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Analysis of sentinel node biopsy – a single institution experience supporting the use of serial sectioning and immunohistochemistry for detection of micrometastases by comparing four different histopathologic laboratory protocols

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Abstract

Aims. Detecting micrometastases (>0.2 mm and ≤ 2 mm/ >200 cells) and isolated tumor cells (ITC) (≤ 0.2 mm/ <200 cells) is important for staging of breast cancer patients. The aim of this study was to systematically compare several laboratory protocols used to detect metastases after initial intraoperative frozen section examination.

Methods and results. Four different protocols for the work-up of sentinel lymph nodes (SLN) after frozen sectioning were applied in the routine diagnostic process from 2001-2009. In addition team work with a limited number of laboratory technicians and pathologists handling SLN was introduced in 2008.

The present study shows overall significantly more node positive patients in the period where team work and intensive step sections including immunohistochemistry (IHC) was used ($p=0.01$). This resulted in 13% more patients found to have ITC and micrometastases compared with the result from a time period when only step sections were done. No rise in false negative frozen sections was seen.

Conclusions. Future guidelines for pathologic work-up of sentinel nodes in women with breast cancer might include team work and IHC if frozen sections are used intraoperatively.

Key words: Sentinel node biopsy, breast cancer, immunohistochemistry, micrometastases, ITC

Abbreviations: ALND, axillary lymph node dissection; FFPE, formalin fixed paraffin embedded; HE, haematoxylin and eosin; IHC, immunohistochemistry; ITC, isolated tumor cells; SLN, sentinel lymph node

Introduction

During the last 10 years sentinel lymph node (SLN) biopsy has gained acceptance as the routine method for staging the ipsilateral axilla in women with early breast cancer. Preoperative procedures including the injection of radioactive colloidal substance and/or blue dye, scintigrams and gammaprobe handling and the intraoperative surgical technique is standardized and largely agreed upon. In the beginning of the 2000's Swedish guidelines for surgeons included SLN biopsy as standard procedure after a period of validation with respect to the false negative rate of axillary staging¹.

On the other hand the work-up of sentinel nodes in the laboratory is still under debate. The 6th edition of the pTNM classification introduced the concept of ITC in 2002^{2, 3} and stated that these should be considered as node negative for staging and treatment purposes. The 7th edition^{4, 5} clarifies the concept and recommends finding all metastases >2 mm by macroscopically slicing the SLN in sections of 2 mm and to state the accuracy of the subsequent formalin fixed paraffin embedded (FFPE) protocol in finding micrometastases and ITC. In 2006 Swedish guidelines for SLN handling was largely adapted from the European guidelines⁶.

The risk of upstaging axillary status by a more thorough examination of SLNs has been considered an issue to be avoided because clinical data was based on only one histologic slice from each axillary lymph node⁷. Nevertheless, a recent paper on women with early stage breast cancer who underwent sentinel node biopsy reports that isolated tumor cells or micrometastases in regional lymph nodes were associated with a decreased disease free survival in women without adjuvant therapy and that disease free survival was improved in this patients group if they received adjuvant therapy⁸. Another review concludes that patients with metastases of 2 mm or smaller in axillary lymph nodes examined by a single HE section have a worse outcome compared to node negative patients⁹, but for patients treated by SLN the data is still inconclusive.

A further review of SLN micrometastases concludes that SLN micrometastases are likely to represent an incremental detriment to prognosis and increased risk of non-SLN involvement¹⁰. In addition metastases uncovered in formerly negative axillary lymph node dissection (ALND) with a SLN protocol including IHC have prognostic significance in breast cancer patients¹¹. The Swedish national figures show a worse outcome of patients with micrometastases in SLN compared to node negative patients¹², but other reports have shown no difference in survival between SLN negative and those with micrometastases¹³.

These findings emphasize the need to optimize the accuracy of SLN pathology. At the same time, whatever method is chosen must not dramatically increase the cost and time spent for analysis. In histopathological practice the technical quality of HE slides prepared from formerly frozen material occasionally can be inferior to material which was not frozen; freeze artifacts still show in the paraffin sections. This study address these problems by comparing the results of routine histopathologic work-up over four different time periods at the same institution using different protocols, all including frozen sections but with diverse methods for step sectioning and use of immunohistochemistry. In the last time period, laboratory organization was changed such that fewer laboratory technicians and pathologists were involved in frozen section diagnosis and in the subsequent work-up of the SLN.

Material and methods

The study was conducted on the consecutive series of the 1576 women with a first primary operable breast cancer treated at the University Hospital of Lund from January 1, 2001 to December 31, 2009 (Figure 1). Excluded were 25 women diagnosed in October 2008 because these women were analyzed by SLN using method 4 but before the laboratory was reorganized into work teams. Patients were selected by extraction from the data files of the pathologic

department diagnosed with topography code for breast in the SNOMED system. By manual examination of the files patients with a previous history in the pathology files of invasive carcinoma in the breasts were excluded as were patients with previous malignancy elsewhere. Men with breast cancer, benign cases and cases with in situ carcinoma were also excluded. A total of 27 women with synchronous bilateral breast cancer were included using the side showing the worse axillary status. The other side was excluded in these women.

The surgical procedure of SLN biopsy was preceded by subdermal injection with ^{99}Tc labeled nanocolloid followed by a scintigramme. Injection of 0.5 ml of Patent V Blue was performed intradermally after anesthesia in the operating room. Any node that was hot, blue, or palpable was considered a SLN and removed. The surgical procedure, instrument and surgical staff did not differ in the cohort period.

As a general rule frozen section examination was undertaken in up to four nodes with following examination of FFPE sections stained with HE and/or IHC with pan-anticytokeratins according to different protocols. Throughout the study period four different protocols of routine histopathological work-up of SLN were used (Figure 2). Generally SLNs of 4 mm or more were cut through the longitudinal axis and both halves examined. Smaller nodes were examined in toto. All specimens were examined by one or two HE stained frozen sections and no staining with IHC were performed intraoperatively. After formalin fixation and paraffin embedding the protocols differed. During the first period the sections were examined by one paraffin section and by immunohistochemical analysis with anticytokeratin in frozen negative cases (n=287) (Method 1). In the second period the SLNs were analyzed by step sectioning at three levels randomly chosen and by immunohistochemistry at the first level in frozen negative cases (n=148) (Method 2). The third period introduced step sectioning of SLNs at three fixed levels of 0.2 mm and use of immunohistochemistry only in suspicious cases (n=474) (Method 3). The last period included step sectioning of SLNs at three fixed levels of 0.2 mm with immunohistochemistry at every

level in cases which were frozen negative or had a metastasis of less than 2 mm on the frozen sections (n=186). During the last period the laboratory work was done by fewer, specially trained technicians and fewer pathologists. The number of laboratory technicians was concentrated from 12 to 5 and pathologists were decreased from about 14 to about 3. The laboratory technicians were specially trained in doing frozen sections on SLNs with fatty tissue around and inside the lymph nodes. The frozen sections were examined in the microscope by a pathologist looking for metastases together with a laboratory technician who participated in the analysis of the quality of the frozen section. This method is what we refer to as team work.

A review of the slides of all cases with positive SLN was undertaken and the 7th edition of the AJCC and UICC classification was applied^{4, 14}. Women without detectable tumor cells in the nodes were designated node negative equal to cut off zero. Using a cut off of 0.2 mm women were staged as negative when they had no metastases or had ITC of 0.2 mm and smaller or with less than 200 tumor cells. Patients with micrometastases had metastases >0.2 mm or more than 200 tumor cells but not measuring more than 2.0 mm. A cut off of 2.0 mm separated women with metastases larger than 2.0 mm from all those with smaller ones.

The size of the metastatic deposits was determined on the slide where it was largest. In 2004 the 6th edition of the AJCC was implemented and women with deposits of 0.2 mm or smaller or with ITC were hereafter not routinely offered axillary dissection. All other women with metastasis larger than 0.2 mm in at least one SLN were offered axillary dissection either in combination with the surgery for breast cancer due to a positive frozen section or in a second operative procedure as a result of a positive SLN on FFPE sections and thereby false negative frozen section of the SLN.

From 2001 to Oct 2008 axillary lymph nodes from the axillary clearance were handled by isolation of the lymph nodes in formalin fixed surgical specimens and embedding one slice from each node often including several nodes in each block. From Oct 2008 and thereafter axillary

lymph nodes from the axillary clearance were bisected and macroscopically negative nodes were entirely embedded in separate blocks. One slice sufficed for macroscopically metastatic nodes. One HE section from each block was examined and in difficult cases IHC was applied. Details of age of the patients, tumor size, histological grade, estrogen- and progesterone receptor status were recorded from the pathology reports. Histologic grade was assessed using the Nottingham modification¹⁵ of the Scharff, Bloom and Richardson grading system¹⁶. Estrogen and progesterone receptor status was determined by IHC assay and carcinomas with >10% stained nuclei were considered positive. A mammography screening program was conducted in the local area since 1989 and women aged 45 to 74 were invited every 18-24 months.

Statistics

Differences between proportions were examined by chi-square test and $p < 0.05$ was considered significant. Differences between continuous variables were analyzed by the Kruskal-Wallis test.

Results

Details of patients and tumor features are listed in Table 1. Tumor size did not differ between the four different study periods. About one fourth of the patients were 70 years or older and the median age of the patients was slightly increasing throughout the study. Histological grade was more often grade 3 in the last period and fewer patients had a diagnosis of lobular carcinoma in the later part of the study period. Receptor status did not differ among the study periods.

Of 1576 patients included in the study 70% had SLN biopsy, 20% were treated by primary ALND and 10% were allocated to other protocols without adequate determination of axillary status at the time of surgery (Figure 1). Frequency of patients treated by SLN biopsy was 63, 68, 69, and 86% in the four periods with different SLN examination. Using a cut off of zero for classifying SLNs with negative status, significantly more patients were node positive in the last

period both among women treated by SLN and among all women ($p=0.01$) (Table 2a). When the cut off 0.2 mm/ <200 cells was applied for negative status the difference was only borderline significant ($p=0.07$) (Table 2b). IHC was used in 72, 74, 21, and 92% of the cases treated by SLN biopsy. The median number of analyzed SLN per patient was 2 and did not differ between the periods.

Concentrating on the SLN totally negative and those with ITC of ≤ 0.2 mm/ <200 cells or micrometastases of 2 mm or smaller some striking differences appeared. In the last period using method 4 with step sections at fixed intervals and IHC at every level, where frozen sections and step sections were performed by trained laboratory technicians, significant more patients were diagnosed with ITC or micrometastases in the SLNs ($p<0.01$). The number of patients with ITC and micrometastases was 9% higher when method 4 was used compared with method 1 and 2 using either just one HE section followed by IHC or one HE section and IHC followed by random step sections. Teamwork and intensive IHC in method 4 resulted in 13% more patients with ITC and micrometastases compared with the result of method 3 with step sections at fixed intervals. Frequency of women with ITC and micrometastases compared with those with macrometastases did not differ significantly between the study periods, which indicate that the ITC and micrometastases were found patients who previously were classified as node negative.

Frozen sections were reported positive to the surgeons if a metastasis of larger than 0.2 mm was encountered. The false negative rate of the frozen section technique was similar during the period for method 4 with intensive IHC of step sections in FFPE slides and team work and during the period for method 1 with IHC but without step sections. When method 2 and 3 without team work was used the false negative rate of frozen sections was higher.

Discussion

The present study shows overall more node positive patients in the period where method 4 was used including step sections at fixed intervals and intense use of IHC in hands of specially trained personal. At the same time more women were diagnosed with ITC and micrometastases in their SLNs without increasing the false negative rate of frozen sections. These results were accomplished by a relatively simple change in laboratory organization. Instead of all the laboratory workers handling all the material which came to the department, groups of workers **was** formed which handled only certain types of specimens, based on organ groups. Thus the number of assistants involved in the process of sentinel nodes was smaller which increased their expertise in this area of laboratory work.

The introduction of team work also made possible a shift from the use of a metal stretch plate in the process of moving tissue sections to glass slides. A temperate water bath was used instead. This improved the quality of the HE section to a degree that the frequency of patient with histologic grade 3 was for the first time within the limit of recommended value in Sweden. The shift to fewer women diagnosed with lobular carcinoma was caused by the introduction of E-cadherin IHC and possibly by a decreased use of hormone replacement therapy which has been seen in the population in recent years. The age of the patients is steadily increasing probably due to more active treatment of the elderly.

By applying step sections and/or IHC to negative regional lymph nodes, the use of step sections increases the harvest of small metastases by about 7-13% and the addition of IHC will find about 15-30% more cases with small metastases^{11, 17}. Our results and the conclusion of results of another report¹⁸ implies that smaller metastases will be missed if step sections and IHC of SLNs is left out. The varying false negative rate of metastasis obtained by frozen sections among the study periods indicates that small metastasis might be missed. The sensitivity of frozen section methodology is within the range found in other studies^{19, 20}.

From the surgical point of view discussion is ongoing as to whether patients with micrometastases benefit from subsequent ALND because the frequency of local recurrence is extremely low^{21, 22}. However systemic therapy might improve survival even in patients with tumor deposits even as small as ITC⁸.

A consensus with regard to an optimal pathologic work-up of SLNs is hard to achieve because viewpoints differ about the objectives of the examination. A more modest work-up favors attempts to avoid stage migration and acknowledges the possibility of benign transportation of epithelial tissue to the SLNs. Up-staging of breast cancer patients implies that a more thorough method of SLN examination results in more positive cases²³. The clinical implication of the additional metastases is not determined in the clinical trials where use of adjuvant systemic therapy is applied. Another caution against taking therapeutic action as a consequence of small metastatic deposits only detectable with IHC, is raised by the possibility of benign transportation of cells to the SLN²⁴ after needle diagnostic procedures or manipulation of the breast²⁵. Malignant cells might even be displaced to the SLNs without being metastatic in nature. Lately benign elements have been identified as elements of papillomas from the breast²⁶.

Different models of handling of SLNs have been presented^{27, 28} and all models shows that using an acceptable number of steps and sections in routine work-up, results in missed deposits of small metastases. Furthermore, pathologists do not agree on what constitutes ITC²⁹. Therefore the 7th AJCC staging recommends slicing the SLN in 2 mm intervals and stating the sensitivity of the step section method used to detect small metastases afterwards. In addition clarifications of the concept of ITC and/or less than 200 cells have been reported⁵. The lymph node slices of 2 mm is obtained in practice by dividing the SLN by the longitudinal axis and this will always be an approximation since lymph nodes are of different size. One survey of routine practice in Europe revealed that 73% of participating institutions did IHC on SLNs³⁰. More intensive

protocols using IHC reveal more metastasis²⁸ but method 4 in this study seems to combine an acceptable level of work with an acceptable cost.

Both AJCC and European guidelines recommend step microscopic sections of SLN but both determine the size of the largest metastases on one section in two dimensions and do not take into account the third dimension that emerges from the step sectioning. Most important is ITC appearing in the deepest section. If another level is not examined this ITC might in fact be a macrometastasis. When the SLNs are step sectioned at fixed intervals the size of the metastases can be determined in three dimensions but no guidelines include size in the third dimension and this might underestimate the actual size of the metastasis.

Intraoperative procedures are considered optional and so far no guidelines deals with the fact that frozen section methodology also may introduce freeze artifacts in the FFPE sections. This makes routine examination of HE sections more difficult and the pathologist might miss metastases that easily would have been seen in sections from tissue which had not been frozen. In order to minimize these artifacts the training of the laboratory assistants is crucially important. The preparation of frozen sections is meticulous handwork and must be handled by specially trained laboratory assistants with have the opportunity to do the work often. The present work in the first three periods was carried out at a time when all the laboratory assistants prepared frozen sections as did most of the pathologists. In the last period team work was introduced and a larger fraction of SLN patients were diagnosed as positive with a larger fraction having smaller metastases. There was still an acceptable level of false negative frozen section examinations, however.

This study has some draw backs. As it is a single institution study design, the different methods compared are based on examination of SLN in successive time intervals and comparison of the methods was not randomized during a fixed time interval. Also the frequency of patients treated by SLN was increasing during the study period, and thereby the frequency of positive nodes in the SLN population would be expected to increase. Therefore data from all breast cancer patients

in the institution is considered and because the increase is general in the entire study population the increase in node positivity among patients with SLN is interpreted as being method related and not a result of selection bias. With the present study design reported here it will never be possible to prove that the increase in ITC and micrometastases found using method 4 is not simply a result of more patients having SLN performed. During the study period the indication for axillary clearance also changed so that only patients with a suspicion of, or preoperatively diagnosed regional metastases, would receive axillary clearance primarily. The protocol for the handling of nodes from axillary clearance was also altered before method 4 was used. The frequency of screening detected breast cancer in the population was not changed during the study. In patients undergoing SLN the increased number of ITC's and micrometastases were found in patients who had previously been classified as node negative and therefore the increase might be method related.

To our knowledge the results of work by a specialised team including laboratory technicians have not been studied earlier. Therefore we cannot state that the increase of 13% in ITC and micrometastases which we documented during the time period when method 4 was used is solely based on the result of the specialised team work or just the result of more intensive step sectioning and IHC.

If the handling of SLN is reserved to a small specialized group of laboratory technicians and pathologists it may be expected that the rate of false positive frozen sections be decreased. In fact one of the three false positive cases in the present series were due to the presence of benign nevus in the lymph node capsule, a condition of which not all pathologist are aware³¹.

In conclusion future guidelines for pathologic work-up of sentinel nodes in women with breast cancer might include the recommendation of team work and IHC if frozen sections are to be used intraoperatively. This study shows that a method with team work and intensive step sections including IHC detected 13% more women with ITC or micrometastases.

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Table 1. Patients and tumor characteristics of all women.

	Method 1 n=457	Method 2 n=219	Method 3 n=683	Method 4 n=217	p
Tumor size, mm median (range)	17 (1-80)	18 (0-90)	16 (0-90)	17,5 (0-80)	0.40
Age median (range)	58 (23-99)	59 (30-91)	61 (27-94)	64 (25-91)	<0.01
	n (%)	n (%)	n (%)	n (%)	
Histologic grade					
1	106 (23)	57 (26)	154 (23)	48 (22)	0.003
2	256 (56)	106 (48)	360 (53)	92 (43)	
3	84 (18)	47 (21)	156 (23)	72 (33)	
No grade	11 (2)	9 (4)	13 (2)	5 (2)	
Histologic type					
Ductal	353 (77)	170 (78)	574 (84)	182 (84)	0.04
Lobular	77 (17)	34 (16)	74 (11)	22 (10)	
Other type	27 (6)	15 (7)	35 (5)	13 (6)	
Estrogen receptor					
Negative	83 (18)	43 (20)	101 (15)	30 (14)	0.18
Positive	364 (80)	174 (79)	575 (84)	185 (85)	
Unknown	10 (2)	2 (1)	7 (1)	2 (1)	
Progesterone receptor					
Negative	168 (37)	75 (34)	221 (32)	61 (28)	0.14
Positive	279 (61)	142 (65)	455 (67)	154 (71)	
Unknown	10 (2)	2 (1)	7 (1)	2 (1)	

Post operative tumor size was zero in a few patients included in a study using laser therapy³².

Table 2.a. Frequency of positive nodes in women primarily treated by SLN, ALND and other during the different methodologies of SLN handling, with a cut off for metastases at zero.

	Method 1		Method 2		Method 3		Method 4		
Primary surgery	n	n Npos (%)	n	n Npos (%)	N	n Npos (%)	n	n Npos (%)	
SLN cut off 0	287	123 (43)	148	52 (35)	474	168 (35)	186	92 (49)	p<0.01
ALND	117	68 (58)	51	28 (55)	138	84 (61)	16	14 (88)	p=0.12
Other	53	46 (87)	20	16 (80)	71	57 (80)	15	15 (100)	p=0.53
Total cut off 0	457	237 (52)	219	96 (44)	683	309 (45)	217	121 (56)	p=0.01

* Other se figure 1.

Table 2.b. Frequency of positive nodes in women primarily treated by SLN, ALND and other during the different methodologies of SLN handling, with a cut off for metastases at 0.2 mm/200 cells.

	Method 1		Method 2		Method 3		Method 4		
Primary surgery	n	n Npos (%)	n	n Npos (%)	n	n Npos (%)	n	n Npos (%)	
SLN cut off 0.2 mm/ <200 cells	287	112 (39)	148	45 (30)	474	154 (32)	186	76 (41)	p=0.06
Total cut off 0.2 mm/ 200 cells	457	226 (49)	219	89 (41)	683	295 (43)	217	105 (48)	p=0.07

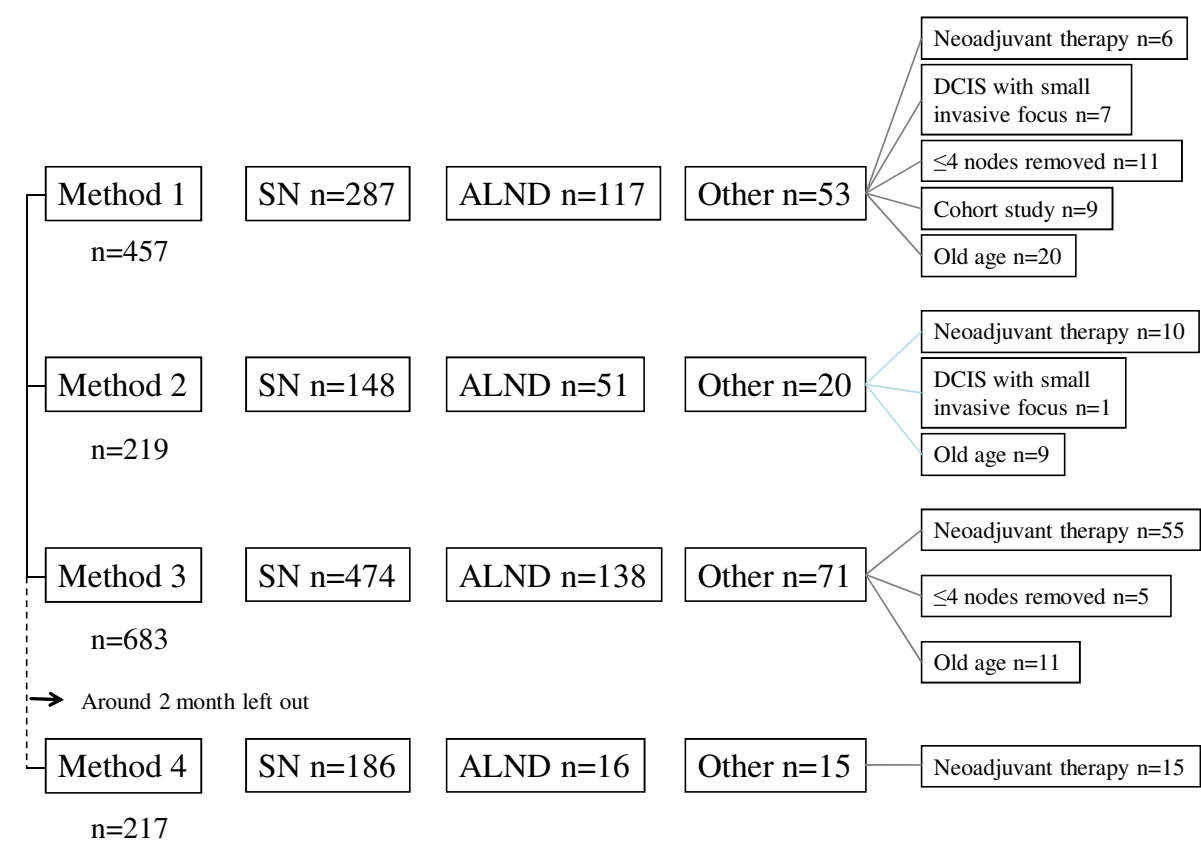
Table 3. Frequency of isolated tumor cells, micro-, and macrometastases in the intervals with different methodology of SLN handling.

	Method 1	Method 2	Method 3	Method 4	
	n (%)	n (%)	n (%)	n (%)	
SLN Negative	165 (57)	97 (66)	311 (66)	94 (51)	p=0.01
SLN ITC ≤ 0.2 mm/<200 cells	11 (4)	7 (5)	14 (3)	16 (9)	
SLN positive > 0.2 and ≤ 2.0 mm	25 (9)	14 (9)	36 (8)	18 (10)	
SLN positive > 2.0 mm	86 (30)	30 (20)	113 (24)	58 (31)	
Total	287	148	478	186	

Table 4. False negative results of frozen sections of SLN compared with FFPE sections using cut off 0.2 mm.

	Method 1 n (%)	Method 2 n (%)	Method 3 n (%)	Method 4 n (%)
SLN total negative	164 (57)	94 (64)	308 (65)	91 (49)
ITC \leq 0.2 mm/200 cells	11 (4)	7 (5)	14 (3)	15 (8)
positive > 0.2 mm/200 cells				
positive > 0.2 and \leq 2 mm	12 (4)	4 (3)	10 (2)	4 (2)
positive >2 mm	73 (25)	25 (17)	96 (20)	49 (26)
Frozen false negative met > 0.2 mm				
positive > 0.2 and \leq 2 mm	13 (5)	9 (6)	25 (5)	13 (7)
positive >2 mm	13 (5)	5 (3)	15 (3)	3 (2)
Frozen false positive	1 (0)	2 (1)	0	0
No frozen section	0	2 (1)	6 (1)	11 (6)
	287	148	474	186
False negative rate	0,23	0,33	0,27	0,23
Accuracy	0,91	0,89	0,92	0,91

Figure 1. Composition of material.



Method 1 was used January 2001 to September 2003
Method 2 was used October 2003 to December 2004
Method 3 was used January 2005 to September 2008
Method 4 was used November 2008 to December 2009

In the first period a cohort study included women with early breast cancer with an expected risk of loco-regional recurrence of less than 1% in a protocol without axillary staging (N=9).

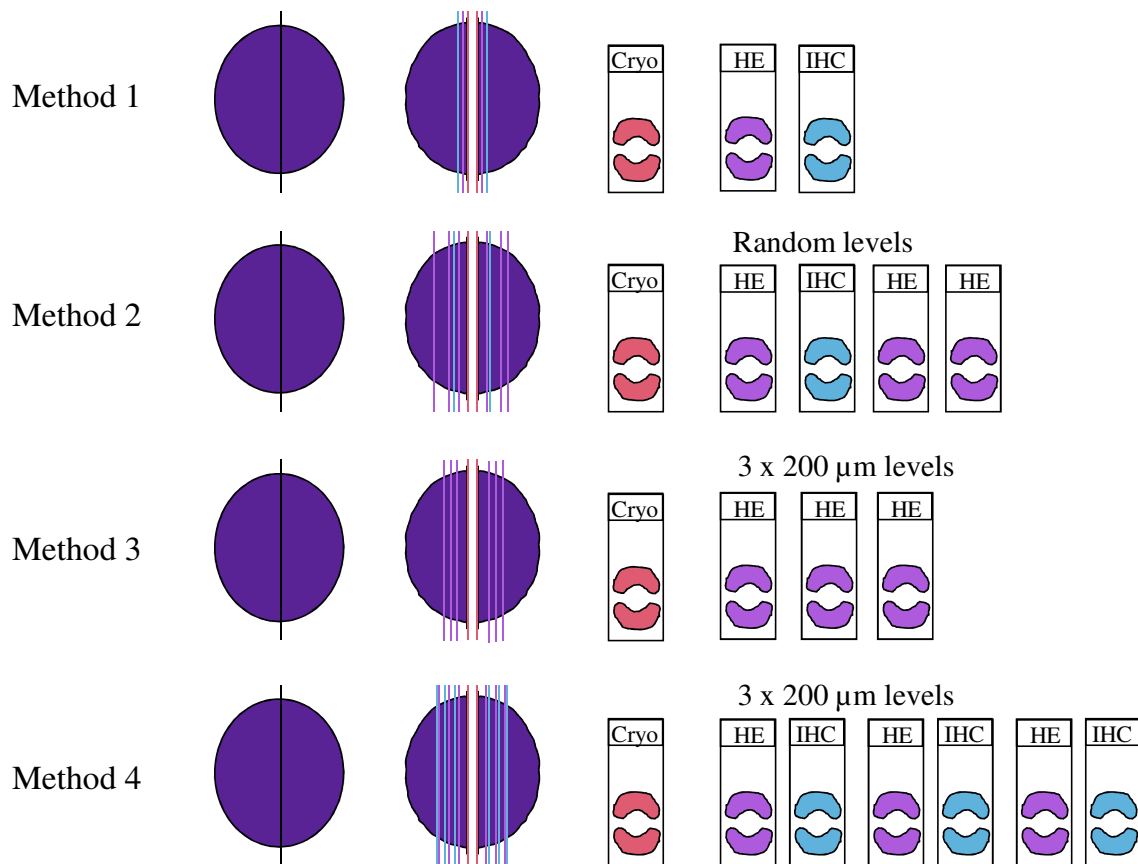
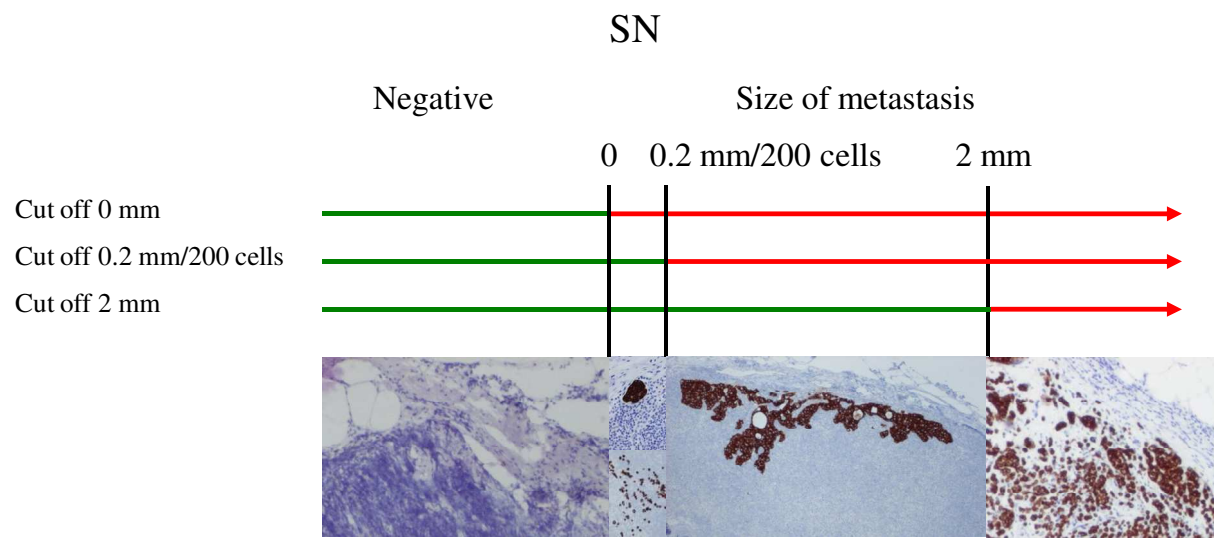
Figure 2. Method of sentinel node work up in the four different periods

Figure 3. Different cut offs of metastatic deposits used in sentinel nodes. The status of the sentinel node is determined at the level with most deposits/tumour cells.



Review

Analysis of sentinel node biopsy – a single institution experience supporting the use of serial sectioning and immunohistochemistry for detection of micrometastases by comparing four different histopathologic laboratory protocols

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Abstract

Aims. Detecting micrometastases (>0.2 mm and ≤ 2 mm/ >200 cells) and isolated tumor cells (ITC) (≤ 0.2 mm/ <200 cells) is important for staging of breast cancer patients. The aim of this study was to systematically compare several laboratory protocols used to detect metastases after initial intraoperative frozen section examination.

Methods and results. Four different protocols for the work-up of sentinel lymph nodes (SLN) after frozen sectioning were applied in the routine diagnostic process from 2001-2009. In addition team work with a limited number of laboratory technicians and pathologists handling SLN was introduced in 2008.

The present study shows overall significantly more node positive patients in the period where team work and intensive step sections including immunohistochemistry (IHC) was used ($p=0.01$). This resulted in 13% more patients found to have ITC and micrometastases compared with the result from a time period when only step sections were done. No rise in false negative frozen sections was seen. The increase in the detection of metastases was economically neutral because the same work was just performed by fewer people.

Conclusions. Future guidelines for pathologic work-up of sentinel nodes in women with breast cancer might include team work and IHC if frozen sections are used intraoperatively.

Key words: Sentinel node biopsy, breast cancer, immunohistochemistry, micrometastases, ITC

Abbreviations: ALND, axillary lymph node dissection; FFPE, formalin fixed paraffin embedded; HE, haematoxylin and eosin; IHC, immunohistochemistry; ITC, isolated tumor cells; SLN, sentinel lymph node

Introduction

During the last 10 years sentinel lymph node (SLN) biopsy has gained acceptance as the routine method for staging the ipsilateral axilla in women with early breast cancer. Preoperative procedures including the injection of radioactive colloidal substance and/or blue dye, scintigrammes and gammaprobe handling and the intraoperative surgical technique is standardized and largely agreed upon. In the beginning of the 2000's Swedish guidelines for surgeons included SLN biopsy as standard procedure after a period of validation with respect to the false negative rate of axillary staging¹.

On the other hand the work-up of sentinel nodes in the laboratory is still under debate. The 6th edition of the pTNM classification introduced the concept of ITC in 2002^{2, 3} and stated that these should be considered as node negative for staging and treatment purposes. The 7th edition^{4, 5} clarifies the concept and recommends finding all metastases >2 mm by macroscopically slicing SLN in 2 mm sections. It is also recommended that the accuracy of the subsequent formalin fixed paraffin embedded (FFPE) protocol in finding micrometastases and ITC be defined. In 2006 Swedish guidelines for SLN handling largely was adapted from the European guidelines⁶.

The risk of upstaging axillary status by a more thorough examination of SLNs has been considered an issue to be avoided because clinical data was based on only one histologic slice from each axillary lymph node⁷. Nevertheless, a recent paper on women with early stage breast cancer who underwent sentinel node biopsy reports that isolated tumor cells or micrometastases in regional lymph nodes were associated with a decreased disease free survival in women without adjuvant therapy and that disease free survival was improved in this patients group if they received adjuvant therapy⁸. Another review concludes that patients with metastases of 2 mm or smaller in axillary lymph nodes examined by a single HE section have a worse outcome compared to node negative patients⁹, but for patients treated by SLN the data is still inconclusive.

A further review of SLN micrometastases concludes that SLN micrometastases are likely to represent an incremental detriment to prognosis and increased risk of non-SLN involvement¹⁰. In addition metastases uncovered in formerly negative axillary lymph node dissection (ALND) with a SLN protocol including IHC have prognostic significance in breast cancer patients¹¹. The Swedish national figures show a worse outcome of patients with micrometastases in SLN compared to node negative patients¹², but other reports have shown no difference in survival between SLN negative and those with micrometastases¹³.

These findings emphasize the need to optimize the accuracy of SLN pathology. At the same time, whatever method is chosen must not dramatically increase the cost and time spent for analysis. In histopathological practice the technical quality of HE slides prepared from formerly frozen material occasionally can be inferior to material which was not frozen; freeze artifacts still show in the paraffin sections. This study address these problems by comparing the results of routine histopathologic work-up over four different time periods at the same institution using different protocols, all including frozen sections but with diverse methods for step sectioning and use of immunohistochemistry. In the last time period, laboratory organization was changed such that fewer laboratory technicians and pathologists were involved in frozen section diagnosis and in the subsequent work-up of the SLN.

Material and methods

The study was conducted on the consecutive series of the 1576 women with a first primary operable breast cancer treated at the University Hospital of Lund from January 1, 2001 to December 31, 2009 (Figure 1). Excluded were 25 women diagnosed in October 2008 because these women were analyzed by SLN using method 4 but before the laboratory was reorganized into work teams. Patients were selected by extraction from the data files of the pathologic

department diagnosed with topography code for breast in the SNOMED system. By manual examination of the files patients with a previous history in the pathology files of invasive carcinoma in the breasts were excluded as were patients with previous malignancy elsewhere. Men with breast cancer, benign cases and cases with in situ carcinoma were also excluded. A total of 27 women with synchronous bilateral breast cancer were included using the side showing the worse axillary status. The other side was excluded in these women.

The surgical procedure of SLN biopsy was preceded by subdermal injection with ^{99}Tc labeled nanocolloid followed by a scintigramme. Injection of 0.5 ml of Patent V Blue was performed intradermally after anesthesia in the operating room. Any node that was hot, blue, or palpable was considered a SLN and removed. The surgical procedure, instrument and surgical staff did not differ in the cohort period.

As a general rule frozen section examination was undertaken in up to four nodes with following examination of FFPE sections stained with HE and/or IHC with pan-anticytokeratins according to different protocols. Throughout the study period four different protocols of routine histopathological work-up of SLN were used (Figure 2). Generally SLNs of 4 mm or more were cut through the longitudinal axis and both halves examined. Smaller nodes were examined in toto. All specimens were examined by one or two frozen sections. After formalin fixation and paraffin embedding the protocols differed. During the first period the sections were examined by one paraffin section and by immunohistochemical analysis with anticytokeratin in frozen negative cases (n=287) (Method 1). In the second period the SLNs were analyzed by step sectioning at three levels randomly chosen and by immunohistochemistry at the first level in frozen negative cases (n=148) (Method 2). The third period introduced step sectioning of SLNs at three fixed levels of 0.2 mm and use of immunohistochemistry only in suspicious cases (n=474) (Method 3). The last period included step sectioning of SLNs at three fixed levels of 0.2 mm with immunohistochemistry at every level in cases which were frozen negative or had a

metastasis of less than 2 mm on the frozen sections (n=186). During the last period the laboratory work was done by fewer, specially trained technicians and fewer pathologists. The number of laboratory technicians was concentrated from 12 to 5 and pathologists were decreased from about 14 to about 3. The laboratory technicians were specially trained in doing frozen sections on SLNs with fatty tissue around and inside the lymph nodes.

A review of the slides of all cases with positive SLN was undertaken and the 7th edition of the AJCC and UICC classification was applied^{4, 14}. Women without detectable tumor cells in the nodes were designated node negative equal to cut off zero. Using a cut off of 0.2 mm women were staged as negative when they had no metastases or had ITC of 0.2 mm and smaller or with less than 200 tumor cells. Patients with micrometastases had metastases >0.2 mm or more than 200 tumor cells but not measuring more than 2.0 mm. A cut off of 2.0 mm separated women with metastases larger than 2.0 mm from all those with smaller ones.

The size of the metastatic deposits was determined on the slide where it was largest. In 2004 the 6th edition of the AJCC was implemented and women with deposits of 0.2 mm or smaller or with ITC were hereafter not routinely offered axillary dissection. All other women with metastasis larger than 0.2 mm in at least one SLN were offered axillary dissection either in combination with the surgery for breast cancer due to a positive frozen section or in a second operative procedure as a result of a positive SLN on FFPE sections and thereby false negative frozen section of the SLN.

From 2001 to Oct 2008 axillary lymph nodes were handled by isolation of the lymph nodes in formalin fixed surgical specimens and embedding one slice from each node often including several nodes in each block. From Oct 2008 and thereafter axillary lymph nodes were bisected and macroscopically negative nodes were entirely embedded in separate blocks. One slice sufficed for macroscopically metastatic nodes. One HE section from each block was examined and in difficult cases IHC was applied. Details of age of the patients, tumor size, histological

grade, estrogen- and progesterone receptor status were recorded from the pathology reports. Histologic grade was assessed using the Nottingham modification¹⁵ of the Scharff, Bloom and Richardson grading system¹⁶. Estrogen and progesterone receptor status was determined by IHC assay. A mammography screening program was conducted in the local area since 1989 and women aged 45 to 74 were invited every 18-24 months.

Statistics

Differences between proportions were examined by chi-square test and $p < 0.05$ was considered significant. Differences between continuous variables were analyzed by the Kruskal-Wallis test.

Results

Details of patients and tumor features are listed in Table 1. Tumor size did not differ between the four different study periods. About one fourth of the patients were 70 years or older and the median age of the patients was slightly increasing throughout the study. Histological grade was more often grade 3 in the last period and fewer patients had a diagnosis of lobular carcinoma in the later part of the study period. Receptor status did not differ among the study periods.

Of 1576 patients included in the study 70% had SLN biopsy, 20% were treated by primary ALND and 10% were allocated to other protocols without adequate determination of axillary status at the time of surgery (Figure 1). Frequency of patients treated by SLN biopsy was 63, 68, 69, and 86% in the four periods with different SLN examination. Using a cut off of zero for classifying SLNs with negative status, significantly more patients were node positive in the last period both among women treated by SLN and among all women ($p = 0.01$) (Table 2a). When the cut off 0.2 mm/ < 200 cells was applied for negative status the difference was only borderline significant ($p = 0.07$) (Table 2b). IHC was used in 72, 74, 21, and 92% of the cases treated by SLN

biopsy. The median number of analyzed SLN per patient was 2 and did not differ between the periods.

Concentrating on the SLN totally negative and those with ITC of ≤ 0.2 mm/ <200 cells or micrometastases of 2 mm or smaller some striking differences appeared. In the last period using method 4 with step sections at fixed intervals and IHC at every level, where frozen sections and step sections were performed by trained laboratory technicians, significant more patients were diagnosed with ITC or micrometastases in the SLNs ($p < 0.01$). The number of patients with ITC and micrometastases was 9% higher when method 4 was used compared with method 1 and 2 using either just one HE section followed by IHC or one HE section and IHC followed by random step sections. Teamwork and intensive IHC in method 4 resulted in 13% more patients with ITC and micrometastases compared with the result of method 3 with step sections at fixed intervals. Frequency of women with ITC and micrometastases compared with those with macrometastases did not differ between the study periods.

Frozen sections were reported positive to the surgeons if a metastasis of larger than 0.2 mm was encountered. The false negative rate of the frozen section technique was similar during the period for method 4 with intensive IHC of step sections in FFPE slides and team work and during the period for method 1 with IHC but without step sections. When method 2 and 3 without team work was used the false negative rate of frozen sections was higher.

Discussion

The present study shows overall more node positive patients in the period where method 4 was used including step sections at fixed intervals and intense use of IHC in hands of specially trained personal. At the same time more women were diagnosed with ITC and micrometastases in their SLNs without increasing the false negative rate of frozen sections. These results were

accomplished by a relatively simple change in laboratory organization. Instead of all the laboratory workers handling all the material which came to the department, groups of workers were formed which handled only certain types of specimens, based on organ groups. Thus the number of assistants involved in the process of sentinel nodes was smaller which increased their expertise in this area of laboratory work.

The introduction of team work also made possible a shift from the use of a metal stretch plate in the process of moving tissue sections to glass slides. A temperate water bath was used instead. This improved the quality of the HE section to a degree that the frequency of patient with histologic grade 3 was for the first time within the limit of recommended value in Sweden. The shift to fewer women diagnosed with lobular carcinoma was caused by the introduction of E-cadherin IHC and possibly by a decreased use of hormone replacement therapy which has been seen in the population in recent years. The age of the patients is steadily increasing probably due to more active treatment of the elderly.

By applying step sections and/or IHC to negative regional lymph nodes, the use of step sections increases the harvest of small metastases by about 7-13% and the addition of IHC will find about 15-30% more cases with small metastases^{11, 17}. Our results and the conclusion of results of another report¹⁸ implies that smaller metastases will be missed if step sections and IHC of SLNs is left out. The varying false negative rate of metastasis obtained by frozen sections among the study periods indicates that small metastasis might be missed. The sensitivity of frozen section methodology is within the range found in other studies^{19, 20}.

From the surgical point of view discussion is ongoing as to whether patients with micrometastases benefit from subsequent ALND because the frequency of local recurrence is extremely low^{21, 22}. However systemic therapy might improve survival even in patients with tumor deposits even as small as ITC⁸.

A consensus with regard to an optimal pathologic work-up of SLNs is hard to achieve because viewpoints differ about the objectives of the examination. A more modest work-up favors attempts to avoid stage migration and acknowledges the possibility of benign transportation of epithelial tissue to the SLNs. Up-staging of breast cancer patients implies that a more thorough method of SLN examination results in more positive cases²³. The clinical implication of the additional metastases is not determined in the clinical trials where use of adjuvant systemic therapy is applied. Another caution against therapeutic action as a consequence of small metastatic deposits only detectable with IHC raise the possibility of benign transportation to the SLN²⁴ after needle diagnostic procedures or manipulation of the breast²⁵ and even malignant cells might be displaced to the SLNs without being metastatic in nature. Lately benign elements have been identified as elements of papillomas from the breast²⁶.

Different models of handling of SLNs have been presented^{27, 28} and all models shows that using an acceptable number of steps and sections in routine work-up, results in missed deposits of small metastases. Furthermore, pathologists do not agree on what constitutes ITC²⁹. Therefore the 7th AJCC staging recommends slicing the SLN in 2 mm intervals and stating the sensitivity of the step section method used to detect small metastases afterwards. In addition clarifications of the concept of ITC and/or less than 200 cells have been reported⁵. The lymph node slices of 2 mm is obtained in practice by dividing the SLN by the longitudinal axis and this will always be an approximation since lymph nodes are of different size. One survey of routine practice in Europe revealed that 73% of participating institutions did IHC on SLNs³⁰. More intensive protocols using IHC reveal more metastasis²⁸ but method 4 in this study seems to combine an acceptable level of work with an acceptable cost.

Both AJCC and European guidelines recommend step microscopic sections of SLN but both determine the size of the largest metastases on one section in two dimensions and do not take into account the third dimension that emerges from the step sectioning. Most important is ITC

appearing in the deepest section. If another level is not examined this ITC might in fact be a macrometastasis. When the SLNs are step sectioned at fixed intervals the size of the metastases can be determined in three dimensions but no guidelines include size in the third dimension and this might underestimate the actual size of the metastasis.

Intraoperative procedures are considered optional and so far no guidelines deals with the fact that frozen section methodology also may introduce freeze artifacts in the FFPE sections. This makes routine examination of HE sections more difficult and the pathologist might miss metastases that easily would have been seen in sections from tissue which had not been frozen. In order to minimize these artifacts the training of the laboratory assistants is crucially important. The preparation of frozen sections is meticulous handwork and must be handled by specially trained laboratory assistants with have the opportunity to do the work often. The present work in the first three periods was carried out at a time when all the laboratory assistants prepared frozen sections as did most of the pathologists. In the last period team work was introduced and a larger fraction of SLN patients were diagnosed as positive with a larger fraction having smaller metastases. There was still an acceptable level of false negative frozen section examinations, however.

This study has some draw backs. As it is a single institution study design, the different methods compared are based on examination of SLN in successive time intervals and comparison of the methods was not randomized during a fixed time interval. Also the frequency of patients treated by SLN was increasing during the study period, and thereby the frequency of positive nodes in the SLN population would be expected to increase. Therefore data from all breast cancer patients in the institution is considered and because the increase is general in the entire study population the increase in node positivity among patients with SLN is interpreted a being method related and not a result of selection bias.

If the handling of SLN is reserved to a small specialized group of laboratory technicians and pathologists it may be expected that the rate of false positive frozen sections be decreased. In fact

one of the three false positive cases in the present series were due to the presence of benign nevus in the lymph node capsule, a condition of which not all pathologist are aware³¹.

In conclusion future guidelines for pathologic work-up of sentinel nodes in women with breast cancer might include the recommendation of team work and IHC if frozen sections are to be used intraoperatively. This study shows that a method with team work and intensive step sections including IHC detected 13% more women with ITC or micrometastases.

For Peer Review

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Table 1. Patients and tumor characteristics of all women.

	Method 1 n=457	Method 2 n=219	Method 3 n=683	Method 4 n=217	p
Tumor size median (range)	17 (1-80)	18 (0-90)	16 (0-90)	17,5 (0-80)	0.40
Age median (range)	58 (23-99)	59 (30-91)	61 (27-94)	64 (25-91)	<0.01
	n (%)	n (%)	n (%)	n (%)	
Histologic grade					
1	106 (23)	57 (26)	154 (23)	48 (22)	0.003
2	256 (56)	106 (48)	360 (53)	92 (43)	
3	84 (18)	47 (21)	156 (23)	72 (33)	
No grade	11 (2)	9 (4)	13 (2)	5 (2)	
Histologic type					
Ductal	353 (77)	170 (78)	574 (84)	182 (84)	0.04
Lobular	77 (17)	34 (16)	74 (11)	22 (10)	
Other type	27 (6)	15 (7)	35 (5)	13 (6)	
Estrogen receptor					
Negative	83 (18)	43 (20)	101 (15)	30 (14)	0.18
Positive	364 (80)	174 (79)	575 (84)	185 (85)	
Unknown	10 (2)	2 (1)	7 (1)	2 (1)	
Progesterone receptor					
Negative	168 (37)	75 (34)	221 (32)	61 (28)	0.14
Positive	279 (61)	142 (65)	455 (67)	154 (71)	
Unknown	10 (2)	2 (1)	7 (1)	2 (1)	

Post operative tumor size was zero in a few patients included in a study using laser therapy³².

Table 2.a. Frequency of positive nodes in women primarily treated by SLN, ALND and other during the different methodologies of SLN handling, with a cut off for metastases at zero.

	Method 1		Method 2		Method 3		Method 4		
Primary surgery	n	n Npos (%)	n	n Npos (%)	N	n Npos (%)	n	n Npos (%)	
SLN cut off 0	287	123 (43)	148	52 (35)	474	168 (35)	186	92 (49)	p<0.01
ALND	117	68 (58)	51	28 (55)	138	84 (61)	16	14 (88)	p=0.12
Other	53	46 (87)	20	16 (80)	71	57 (80)	15	15 (100)	p=0.53
Total cut off 0	457	237 (52)	219	96 (44)	683	309 (45)	217	121 (56)	p=0.01

* Other se figure 1.

Table 2.b. Frequency of positive nodes in women primarily treated by SLN, ALND and other during the different methodologies of SLN handling, with a cut off for metastases at 0.2 mm/200 cells.

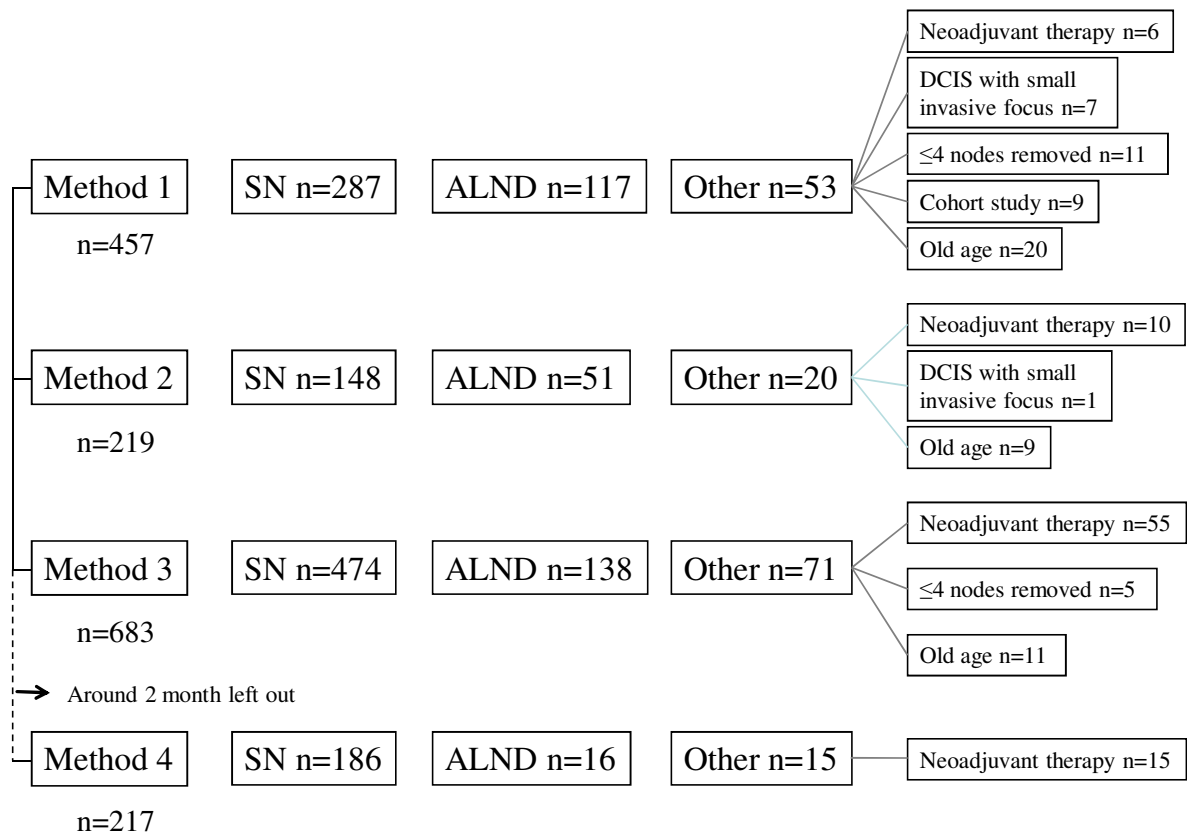
	Method 1		Method 2		Method 3		Method 4		
Primary surgery	n	n Npos (%)	n	n Npos (%)	n	n Npos (%)	n	n Npos (%)	
SLN cut off 0.2 mm/ <200 cells	287	112 (39)	148	45 (30)	474	154 (32)	186	76 (41)	p=0.06
Total cut off 0.2 mm/ 200 cells	457	226 (49)	219	89 (41)	683	295 (43)	217	105 (48)	p=0.07

Table 3. Frequency of isolated tumor cells, micro-, and macrometastases in the intervals with different methodology of SLN handling.

	Method 1 n (%)	Method 2 n (%)	Method 3 n (%)	Method 4 n (%)	
SLN Negative	165 (57)	97 (66)	311 (66)	94 (51)	p=0.01
SLN ITC ≤ 0.2 mm/<200 cells	11 (4)	7 (5)	14 (3)	16 (9)	
SLN positive > 0.2 and ≤ 2.0 mm	25 (9)	14 (9)	36 (8)	18 (10)	
SLN positive > 2.0 mm	86 (30)	30 (20)	113 (24)	58 (31)	
Total	287	148	478	186	

Table 4. False negative results of frozen sections of SLN compared with FFPE sections using cut off 0.2 mm.

	Method 1	Method 2	Method 3	Method 4
	n (%)	n (%)	n (%)	n (%)
SLN total negative	164 (57)	94 (64)	308 (65)	91 (49)
ITC ≤ 0.2 mm/200 cells	11 (4)	7 (5)	14 (3)	15 (8)
positive > 0.2 mm/200 cells				
positive > 0.2 and ≤ 2 mm	12 (4)	4 (3)	10 (2)	4 (2)
positive >2 mm	73 (25)	25 (17)	96 (20)	49 (26)
Frozen false negative met > 0.2 mm				
positive > 0.2 and ≤ 2 mm	13 (5)	9 (6)	25 (5)	13 (7)
positive >2 mm	13 (5)	5 (3)	15 (3)	3 (2)
Frozen false positive	1 (0)	2 (1)	0	0
No frozen section	0	2 (1)	6 (1)	11 (6)
	287	148	474	186
False negative rate	0,23	0,33	0,27	0,23
Accuracy	0,91	0,89	0,92	0,91

Figure 1. Composition of material.

Method 1 was used January 2001 to September 2003

Method 2 was used October 2003 to December 2004

Method 3 was used January 2005 to September 2008

Method 4 was used November 2008 to December 2009

In the first period a cohort study included women with early breast cancer with an expected risk of loco-regional recurrence of less than 1% in a protocol without axillary staging (N=9).

Figure 2. Method of sentinel node work up in the four different periods

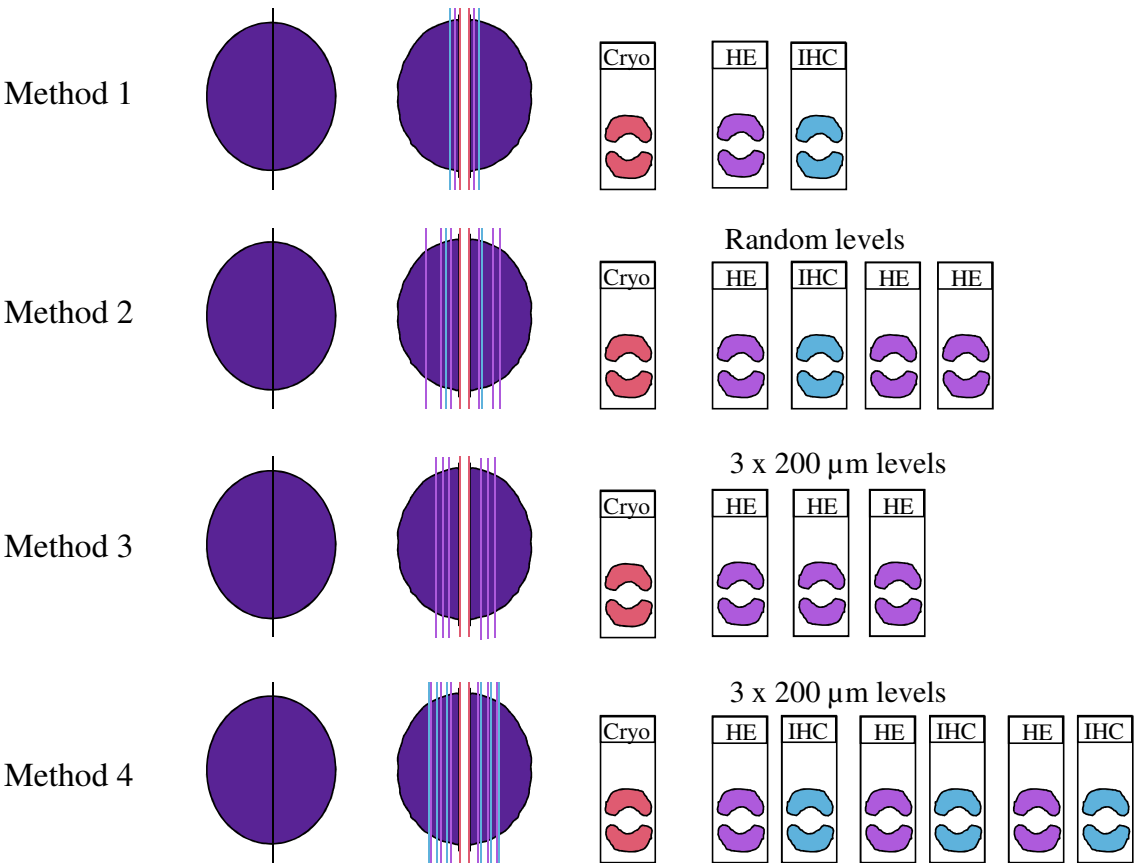


Figure 3. Different cut off of metastatic level used in sentinel nodes