
<th>Factor (n=200)</th>
<th>Variable</th>
<th>Number (percentage / range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site of tumour</td>
<td>Caecum</td>
<td>38/200 (19.0%)</td>
</tr>
<tr>
<td></td>
<td>Ascending colon</td>
<td>18/200 (9.0%)</td>
</tr>
<tr>
<td></td>
<td>Hepatic flexure</td>
<td>7/200 (3.5%)</td>
</tr>
<tr>
<td></td>
<td>Transverse colon</td>
<td>11/200 (5.5%)</td>
</tr>
<tr>
<td></td>
<td>Splenic flexure</td>
<td>4/200 (2.0%)</td>
</tr>
<tr>
<td></td>
<td>Descending colon</td>
<td>9/200 (4.5%)</td>
</tr>
<tr>
<td></td>
<td>Sigmoid colon</td>
<td>49/200 (24.5%)</td>
</tr>
<tr>
<td></td>
<td>Rectum</td>
<td>64/200 (32.0%)</td>
</tr>
<tr>
<td>Neoadjuvant therapy</td>
<td>None</td>
<td>166/200 (83.0%)</td>
</tr>
<tr>
<td></td>
<td>Long course</td>
<td>27/200 (13.5%)</td>
</tr>
<tr>
<td></td>
<td>Short course</td>
<td>7/200 (3.5%)</td>
</tr>
<tr>
<td>Dissector group for primary dissection</td>
<td>Consultant (n=5)</td>
<td>66/200 (33.0%)</td>
</tr>
<tr>
<td></td>
<td>Advanced practitioner (n=1)</td>
<td>64/200 (32.0%)</td>
</tr>
<tr>
<td></td>
<td>Senior trainee&lt;sup&gt;b&lt;/sup&gt; (n=9)</td>
<td>48/200 (24.0%)</td>
</tr>
<tr>
<td></td>
<td>Junior trainee&lt;sup&gt;c&lt;/sup&gt; (n=7)</td>
<td>22/200 (11.0%)</td>
</tr>
<tr>
<td>Mean time for primary dissection&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Overall</td>
<td>51 mins (14-150)</td>
</tr>
<tr>
<td></td>
<td>Consultant</td>
<td>32 mins (14-92)</td>
</tr>
<tr>
<td></td>
<td>Advanced practitioner</td>
<td>58 mins (25-105)</td>
</tr>
<tr>
<td></td>
<td>Senior trainee&lt;sup&gt;e&lt;/sup&gt;</td>
<td>52 mins (15-110)</td>
</tr>
<tr>
<td></td>
<td>Junior trainee&lt;sup&gt;e&lt;/sup&gt;</td>
<td>84 mins (30-150)</td>
</tr>
<tr>
<td>Intervention chemical</td>
<td>Further fixation</td>
<td>101/200 (50.5%)</td>
</tr>
<tr>
<td></td>
<td>GEWF</td>
<td>99/200 (49.5%)</td>
</tr>
<tr>
<td>Mean time spent in intervention chemical</td>
<td>Further fixation</td>
<td>25 hours (24-29)</td>
</tr>
<tr>
<td></td>
<td>GEWF</td>
<td>25 hours (24-32)</td>
</tr>
<tr>
<td>Mean time for secondary dissection</td>
<td>Further fixation</td>
<td>15.3 mins (4-45)</td>
</tr>
<tr>
<td></td>
<td>GEWF</td>
<td>15.2 mins (1-35)</td>
</tr>
<tr>
<td>TNM classification</td>
<td>(yp)T0</td>
<td>8/200 (4.0%)</td>
</tr>
<tr>
<td></td>
<td>(yp)T1</td>
<td>15/200 (7.5%)</td>
</tr>
<tr>
<td></td>
<td>(yp)T2</td>
<td>39/200 (19.5%)</td>
</tr>
<tr>
<td></td>
<td>(yp)T3</td>
<td>93/200 (46.5%)</td>
</tr>
<tr>
<td></td>
<td>(yp)T4</td>
<td>45/200 (22.5%)</td>
</tr>
</tbody>
</table>

<sup>a</sup>placed within the non-neoadjuvant group; <sup>b</sup>in second year or more of histopathology training; <sup>c</sup>in first year of histopathology training; <sup>d</sup>not measured in 2/200 cases; <sup>e</sup>not measured in 5/200 cases.

The mean and median numbers of lymph nodes identified at primary dissection were 19.3 and 18.0 respectively (Table 2). The median number of lymph nodes retrieved at primary dissection for the neoadjuvant group was lower than that of the non-neoadjuvant group (13 vs. 19). The mean size of the smallest lymph nodes found at primary dissection was similar in both the neoadjuvant group (1.3mm) and the entire sample (1.5mm), although there was a difference in the mean size of the largest lymph nodes found at primary dissection between neoadjuvant group (5.6mm) and the entire sample (8.9mm).
Table 2. Lymph nodes harvested at primary and secondary dissection for the entire sample and the subset in the neoadjuvant group.

<table>
<thead>
<tr>
<th>Demographic</th>
<th>Primary dissection (n=200)</th>
<th>Secondary dissection (n=200)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sample (n=200)</td>
<td>Neoadjuvant (n=27)</td>
</tr>
<tr>
<td>Total no. of nodes at primary dissection</td>
<td>3850</td>
<td>379</td>
</tr>
<tr>
<td>Mean no. of nodes at primary dissection</td>
<td>19.3 (3-47)</td>
<td>14.0 (3-27)</td>
</tr>
<tr>
<td>Mean size of smallest-largest lymph nodes at primary dissection</td>
<td>1.5-8.9mm (0.5-28.0)</td>
<td>1.3-5.6mm (0.5-10.0)</td>
</tr>
<tr>
<td>Median no. of nodes at primary dissection</td>
<td>18 (3-47)</td>
<td>13 (3-27)</td>
</tr>
<tr>
<td>No. of cases with only negative nodes at primary dissection</td>
<td>121/200 (60.5%)</td>
<td>22/27 (81.5%)</td>
</tr>
<tr>
<td>No. of negative nodes at primary dissection</td>
<td>3561</td>
<td>361</td>
</tr>
<tr>
<td>No. of cases with positive nodes at primary dissection</td>
<td>79/200 (39.5%)</td>
<td>5/27 (18.5%)</td>
</tr>
<tr>
<td>No. of positive nodes at primary dissection</td>
<td>289</td>
<td>18</td>
</tr>
<tr>
<td>Mean size of largest positive nodes at primary dissection</td>
<td>8.6mm (1.0-28.0)</td>
<td>4.6mm (2.0-9.0)</td>
</tr>
</tbody>
</table>
At secondary dissection 1555 additional lymph nodes were harvested, equating to a mean of 7.8 nodes per case overall, and a mean of 6.3 in the neoadjuvant group (Table 2). In the non-neoadjuvant group the mean increase in number of lymph nodes retrieved was 49.9%. In the neoadjuvant group this increased to 65.5%. When comparing further fixation and GEWF, there was no statistically significant difference in the number of lymph nodes retrieved at secondary dissection in either the entire sample (p=0.211) or the neoadjuvant group (p=0.614) (Table 3).

Table 3. Statistical analysis with independent samples t-test.

<table>
<thead>
<tr>
<th>Research variable</th>
<th>Further fixation [n=101 (SD)]</th>
<th>GEWF [n=99 (SD)]</th>
<th>Mean difference (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean no. of lymph nodes</td>
<td>8.27 (5.34)</td>
<td>7.25 (6.18)</td>
<td>1.02 (-0.58 - 2.63)</td>
<td>0.211</td>
</tr>
<tr>
<td>Mean size of lymph nodes</td>
<td>2.56 (1.53)</td>
<td>2.43 (1.44)</td>
<td>0.13 (-0.02 - 0.27)</td>
<td>0.093</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Research variable</th>
<th>Further fixation [n=15 (SD)]</th>
<th>GEWF [n=12 (SD)]</th>
<th>Mean difference (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean no. of lymph nodes</td>
<td>6.73 (4.32)</td>
<td>5.83 (4.82)</td>
<td>0.9 (-2.73 – 4.53)</td>
<td>0.614</td>
</tr>
<tr>
<td>Mean size of lymph nodes</td>
<td>1.81 (1.04)</td>
<td>1.76 (1.0)</td>
<td>0.05 (-0.26 – 0.37)</td>
<td>0.730</td>
</tr>
</tbody>
</table>

The mean size of the smallest lymph nodes found at secondary dissection was similar in both the neoadjuvant group (0.9mm) and the entire sample (1.2mm), although there was a difference in the mean size of the largest lymph nodes found at secondary dissection between the neoadjuvant group (2.9mm) and the entire sample (4.1mm) (Table 2). When comparing further fixation and GEWF, there was minimal difference between the size of nodes found in either the entire sample (2.6mm with further fixation vs 2.4mm with GEWF) or neoadjuvant group (1.8mm for both interventions) (Table 4), with no statistically significant difference in the size of lymph nodes retrieved at secondary dissection in either the entire sample (p=0.093) or the neoadjuvant group (p=0.730) (Table 3).
Fewer specimens elicited positive lymph nodes than at primary dissection (9.7% vs 39.5%), with only 29 positive nodes identified at secondary dissection (Table 2).

Most positive nodes were retrieved in the non-neoadjuvant cases (93.1%).

The mean size of positive nodes identified at secondary dissection was smaller (Table 2). The mean time spent performing the secondary dissection for each group was almost identical - 15.3 in the further fixation group vs 15.2 minutes in the GEWF group (Table 1).

Upstaging that would lead to a possible change in treatment (pN0 to pN1/pN2) occurred in only two cases (1.0%), both of which were in the non-neoadjuvant group and had primary dissections performed by experienced dissectors. The first case
was in the GEWF group (primary nodal count 0/11). Two of the 22 lymph nodes identified at secondary dissection were positive (4mm and 8mm in size), giving a final nodal count of 2/33. The second case was in the further fixation group (primary nodal count 0/43). A further 12 nodes were identified at secondary dissection, and one of these was positive (3mm in size), giving a final nodal count of 1/55.

There was a clearly detectable macroscopic difference between the two chemicals. GEWF made the fat nodular and hard, consistent with dehydration by the alcohol, making palpation for nodes more difficult. On the other hand, GEWF improved the visibility of lymph nodes as they became white in colour (Figure 2).

**DISCUSSION**

Secondary dissection resulted in the detection of more lymph nodes, in keeping with previous studies.[9,16,37-43,45-46] We found that GEWF was not superior to formalin fixation in this respect. Only 29 (1.9%) of additional lymph nodes found at secondary dissection contained metastases; this small number may be a reflection of high quality underlying dissection practice, with staff meeting appropriate national standards[51-52] allowing positive lymph nodes to be appropriately identified at primary dissection.

The mean size of positive nodes identified at secondary dissection was smaller in the entire sample, and also in the neoadjuvant group - most likely because the larger positive nodes had already been identified at primary dissection. Our findings are in keeping with previous studies which have described the retrieval of smaller lymph nodes in the specimens of patients who have received neoadjuvant therapy.[53-57]
Nevertheless, the mean number of nodes retrieved at primary dissection in the neoadjuvant group achieved recommended targets in our study, contradicting the suggestion that the retrieval of 12 lymph nodes is unrealistic in these specimens,[49,54] We found the target of 12 nodes is achievable without adjunct techniques, but relies upon high quality dissection practice; in our experience tiny lymph nodes may be identified in this way.

Upstaging following secondary dissection from node negative (pN0) to node positive (pN1) was very infrequent, being observed in only two cases (1.0%). Two previous studies found much higher levels of upstaging,[42-43] but were small and open to detection and analysis bias, e.g. unclear statistical methodology.

Although GEWF made nodes more visible, distinction between nodes and nodular fat or blood vessels by palpation was more difficult. In contrast, formalin is water-based and so the fat remained soft. Lymph nodes may not be so easily visible, but as only small lymph nodes remained in the fat at secondary dissection the ability to detect them by touch may have been more important in our specimens. It is therefore essential that during training dissectors develop appropriate techniques in both vision and palpation in order to identify smaller nodes, as this may be important, especially for patients who have received long-course neoadjuvant therapy.

This study attempted to remove and minimise bias, however, it was not entirely achievable.[58] The main issue was the ability to detect the adjunct chemical, due to the distinctive smell and texture of GEWF making it instantly recognisable. It has to be accepted that potential bias from the secondary dissector could be introduced
because blinding to the secondary fixation solution is not possible. However, the equivalence in the time spent on the secondary dissection for each adjunct chemical may be evidence that equal effort was used for each. A further limitation of this study is the small size of the neoadjuvant group (n=27), which may put the findings at risk of type 1 statistical error.

In conclusion, we have shown that secondary dissection retrieves more lymph nodes, irrespective of whether GEWF or formalin is used as the fixative. Although GEWF might be of benefit to practitioners who rely more on vision than palpation, we recommend further fixation in formalin in difficult cases – not only because it yields an equivalent number of lymph nodes, but also because it is cheaper, easier to prepare and more readily available. Furthermore, adequate fixation combined with high quality training of staff and an appropriate time spent retrieving lymph nodes should allow standards to be achieved without the need for adjunct techniques in the great majority of cases.

**TAKE HOME MESSAGES**

GEWF is no better than further fixation in formalin in terms of number and size of lymph nodes harvested at secondary dissection.

The use of adjunct techniques followed by secondary dissection only leads to upstaging in a small percentage of cases, but in these it may be clinically significant.
Adjunct techniques are not required in the majority of cases if an appropriate primary dissection has been performed on an adequately fixed specimen by an appropriately trained person who spends appropriate time performing the task.

In difficult cases adjunct techniques and/or a secondary dissection may be useful, but further fixation in formalin is recommended as it is cheaper, more readily available and more efficient at detecting lymph nodes.

ACKNOWLEDGEMENTS

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CONTRIBUTORS

JH had the original research idea, led the study and performed all secondary dissections. IR, ACB and NJC helped develop the research idea, contributed to the study and helped structure the paper. NK provided statistical support. JA and SS provided scientific and technical leadership and support within the dissection laboratory during the data collection period.

COMPETING INTERESTS AND FUNDING

None.

PROVENANCE AND PEER REVIEW
Not commissioned; externally peer reviewed. This work formed part of the Professional Doctorate thesis of JH.

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Figure 1. Groups within the study.

- **Group 1**
  - 24 hours GEWF
  - n = 99
  - **Group 1A** GEWF
    - Non-neoadjuvant cases
    - (n = 87)
  - **Group 1B** GEWF
    - Neoadjuvant cases
    - (n = 12)

- **Group 2**
  - 24 hours further fixation
  - n = 101
  - **Group 2A** Further fixation
    - Non-neoadjuvant cases
    - (n = 86)
  - **Group 2B** Further fixation
    - Neoadjuvant cases
    - (n = 15)

Figure 2. Characteristic white colour of lymph nodes after GEWF use.

LN – lymph node; A – adipose tissue; BV – blood vessel.
Figure 3. Normal distribution plots of data.

a. no. of additional nodes at secondary dissection (all cases); b. no. of additional nodes at secondary dissection (neoadjuvant cases); c. size of nodes at secondary dissection (all cases); d. size of nodes at secondary dissection (neoadjuvant cases).