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# Treatment influencing down-staging in EORTC Melanoma Group sentinel node histological protocol compared with complete step-sectioning: A national multicentre study

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ABSTRACT

*Aim:* Metastasis size in melanoma sentinel lymph nodes (SLNs) is an emerging prognostic factor. Two European melanoma treatment trials include SLN metastasis diameters as inclusion criteria. Whilst diameter estimates are sensitive to the number of sections examined, the level of this bias is largely unknown. We performed a prospective multicentre study to compare the European Organisation for Research and Treatment of Cancer (EORTC) recommended protocol with a protocol of complete step-sectioning.

*Methods:* One hundred and thirty-three consecutive SLNs from seven SLN centres were analysed by five central sections 50 μm apart (*EORTC Protocol*) followed by complete 250 μm step-sectioning.

*Results:* Overall, 29 patients (21.8%) were SLN-positive. The *EORTC Protocol* missed eight of these metastases (28%), one metastasis measuring less than 0.1 mm in diameter, seven measuring between 0.1 and 1 mm. Complete step-sectioning at 250 μm intervals (*Extensive Protocol*) missed one metastasis (3%) that measured less than 0.1 mm. Thirteen treatment courses (34%) performed if inclusion was based on the *Combined Protocol* would not be performed if assessed by the *EORTC Protocol*. Thus, 10 patients would be without completion lymph node dissection (*EORTC MINITUB study*), whilst three patients would not be eligible for anti-CTLA4 trial (*EORTC protocol 18071*). The corresponding number with the *Extensive Protocol* would be three; one patient for the MINITUB registration study and two patients for the anti-CTLA4 study.

*Conclusions:* Examining SLNs by close central sectioning alone (*EORTC Protocol*) misses a substantial number of metastases and underestimates the maximum metastasis diameter, leading to important changes in patient eligibility for various treatment protocols.



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## 1. Introduction

Melanoma sentinel lymph node (SLN) positivity rates vary according to the histopathological protocol used, and appear to be strongly dependent on the number of tissue sections examined from each node.[1,2] Growing evidence for this fact has spurred much of the current debate about both the clinical relevance of the additional metastases found and the optimal way to assess metastatic burden in SLNs.[3,4]

Early studies suggested that melanoma metastases are predominantly found in the central part of the lymph nodes, close to the hilar region in the longitudinal plane.[5] As a result, many histological protocols focus on this region, whilst the peripheral parts of the SLNs are often left unexamined.[3,6,7] However, we have recently shown that melanoma metastases are evenly distributed in positive SLNs, showing no predilection for the central nodal region.[8] Furthermore, we have shown that a protocol of complete step sectioning of SLNs at 250 μm levels will detect a high number of metastases.[1]

In addition to the effect on metastasis detection rates, the histological protocol chosen will also affect semi-quantitative tumour burden measurements.[9] First and foremost, metastases not found by a given protocol will of course not be measured. Second, and of almost equal importance, protocol dependent measurement bias will also be introduced. Thus, we have shown that semi-quantitative metastasis size estimates are greatly influenced by the number of sections examined.[9] This is especially true when the measurements obtained are used to estimate the observed maximum value of a given parameter, e.g. the maximum metastasis diameter. Put simply, increasing the number of sections examined can never lead to a decrease in the maximum metastasis diameter observed, since this can only ever increase or stay the same.

In an important effort to optimise diagnosis and treatment for melanoma patients, the Melanoma Group of the European Organization for Research and Treatment of Cancer (EORTC) has suggested a standardised protocol for the histological assessment of melanoma SLNs.[6] Although this protocol is quite extensive, it limits the examination to the most central regions of the SLNs. Several European treatment trials now require melanoma SLNs from participating patients to be sectioned according to the EORTC protocol. As a consequence, the EORTC Melanoma Group protocol has been adopted as the recommended national protocol for melanoma SLN examination in Denmark. In order to evaluate the effectiveness of the EORTC protocol in comparison with the previous National Danish protocol (*DMG protocol*) and with a protocol of complete step sectioning (*Extensive protocol*) previously performed at Aarhus University Hospital, we performed a prospective national multicentre study with the participation of seven Danish pathology laboratories examining melanoma SLN biopsies.

## 2. Patients and methods

### 2.1. Patients

A total of 140 patients undergoing SLN biopsy were initially included, comprising 20 consecutive patients from each of the seven participating centres. The patients were included during the period of February 26th 2009 to September 2nd 2009. Patients were eligible for SLN biopsy if they had a primary melanoma that was either more than 1 mm in thickness, or was Clark's level IV/V, or was ulcerated, and if they were without clinical evidence of metastases. The study was conducted after approval by the Southern Denmark Region Committee on Biomedical Research Ethics.

### 2.2. Diagnostic procedures

The diagnostic procedures were performed according to the National Danish guidelines as previously described.[1] In short, 0.2 mL $^{99m}$Tc human albumin colloid (Nanocoll, GE Healthcare, Denmark) was injected intradermally at four sites around the tumour or biopsy scar. At four of the seven centres, static lymphoscintigraphy was carried out 2 h after injection on the day before surgery, in one centre this was done on the day of surgery. Two centres performed dynamic lymphoscintigraphy on the day of surgery. The location and number of draining lymph node basins were determined. SLNs were detected during the operation with a handheld gamma probe. Blue dye (Patent Blue V) was used in all centres but one. Hot nodes were designated as SLNs when they had an *in vivo* gamma count $\geqslant$10% of the level found in the node with the highest count in the same lymph node basin. The SLNs from the 140 patients were mostly bisected, although in a few instances SLNs were divided into three pieces or embedded *in toto* before being completely step-sectioned. The first six levels were obtained at intervals of 50 μm, the remaining part of the nodes being sectioned at 250 μm intervals. From the first five levels (*EORTC protocol*), three sections were stained, one with standard haematoxylin-eosin (H&E), the remaining two with standard immunohistochemistry for S-100 protein and MART-1. In addition, an unstained section was obtained from each of these five levels. From the remaining levels, two sections were stained, one with H&E, and one with MART-1.

### 2.3. Excluded patients

Of the 140 patients who underwent biopsy, two patients had mucosal melanomas, one conjunctival and one from the female genital tract. Both these patients were SLN negative. Five additional patients did not have their SLN sectioned or stained according to protocol. Having excluded these seven patients, 133 patients were left for study analysis.

### 2.4. Metastasis detection rate

As shown in Fig. 1, sections from all SLN-positive patients were reviewed and the number of metastases found with each of three different protocols: (1) the *EORTC protocol*: examining five centrally located steps 50 μm apart; (2) the *DMG protocol*: examining three centrally located steps 500 μm apart; and (3) the *Extensive protocol*: examining the SLN sectioned to extinction at an interval of 250 μm. These protocols were compared with the results obtained from assessing all the sections obtained (*Combined protocol*).

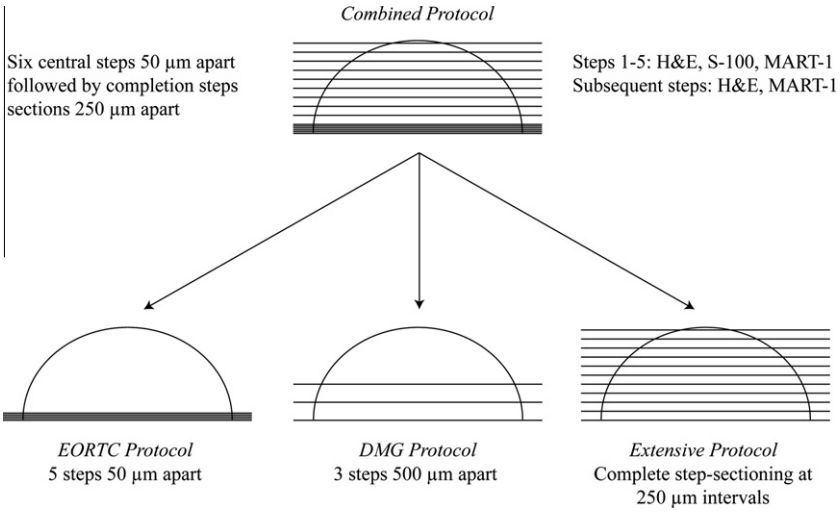


**Fig. 1 – The four histological study protocols.**

### 2.5. *Metastasis size estimation*

A single metastasis was defined and measured according to EORTC recommendations, as the maximum diameter of any group of coherent melanoma cells not separated by lymphocytes in the plane visualised.[10]

### 2.6. *Treatment classification*

To evaluate the clinical significance of differences in the number and size of metastases found using the various study protocols, we compared the number of patients eligible for one of the two potential treatment studies: (1) the *EORTC MINITUB registration study* in which patients with exclusively subcapsular metastases with a maximum diameter of 0.4 mm and patients with parenchymal metastases with a maximum diameter of 0.1 mm may be given the choice of omitting completion lymph node dissection (CLND), and (2) the *EORTC anti-CTLA4 Trial*; *Ipi Trial* (EORTC 18071 study) in which patients with lymph node metastases larger than 1 mm in diameter are eligible for randomisation to anti-CTLA4 treatment versus placebo. Inclusion in either of these two studies is based on the metastasis size obtained with the EORTC histological protocol.

## 3. Results

We examined 346 SLNs from 162 nodal basins in 133 patients. The mean number of SLNs submitted per patient was 2.6 (range, 1–10) and per basin 2.1 (range 1–10). A total of 5295 steps were analysed, corresponding to a mean of 15.3 steps per SLN (*Combined protocol*).

In some cases, the SLNs were embedded in more than one paraffin block. Thus, 57 were embedded in two blocks, five in three blocks, and six in four blocks. A total of four SLNs were embedded with two SLNs in each block. The mean number of steps per SLN was 3.7 for the *DMG protocol*, 6.2 for the *EORTC protocol* and 10.3 for the *Extensive protocol*. The clinical and pathological characteristics of the 133 included patients are shown in Table 1.

A total of 29 patients (21.8%) had melanoma metastases in 31 regions when all sections stained were included (*Combined protocol*). The metastasis detection rates and the size of metastases missed using each of the four different study protocols are shown in Table 2. After a median follow-up time of 23.2 months, none of the nine patients with metastases completely missed either by the *EORTC protocol* ($n = 8$) or the *Extensive protocol* ($n = 1$) experienced a recurrence, whereas one patient with a metastasis missed by the *DMG protocol* developed a local recurrence.

The size of the metastases detected by the *Combined protocol* would have led to a total of 28 CLNDs if evaluated by the criteria determined by the *EORTC MINITUB registration study*. Ten patients had metastases larger than 1 mm in diameter; the metastasis size in the central steps acting as an inclusion criteria for the *EORTC anti-CTLA4 Trial*.

Up to 13 of 38 potential treatment courses (34%) when assessing all sections obtained, would not have been performed if evaluated by one of the less extensive histological protocols (Figs. 2 and 3). Of these, six treatments would not have been performed because the metastases were missed by the *EORTC Protocol* and five because missed by the *DMG Protocol*, whilst a further seven (*EORTC Protocol*) and six (*DMG Protocol*) treatments would not be performed because of the treatment influencing changes in the maximum metastasis diameter measurements. Two SLN metastases missed by the *EORTC Protocol* were small enough to have entered the *MINITUB Registration study*, one measuring 0.051 mm and one measuring 0.21 mm but exclusively located in the subcapsular space. The same was true for three metastases missed by the *DMG Protocol* (0.051 mm, 0.053 mm, and the subcapsular metastasis measuring 0.21 mm)

All of the three potential treatments brought forth by the *Combined Protocol* compared with the *Extensive Protocol* were caused by the treatment influencing changes in the metastasis diameter measurements. The single metastasis missed by the *Extensive Protocol* measured 0.053 mm and the patient would thus have been eligible for the *MINITUB Registration Study*.

**Table 1 – The clinical and pathological features of the 133 study patients.**

| Feature | No. of patients (%) |
|---|---|
| *Gender* | |
| Male | 58 (44) |
| Female | 75 (56) |
| Median age (years) | 61 |
| Age range (years) | 19–85 |
| *Primary tumour site* | |
| Trunk | 64 (48) |
| Upper extremity | 24 (18) |
| Lower extremity | 33 (25) |
| Head and neck | 12 (9) |
| *Breslow thickness*[a] | |
| <1.00 mm | 16 (12) |
| 1.00–2.00 mm | 54 (41) |
| 2.0–4.00 mm | 27 (20) |
| >4.00 mm | 11 (8) |
| Unclassified | 24 (18) |
| Unknown | 1 (1) |
| Mean tumour thickness, mm[a] | 2.20 |
| Median tumour thickness, mm[a] | 1.41 |
| *Clark's level*[a] | |
| II | 5 (4) |
| III | 30 (22) |
| IV | 73 (55) |
| V | 4 (3) |
| Unclassified | 20 (15) |
| Unknown | 1 (1) |
| *Ulceration*[a] | |
| Yes | 29 (22) |
| No | 96 (72) |
| Unclassified | 7 (5) |
| Unknown | 1 (1) |
| *Histological subtype*[a] | |
| Superficial spreading | 87 (65) |
| Nodular | 28 (21) |
| Acral-lentiginous | 2 (2) |
| Unknown potential | 4 (3) |
| Unknown primary or metastasis | 4 (3) |
| Unclassified | 7 (5) |
| Unknown | 1 (1) |

[a] The primary lesion of one patient was unavailable for histopathological examination and as a consequence its features were unknown.

## 4. Discussion

SLN status is the most important prognostic factor in melanoma,[11] and there is solid evidence to support the prognostic significance of metastasis size in these nodes.[12–16] We have previously shown that semi-quantitative metastasis size estimates (such as maximum metastasis diameter) are sensitive to changes in histological protocol.[9] Thus, since semi-quantitative estimates focus on the largest metastasis diameter present in any of the sections obtained, adding steps will either lead to an increase in the observed diameter or maintain the status quo. Adding extra steps can never reduce the maximum metastasis size observed.

The EORTC Melanoma Group protocol has been reported to have a metastasis detection rate of approximately 30%,[13,17] however, the median primary tumour thickness (2.0 and 2.1 mm) in these studies is far greater than the median melanoma thickness in the cohorts undergoing SLN biopsy, we have previously examined (1.4 and 1.6 mm).[1,18]

This prompted us to investigate how many metastases would actually be detected with this protocol in a consecutive Danish population of melanoma patients. The metastasis detection rate in our study is surprisingly low, even when evaluating all the steps obtained. This may in part be explained by the low median primary tumour thickness, although one of our previous studies comprising patients with melanomas with comparable tumour thicknesses found a much higher metastasis detection rate of 31%.[1]

An alternative explanation for the lower metastasis detection rate in the current study may be the rather high median age of our study cohort, since several previous studies have shown that older patients are less likely to be SLN positive.[19,20] Furthermore, several of the study patients underwent SLN biopsy on the basis of either a recurrent melanoma ($n = 2$), a lesion with unknown malignant potential ($n = 4$), or a lesion unknown to be primary or metastatic ($n = 4$), all of which represent lesions that are probably less likely to be SLN positive.

We found that a substantial number of metastases were missed by the *EORTC protocol* (eight of 31 positive basins detected by the *Combined protocol*), a finding in line with our previous results in a larger patient cohort, although the study was performed without close step-sections in the central part of the SLNs.[1] In contrast, the four central sections not included in the *Extensive protocol* analysis only identified one extra patient with metastases, indicating that close step sections in the central SLN region will not compensate for the metastases missed in the node periphery. None of the

**Table 2 – Metastases detected and missed by each of the four protocols.**

| | Combined | EORTC | DMG | Extensive |
|---|---|---|---|---|
| SLN-positive: number (percentage) | 29 (21.8%) | 21 (15.8%) | 21 (15.8%) | 28 (21.1%) |
| Median metastasis diameter (mm) | 0.43 | 0.17 | 0.35 | 0.43 |
| Mean metastasis diameter (mm) | 1.40 (SD 2.5) | 0.94 (SD 2.2) | 1.21 (SD 2.5) | 1.35 (SD2.6) |
| Metastasis diameter range (mm) | 0.05–12 | 0.04–12 | 0.04–12 | 0.04–12 |
| Small (<0.1 mm) metastases missed (number) | – | 1 | 2 | 1 |
| Medium-sized (0.1–1 mm) metastases missed (number) | – | 7[a] | 6[a] | 0 |
| Large metastases (>1 mm) missed (number) | – | 0 | 0 | 0 |

[a] One of these metastases measuring 0.21 mm was located solely in the subcapsular space.

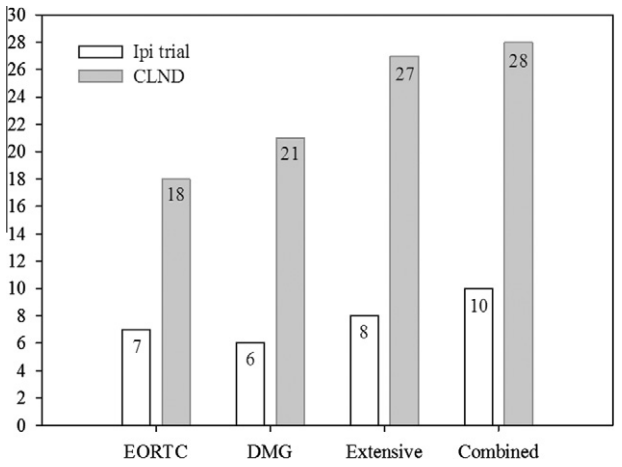


**Fig. 2 – The number of treatments elicited by each of the four study protocols.**

eight patients with metastases missed by the EORTC protocol have developed recurrences. However, all of these patients have had a CLND, potentially sparing them from regional recurrences. Furthermore, the sample size in our SLN cohort is small with a relatively short median follow-up period of 23 months. Thus, the clinical importance of these missed metastases cannot be determined by our study. To our knowledge, location of metastases in the peripheral plane only has not been shown to represent a survival advantage compared with metastases of the same size in the central plane. Therefore, we believe that large metastases must be found regardless of their intra-nodal location.

The true number of metastases missed by the protocols described here can of course only be determined, if all SLNs are subjected to complete serial-sectioning, examining the tissue in its entirety. However, since this would result in more than 1000 sections per lymph node the extra costs and time required for the analysis makes this impracticable. Therefore, some sampling must be performed. We have previously shown that SLN metastases in the central and peripheral parts are equally sized and are distributed throughout the SLNs.[8] This suggests evenly spaced step-sectioning as opposed to close central step-sectioning is the most efficient way to sample SLNs in order to ensure a high metastasis detection rate, with detection of the largest metastases present throughout the node, and not exclusively the centrally located metastases. And, although meticulous orientation of the SLNs before bivalving was strived for in this study, we believe the global location of SLN metastases makes this effort less important. A cost-neutral way of implementing complete step-sectioning in the EORTC countries could be to increase the distance between steps to enable the five-step protocol to cover the entire SLN.

Assessment of metastasis size is required in Europe today if patients are to be included in several of the major melanoma treatment trials. One such trial (*EORTC anti-CTLA4 Trial*) examines the benefit of adjuvant anti-CTLA4 in patients with high-risk regional metastases without distant metastases. Thus, patients with a SLN metastasis diameter greater than 1 mm in the central *EORTC* sections are eligible for inclusion. In a second EORTC study (the *MINITUB registration study*), SLN

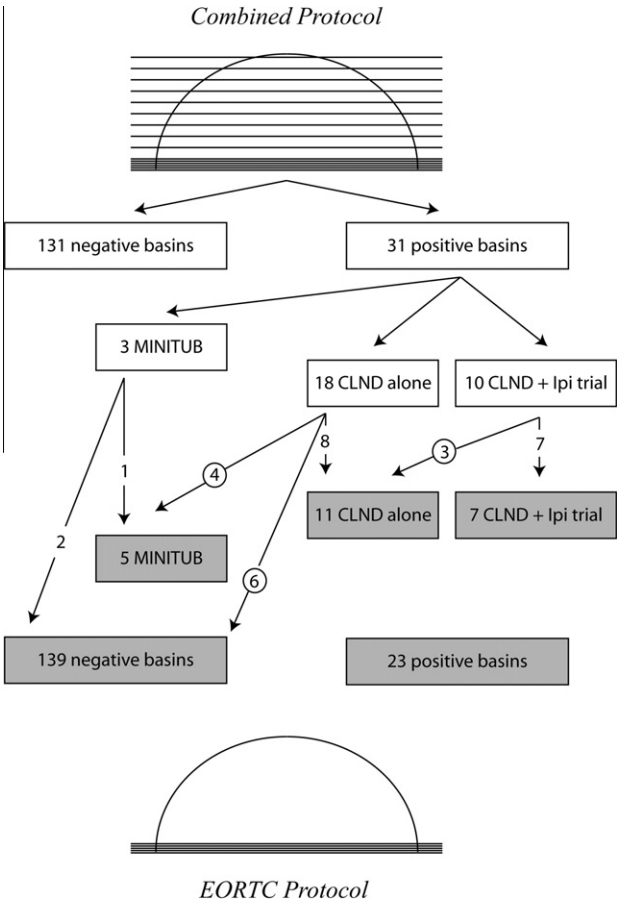


**Fig. 3 – The consequence of omitting the peripheral step-sections in the study population.**

positive patients with small metastasis diameters in the same central *EORTC* sections can elect to forego CLND, after which they are followed and the course of their disease is compared with SLN positive patients undergoing CLND. The outcome analyses in both of these studies are thus dependent on reliable metastasis size estimates.

In our study, 34% of the treatments (CLND or adjuvant anti-CTLA4 treatment) potentially performed if examined by the *Combined Protocol* would not have been performed if the SLNs were evaluated by the *EORTC Protocol*. For approximately half of the treatment courses, this was the result of a missed metastasis in the *EORTC* sections, whilst the remainder was attributable to the classification changes caused by an increase in the metastasis diameter in the supplementary sections. At first sight, it might appear that the latter problem can be solved by adding extra steps to the SLN histological protocol. However, in our opinion, simply increasing the number of sections examined is clearly *not* the answer, since this will not resolve the fundamental problem inherent to the metastasis diameter measurement itself. In an effort to obtain some 'true' estimate, the observer is pushed to keep searching for a parameter that becomes ever larger in size, even though this ultimately results in an overestimate of the actual tumour burden in the SLN, just as the method will tend to underestimate tumour burden if only a few sections are taken from the central region of the SLN, leaving the peripheral parts of the node unexamined.

To truly overcome this bias, stereological measurements of tumour burden such as the total metastatic volume (TMV) will have to be adopted. Until now, this has not been of major importance, since metastasis size estimates have only been used to allocate patients into prognostic groups. However, this situation has now changed, and melanoma metastasis size estimates are being used increasingly to allocate patients to the particular forms of treatment. Accurate tumour burden estimation is now of practical importance for the individual patient.

We have previously shown that TMV estimation is of prognostic importance in melanoma SLNs.[14] Unfortunately, manual TMV measurements are slow and laborious making the technique unsuitable for routine use. However, current attempts to perform TMV measurements by automatic digital image analysis show promise and may help to solve this logistical problem.[21]

In conclusion, melanoma metastases are distributed throughout positive SLNs and protocols directed at the central nodal region only will miss a substantial number of metastases. Using maximum metastasis diameter as a measurement of tumour burden is associated with inherent bias, since increasing the number of step sections can only ever result in uni-directional up-staging, never down-staging. Our study shows that these are not just theoretical problems. A total of 34% of treatments (CLND and anti-CTLA4 treatment) potentially brought forth by the most comprehensive protocol performed (*Combined Protocol*) would not have been offered to the patients if assessed by the more restricted protocol recommended by the EORTC Melanoma Group that focuses on the central SLN region, either because their metastases would be overlooked or because the metastasis diameter was smaller in the central sections than in the periphery of the node. We recommend a melanoma SLN histological protocol that samples all parts of the lymph nodes and urge that continued efforts be made to develop stereologically correct unbiased measurements aided by digital image analysis for assessing tumour burden in these nodes.

## Conflict of interest statement

None declared.

## Sources of support

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