Staging of women with breast cancer after introduction of sentinel node guided axillary dissection

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- Tvedskov TF, Jensen MB, Balslev E, Ejlertsen B, Kroman N. Stage migration after introduction of sentinel lymph node dissection in breast cancer treatment in Denmark: A nationwide study. Eur J Cancer 2011 Apr; 47(6):872-8
- Tvedskov TF, Jensen MB, Lisse IM, Ejlertsen B, Balslev E, Kroman N. High risk of NSN metastases in a group of breast cancer patients with micrometastases in the sentinel node. Int J Cancer 2012, feb 18 (Epub ahead of print)
- Tvedskov TF, Bartels A, Jensen MB, Paachburg B, Kroman N, Balslev E, Brünner N. Evaluating TIMP-1, Ki67 and HER2 as markers for non-sentinel lymph node metastases in breast cancer patients with micrometastases to the sentinel lymph node. APMIS 2011 Dec;119(12):844-52

BACKGROUND

Breast cancer

Breast cancer is the most common cancer in women worldwide. In Europe the disease accounts for 30 % of all cancer cases in women(1). The breast cancer incidence has until recent years been continuously increasing(2) and every year more than 4500 new cases of breast cancer are registered in the Danish Cancer register(3) (Fig 1). The surgical treatment of breast cancer has changed over the years and has become increasingly more conservative. In 1882 William Halsted performed his first radical mastectomy with en bloc removal of the entire breast, the pectoral muscles and the regional lymph nodes(4). This classic operation, modified by Patey et al. in 1948(5), became widely accepted as the standard surgical treatment of breast cancer during decades. In 1973 a randomized study comparing radical mastectomy to breast conserving surgery was initiated by Umberto Veronesi at the Milan Cancer Institute.

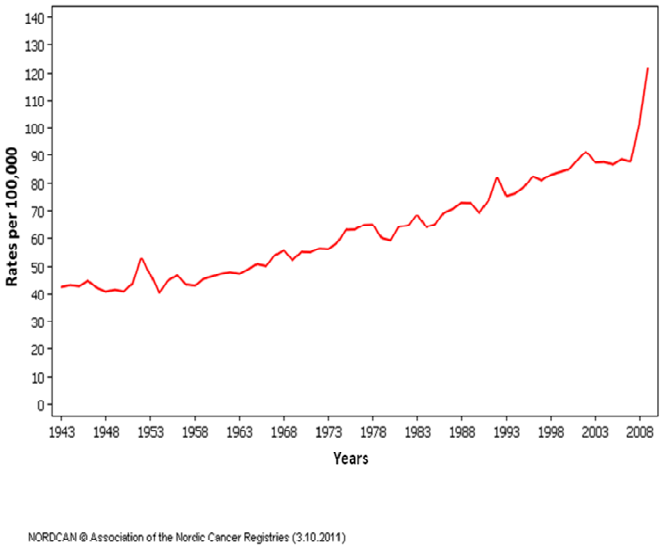

Figure 1: Breast Cancer incidence (ASR) for Danish women from 1943 to 2008.

No difference in survival was seen among women who underwent breast conserving surgery compared to mastectomy(6). Likewise, the randomized National Surgical Adjuvant Bowel and Breast Project (NSABP) B-06 clinical trial, initiated by Bernard Fisher in 1976, showed no difference in survival between patients treated by mastectomy and breast conserving surgery(7). In Denmark, these results were confirmed by Blichert-Toft in the randomized DBCG-82TM trial in 1983-1989(8). Accordingly, breast conserving surgery became the treatment of choice for small breast cancers. By the introduction of sentinel lymph node dissection (SLND) in 1994(9) the surgical treatment of breast cancer continued to move away from the Halstedian paradigm.

Axillary staging

The prognosis of women with primary breast cancer is estimated based on several prognostic factors. These include age at diagnosis, tumor characteristics such as tumor size, malignancy grade and hormonal sensitivity, and presence of metastases in the axillary lymph nodes(10). Axillary nodal status is still the most important prognostic factor. Axillary lymph node dissection (ALND), where about 2/3 of the axillary lymph nodes are removed and examined for metastatic spread, has previously been the standard procedure for staging of the axilla. In addition to staging

of the disease, ALND is an important part of local disease control and may improve survival(11). Results from the randomized NSABP B-04 clinical trial demonstrated a nearly 20% axillary recurrence rate in breast cancer patients not treated by ALND(12). It has furthermore been shown that the risk of relapse increases if less than 10 lymph nodes are removed by ALND, probably because metastatic lymph nodes are left in the axilla(13;14). However, about half of all women with clinically detected breast cancer do not have involvement of axillary lymph nodes(15). Women without nodal involvement have no benefit from ALND which is associated with considerable risk of extensive arm morbidity such as pain, numbness, swelling and reduced mobility(16;17). Therefore, SLND has gradually replaced ALND as standard procedure for staging of the axilla(18).

The sentinel node procedure

The sentinel node procedure was first described in parotid cancer by Gould et al. in 1960(19) and in penile cancer by Cabanas in 1977(20) but not until 1992, when the use of blue dye(21) and isotopes(22) was described for peroperative identification of sentinel nodes, the procedure came into clinical use. In 1994 Armando Giuliano (Fig 2) described SLND as a safe procedure for staging of the axilla in breast cancer patients(9;23). These results were confirmed by Umberto Veronesi (Fig 2) in 2003(24). SLND was introduced in Denmark in 1997 and the procedure was completely implemented in all Danish departments of breast surgery by the end of 2004(25). Today, SLND is standard procedure in Denmark for axillary staging of women with unifocal breast cancers without verified lymph node metastases and without history of surgery in the upper lateral quadrant of the breast(18).

In Denmark, preoperative axillary sonography is performed to identify lymph node metastases. In case of suspicious lymph nodes by sonography, fine needle aspiration cytology (FNAC) is performed. If lymph node metastases are verified by FNAC SLND is redundant and an immediate ALND is offered. A precise preoperative image of the axilla will reduce the number of SLND(26;27).

Figure 2: Armando E. Giuliano (left) and Umberto Veronesi (right)

Surgery

The sentinel nodes are the first lymph nodes receiving lymph from the tumor. In SLND the nodes are identified by radioactive tracer and/or blue dye. In brief the radioactive tracer, 99mTc labeled NanoColl, is injected subareolar and the blue dye, Patent Blue, is injected at the tumor site preoperatively. The tracers drain to the sentinel nodes. Peroperatively, the sentinel nodes are identified guided by the blue staining and by using a hand-held gamma probe, and subsequently removed and send for histopathological examination for metastatic spread. Only in case of metastatic spread to sentinel nodes, patients are recommended an additional ALND, either at the same operation or as a second procedure(28). Accordingly, SLND can accurately stage the axilla by removing only a few lymph nodes(23;24) and as a consequence it causes limited arm morbidity compared to ALND(16;17). Hence, the main purpose of introducing SLND has been to reduce the risk of arm morbidity in patients without metastatic spread to the lymph nodes.

Pathology

Sentinel nodes removed by SLND are examined peroperatively on frozen sections as well as postoperatively on conventional histopathological sections. Sentinel nodes less than 4 mm are embedded without prior section. Sentinel nodes bigger than 4 mm are bisected through the longitudinal axis and larger nodes are cut in slices. On frozen sections, two levels are made for haematoxylin-eosin (HE) staining from every sentinel node. Peroperative frozen sections allow immediate ALND when metastases are found in the sentinel nodes. A supplementary cytokeratin staining may be performed to optimize the identification of metastasis peroperatively and spare patients for a two-stage procedure(29;30), but it is not compulsory. The remaining tissue from the sentinel nodes is formalin fixed, paraffin embedded (FFPE) and HE-stained for standard microscopy. If no metastases are found by HE staining, a section is made for immunohistochemical (IHC) cytokeratin staining and after a 0.5 mm interval another two sections are made for HE and cytokeratin staining.

Metastases in the sentinel node are defined as macrometastases if the diameter is above 2 mm, micrometastases are defined as deposits of tumor cells with a diameter between 0,2 and 2 mm or between 10 and 100 tumor cells (Fig 3), while isolated tumor cells (ITC) are defined as deposits of cells less than 0,2 mm or less than ten tumor cells(31) (Fig 4). Metastases are staged according to the TMN system, where micrometastases are staged as pN1mi and ITC as pN0 (i+).

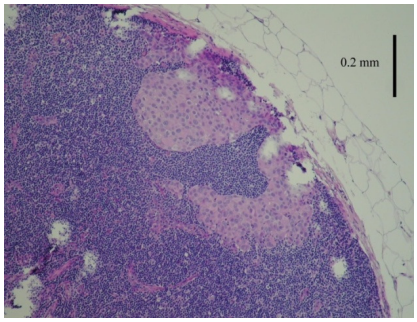

Figure 3: Micrometastasis in the sentinel node.

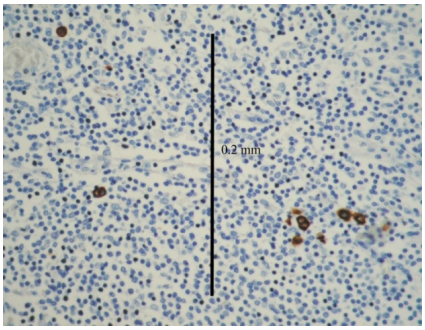

Figure 4: ITC in the sentinel node

Stage migration

Lymph nodes removed by ALND are traditionally examined by standard microscopy of a few histological sections after bisectioning and HE-staining(18). More extensive histopathological examinations using IHC staining on multi-sections of all removed lymph nodes have identified metastasis in 9-31% of cases considered negative by standard examination(32-34). These time-consuming methods are not used routinely in ALND, but the introduction of SLND, where on average only two lymph nodes are removed(18), has made these extensive histopathological examinations possible. As a result more metastases, especially more small metastases, are found(35-38). Patients diagnosed with these metastases are classified in a more advanced stage of the disease. This change in staging is called stage migration(39;40). Some studies have investigated the magnitude of stage migration after introduction of SLND in breast cancer treatment(23;41-48) but only three studies have been population-based(42-44). Most studies do not include the entire period of introduction of SLND, and accordingly the complete size of stage migration caused by SLND is not known. Furthermore, the consequences of this stage migration on the proportion of patients offered adjuvant treatment have not been systematically investigated and remain unknown.

ALND in sentinel node positive patients

The surgical consequences of stage migration with identification of more small metastases may reduce the advantage of SLND due to unnecessary ALND's, because the benefit from ALND in patients with only small metastases in the sentinel node is questionable(49-51). Some studies show that patients with micrometastases or ITC in the sentinel node do not have a worse outcome if ALND is omitted(52-55) while others have shown that ALND is of prognostic importance(56;57), but studies are generally small and with limited follow-up and lack of multivariate analysis. In the NSABP B-32 phase III clinical trial, 3989 breast cancer patients without metastases in the sentinel nodes on HE examination were randomized to either additional ALND or no further axillary surgery. A secondary aim was to evaluate whether patients with metastases identified only on IHC examination and not on HE examination of the sentinel node (occult metastases), had a worse outcome than patients without metastases(53). The impact of ALND was not directly addressed, but a small difference in outcome was found in patients with and without occult metastases if only SLND was performed. In contrast, a recent study, based on the American College of Surgeons Oncology Group Z0010 trial, could not show any difference in survival in 349 breast cancer patients with occult metastases in the sentinel node compared to patients with a negative sentinel node(58). In another recent study, the American College of Surgeons Oncology Group Z0011 trial, no difference in axillary recurrence and survival was found at a median follow-up of 6.3 years if breast cancer patients with positive sentinel nodes were randomized to either ALND or no ALND. About 40% of the included patients had micrometastases in the sentinel node(59;60), 95% received adjuvant systemic treatment and all patients underwent whole breast irradiation. This would to some extend result in irradiation of the lower part of the axilla, which can have affected the outcome. Furthermore, the study was closed early after including less than half of the planned number of patients because of low accrual. Hence, the results cannot be transformed into any breast cancer patient with a positive sentinel node.

Despite the limitations of these earlier studies, they suggest that not all sentinel node positive patients will benefit from an ALND, but will run the risk of considerable arm morbidity(16;17). Accordingly, a tool is needed to select patients who will benefit from an ALND. Metastatic spread beyond the sentinel node can be considered as surrogate end point for axillary recurrence. 38 to 87% of patients with macrometastases in the sentinel node will have further metastatic spread to other lymph nodes(61-63). In case of micrometastases and ITC metaanalyses have shown that only 20%(64) and 12%(65) respectively, will have metastatic spread to non-sentinel node (NSN). Thus, the majority of these patients will probably not benefit from ALND. It would be advantageous to identify these patients in advance to avoid unnecessary ALND.

Risk factors for non-sentinel node metastases

Several authors have tried to predict further metastatic spread to NSN in sentinel node positive patients. Most studies have investigated patients with mainly macrometastases in the sentinel node and several risk factors for NSN metastases have been identified(66-75).

Investigations on patients with only micrometastases or studies on mixed populations of patients with either micrometastases or ITC in the sentinel node are few and based on a limited number of patients and no studies exist where patients with ITC in the

Table 1: Predictive markers for NSN metastases in studies on breast cancer patients with micrometastases or ITC in the sentinel node, where ● is indicating significant markers and o is indicating investigated markers.

Studies	No. of patients		Patient and tumor characteristics										Sentinel node characteristics		
	Micro-metastases	ITC	Age	Tumor size	LVI	Grade	Type	Location in breast	Multi-focality	Hormone receptor status	HER 2 status	Ki67/ mitotic index	Location in SN	Proportion or number of pos SN	Size of metastasis
Den Bakker et al(86)	32			●		●									
Leidenius et al(87)	60	24	o	o	o	o	o	o						●	
Houvenaeghel et al(77)	445	251		●	●	o	●			o					
Cyr et al(78)	41	14	o	●	o	o	o			o	o				
Kumar et al(79)	254	251	o	o	●	o	o		o	o				o	
Carvalho et al(85)	25				o		o	●	o	o	o				
Carcoforo et al(80)	58			●	●		o			o	o	●			
Kraut et al(81)	43	19		o	●	o								o	
Li et al(82)	37	31	o	●	o	o	o		o	o			●		
Schrenk et al83	78	44	o	o	●	o	o	o		o			o	o	●
Gipponi et al(84)	116		o	●	●	o	o					o		o	o
Meretoja et al(76)	278	206	o	●	o	o	o	o	●	o	o	o		o	o

sentinel node are investigated separately. Several traditional prognostic markers like age at diagnosis, histology type, malignancy grade, tumor size, lymphovascular invasion and hormone receptor status, have been investigated, but only tumor size and lymphovascular invasion have been shown to be associated with the risk of NSN metastases in more than one study(76-84) (Table 1). Single studies have found an association between NSN metastases and multifocality, tumors located in the upper lateral quadrant of the breast, histology type and malignancy grade(76;77;85;86).

Regarding characteristics of the sentinel node, the proportion of positive sentinel nodes has been shown to be associated with the risk of NSN metastases(63;87). Furthermore, it has been shown that NSN metastases are more common if metastases are located in the parenchyma of the sentinel node compared to the capsula or sinus(82;88;89), and if micrometastases are larger than 1 mm(83;90).

Extracapsular extension has been shown to be associated with NSN metastases, when macrometastases are found in the sentinel node(66;71;73;75), but extracapsular extension is less common in micrometastases and an association with NSN metastases has not been shown(91). Factors associated with NSN metastases when micrometastases or ITC are found in sentinel node are listed in table 1.

New biomarkers and non-sentinel node metastases

Studies using traditional prognostic markers to predict the presence of NSN metastases in patients with micrometastases or ITC in the sentinel node do not appear to provide a clinically applicable method to identify a subgroup of patients where additional ALND can safely be omitted. Therefore, further attempt has been made to identify additional markers for NSN involvement in breast cancer patients.

It is well known that several proteolytic enzyme systems play a role in cancer cell dissemination(92). Translational research has indicated that many of these proteins may serve as prognostic markers in breast cancer(93-95). It could be hypothesized that biomarkers involved in the process of cancer cell dissemination and associated with poor prognosis(96-98) and positive nodal status(99-101) can be used to predict metastatic spread to NSN. Only few studies have investigated the association between new prognostic markers and NSN metastases and even less have included patients with micrometastases or ITC in the sentinel node(68;76;80;85;102;103).

HER2 has been tested for the ability to predict NSN metastases in studies with a mixed group of patients with micro- or macrometastases in the sentinel node(68;102;104-108). None of these studies found HER2 useful in predicting metastatic spread to NSN. Only four studies exclusively included patients with micrometastases or ITC in the sentinel node(76;78;80;85) and no association between HER2 status and NSN status was found.

Three previous studies have tested if the nuclear antigen and proliferation marker, Ki67, could predict the presence of NSN metastases in patients with micrometastases or ITC in the sentinel node and the results are conflicting(76;80;84). Only one study, by Carcoforo et al., found an association between Ki67 and NSN metastases, but the study size was small and no adjustment for confounders was made(80).

Finally, expression of the tumor suppressor genes p16 and p53 has been investigated as possible biomarkers for NSN metastases. P16 expression has been investigated in 54 breast cancer with macrometastases in the sentinel node and were not found useful as an independent marker for NSN metastases(103). P53 expression was investigated in 58 breast cancer patients with micrometastases in the sentinel node. No association was found between p53 expression and the existence of NSN metastases(80). A recent study including 38 patients with micrometastases and 167 patients with macrometastases in the sentinel node tested the association between NSN metastases and several new biomarkers, but the results were disappointing with no association between the tested biomarkers and NSN metastases(68).

Predictive models for non-sentinel node metastases

Based on risk factors for NSN metastases, scoring systems have been developed(109-115) and validated(116-123) for the prediction of further spread beyond the sentinel node, when macrometastases are found in the sentinel node. The existing scoring systems are listed in Table 2. The Breast Cancer Nomogram (BCN) from the Memorial Sloan Kettering Cancer Centre(109) is the most extensively validated of the existing scoring-systems with an area under the receiver operator characteristic curve (AUC) varying from 0.58 to 0.86(117;124). Both the BCN (http://nomograms.mskcc.org/Breast/BreastAdditionalNonSLNMetastasesPage.aspx) and the Stanford Model (https://www3-hrpdcc.stanford.edu/nsln-calculator/) are available online.

Some of the existing scoring systems have been tested on patients with only micrometastases in the sentinel nodes(76;78;117;122;125;126). Unfortunately, they seem not very well adapted and unreliable for use in such populations(76;120;122;126) where they tend to overestimate the risk of NSN metastases(78;125). One study has shown that the Tenon score perform particularly accurate among women with micrometastases with an AUC on 0.81(117), while others found an AUC on only 0.44 for predicting NSN metastases by the Tenon score in a population of patients with micrometastases in the sentinel node(122). None of the scoring systems have been tested in a population of patients with only ITC in the sentinel node.

Table 2: Existing scoring systems for predicting NSN metastases in breast cancer patients with macrometastases in the sentinel node

Author	Center	Scoring system	AUC
Van Zee et al(109)	Memorial Sloan-Kettering Cancer Center, New York	Breast Cancer Nomogram (BCN)	0.76
Hwang et al (113)	M.D.Anderson Cancer Center, Houston	MDA score	-
Barranger et al (114)	Tenon Hospital, Paris	Tenon score	-
Chagpar et al (112)	Louisville University, Louisville	Louisville model	0.68
Kohrt et al (115)	Stanford University, Stanford	Stanford model	0.74
Pal et al (111)	Cambridge University, Cambridge	Cambridge model	0.84
Degnim et al(110)	Mayo Clinic, Rochester	Mayo nomogram	0.77

Today, no model exists for the prediction of NSN metastases based on patients with either only ITC or only micrometastases in the sentinel node. Only two earlier studies have tried to construct a predictive model based on a mixed population of patients with micrometastases and ITC in the sentinel node(76;127). A study by Hoevanaghel et al. included 909 patients and was based on tumor size, lymphovascular invasion, method of detecting sentinel node

metastasis (HE staining vs. IHC) and histology type (mixed or not). The study was only able to identify about 10% of patients with a risk of NSN metastases less than 5%, and the AUC was only 0.66(127). A study by Meretoja et al. included 484 patients and was based on tumor size and multifocality. The AUC was 0.68 and a low risk group with a risk less than 5% was not found(76). Larger studies are needed to definitely clarify whether a clinically applicable risk-model can be developed to support the decision for omitting ALND in breast cancer patients with micrometastases or ITC in the sentinel node.

HYPOTHESIS AND AIM

The introduction of SLND has lead to stage migration, due to an increased identification of patients with lymph node metastases, especially patients with only micrometastases and ITC, but the size and therapeutic consequences of this stage migration is unknown. The optimal surgical management of patients with micrometastases and ITC in the sentinel node is under debate. ALND may be omitted in some of these patients.

Based on the available literature, we hypothesize that the introduction of SLND in Denmark has increased the number of patients identified with micrometastases or ITC in the axilla and that characteristics of patients, primary tumor and sentinel node metastasis together with measurements of new biomarkers can predict metastatic spread to NSN in these patients.

In Denmark, the DBCG database gives the opportunity of a nation-wide study on a data material of a unique size. Based on information from this database the aim of this PhD thesis was:

- Estimation of the size and therapeutic consequences of stage migration after introduction of SLND in breast cancer treatment in Denmark
- Establishment of a clinically reliable model that can identify a group of patients with micrometastases or ITC in the sentinel node where ALND can safely be omitted due to a minimal risk of NSN metastases and a group of patients where ALND should still be offered because of a high risk of NSN metastases
- Investigation of whether the biomarkers, TIMP-1, Ki67 and HER2, can be used to support this model

MATERIAL AND METHODS

The DBCG database

The thesis was based on data from the DBCG database. DBCG runs the largest clinical cancer database in Denmark. Since 1978 DBCG has registered clinical and histopathological data as well as information on treatment and follow-up status on Danish women with breast cancer. Today, the database contains information on more than 80,000 breast cancer patients(128;129). Furthermore, DBCG describes guidelines for all aspects of breast cancer treatment in Denmark (http://www.dbcg.dk/DBCG%20Retningslinier.htm).

The DBCG database contains among other information on tumor size, histology type, malignancy grade, hormone receptor status and lymphovascular invasion. Tumor size is measured in millimeters by the pathologist as the maximum diameter of the invasive component. Histology type is classified by the WHO classification. Malignancy grading is performed using a modified version of the Scarff, Bloom & Richardson's classification(130). Hormone receptor status used in this study were measured by IHC analysis and defined by percentage of stained tumor cells, where patients with staining for either estrogen or progesterone receptors in ≥ 10% of the cells are considered as being hormone receptor positive. Lymphovascular invasion is defined as tumor cells inside an endothelial cell-lined channel.

In 2001 DBCG started using the first protocol for registration of SLND and the procedure was completely implemented in all Danish departments of breast surgery by the end of 2004(25). Today, more than 3500 new SLND are registered in the DBCG database pr year (Fig 5). Furthermore, DBCG describes guidelines for pathology examinations of the sentinel nodes. Virtually all pathology departments in Denmark have applied these standardized guidelines.

The registration of lymph node metastases has changed over the years. Until the end of 2004 micrometastases and ITC were registered together in the database as micrometastases. In 2005 registrational practice changed and since then micrometastases and ITC have been registered in separate groups according to the 6th edition of the staging manual(31) from the American Joint Committee on Cancer (AJCC) in combination with cell count as described earlier(18).

Apart from size of metastasis, the database contains information on the number of removed sentinel nodes as well as the number of these being positive. Furthermore, the database contains information on additional ALND.

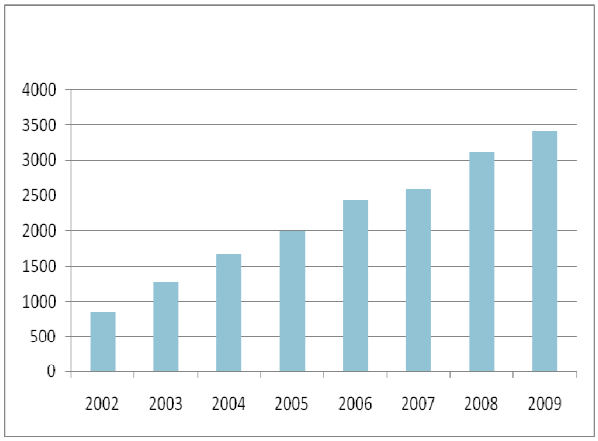

Figure 5: Number of SLND in Danish breast cancer patients registered in the DBCG database from 2002-2009

Study I: Stage migration

Patients

We estimated the size of stage migration after introduction of SLND in breast cancer treatment in Denmark by comparing the distribution of lymph node metastases in breast cancer patients operated in two different periods of four years: from 1993 to 1996 before any department has started using SLND and from 2005 to 2008 after completed introduction of the SLND as standard procedure in all Danish departments of breast surgery. Data on lymph node metastases, age at diagnosis, hormone receptor status, tumor size, histology type and malignancy grade were retrieved from the DBCG database. All registered patients, regardless of inclusion in specific treatment protocols, were included in the study to avoid selection bias. We collected missing information on nodal status manually from the original pathology file when possible. Altogether 1,617 patients were excluded: 53

patients from the first period due to missing information on nodal status, and 1,038 patients from the first and 526 patients from the second period due to missing information of the number of lymph nodes removed or less than 4 lymph nodes removed by ALND.

Table 3: Criteria for risk allocation of breast cancer patients

	Danish high-risk criteria 2007	St Gallen intermediate- or high-risk criteria 2007	High-risk criteria used in the present study of stage migration
Nodal status	Positive	Positive	Positive
Tumor size	>2 cm	>2 cm	>2 cm
Age at diagnosis	<35 years	<35 years	<35 years
Grade	Ductal grade 2 – 3 Lobular grade 3	Grade 2 - 3	Ductal grade 2 - 3
Hormone receptor status	Negative	Negative	Negative
HER2 status	Positive	Positive	Not used
Lymphovascular invasion	Not used	Present	Not used

To investigate if the introduction of SLND had changed the proportion of patients offered adjuvant systemic treatment, we divided patients from the two periods into risk groups according to the risk criteria described at the 10th St. Gallen International Expert Consensus Meeting 2007 (Table 3)(131).

Accordingly, negative nodal status, tumor size ≤ 2 cm, positive or unknown hormone receptor status, age ≥ 35 years, and ductal carcinoma malignancy grade I or unknown grade were considered as low risk criteria. Non-ductal carcinomas were not graded histologically in the first period. Still, non-ductal carcinoma was considered as a low risk criterion. HER2 status and lymphovascular invasion were not included as risk criteria when comparing the two periods because these parameters were only measured in the last period.

Statistical analysis

The DBCG Data Centre was responsible for data collection and data analysis. Associations between pairs of variables were analysed by the $\chi 2$-test (excluding unknowns). Univariate and multivariate logistic regression models were applied to examine the effect of age at diagnosis, tumor size, histology type and malignancy grade, hormone receptor status and period on nodal status. Odds ratios (OR) and 95% confidence intervals (CI) were calculated, and the Wald test was used to test the overall significance of each parameter. For departments of pathology involved in both periods, a multivariate model including interaction terms of departments and period was set up to test heterogeneity using the Wald test. Two-tailed p-values were applied and level of significance was set to 5%. All statistical analyses were done using SAS 9.1 (SAS Institute, Cary, NC, USA).

Study II: Predictive model for NSN metastases

Patients

From the introduction of SLND in 2001 to the end of 2008 a total number of 2293 breast cancer patients had been registered with micrometastases or ITC in the sentinel node in the DBCG database. From 2005 to 2008, metastases were classified according to the American Joint Committee on Cancer (AJCC) staging manual (31) in combination with cell count as described. In that period, a total number of 368 patients had been registered with ITC and 1474 patients with micrometastasis in the sentinel node. This information was validated using original pathology files and a 98% concordance was found. 15 patients had macrometastases, and in 2 patients the pathology file could not be found. These 17 patients were excluded. 11 patients registered with ITC in the sentinel node were identified with micrometastases and one patient registered with micrometastases was identified with ITC. From 2001 to the end of 2004 all tumor cell deposits under 2 mm or less than 100 tumor cells were registered as micrometastases. A total number of 451 patients with micrometastases were registered in that period. A re-evaluation of specimens from the sentinel nodes from this period identified 278 patients with micrometastases and 68 patients with ITC. The remaining 74 patients had macrometastases, 8 patients were node negative and in 23 patients the specimen was missing. These 105 patients were excluded. The re-evaluation was performed by two breast-trained pathologists from the Department of Pathology, Herlev Hospital.

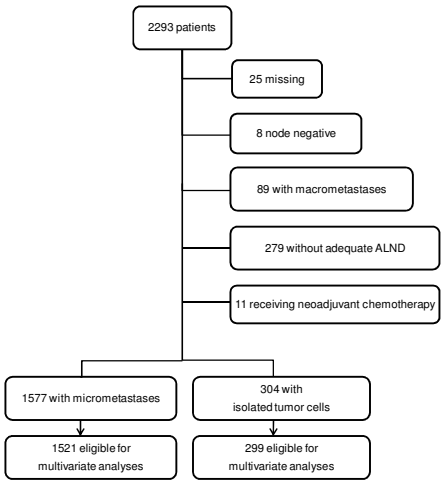

Figure 6: Flowchart for inclusion of patients with micrometastases or ITC from the DBCG database in study II

Missing information on additional ALND was collected from original pathology files. In total, 279 patients did not undergo an adequate ALND with at least 7 lymph nodes removed and were excluded. Another 11 patients were excluded because of neoadjuvant treatment.

A total number of 1577 patients with micrometastases and 304 patients with ITC were eligible for final analysis (Fig. 6).

Variables

From the DBCG database we retrieved information on age at diagnosis, tumor size, hormone receptor status, histology type, malignancy grade, number of removed sentinel nodes, number of positive sentinel nodes, lymphovascular invasion, multifocality, HER2 status, location of tumor in the breast and presence of NSN metastases. Location of tumor in the breast was divided into location in upper lateral quadrant versus located in other quadrants, centrally or on the edge of the upper lateral quadrant.

The Pathology Database(132) was searched for pathology files on HER2 analysis on primary tumor tissue not reported to the DBCG. Information on location of metastasis in the sentinel node was obtained from original pathology files and from the re-evaluation of existing specimens. For micrometatases we furthermore searched pathology files for information on extracapsular extension of the sentinel node metastasis.

Statistical analyses

Associations between presence of NSN metastases and patient, tumor and sentinel node characteristics were analysed by $\chi 2$-test and Fischer's exact test for patients with ITC and patients with micrometastases, respectively, in the sentinel node. Univariate and multivariate logistic regression models were applied to examine the influence of age at diagnosis, tumor size, histology type and malignancy grade, lymphovascular invasion, hormone receptor status, HER2 status, location of tumor in the breast, focality of tumor, location of metastasis in the sentinel node, extracapsular extension (for micrometastases only), number of removed sentinel nodes as well as the proportion of positive sentinel nodes among removed sentinel nodes on the risk of NSN metastases in patients with ITC in the sentinel node and patients with micrometastases in the sentinel node. 56 patients with micrometastases and 5 patients with ITC in the sentinel node were excluded from the multivariate analyses because of missing information on variables. Adjusted odd ratios (OR) and 95% confidence intervals (CI) were calculated and the Wald test was used to test the significance of each parameter. Test for interaction between covariates were performed pairwise. For the multivariate model for micrometastases a ROC curve including the c-statistic (AUC) were produced. Also a score was assigned to each patient by adding the relevant β coefficients from the multivariate logistical regression model, which was supplemented by a simplified score by adding number of risk factors present. SAS version 9.1 (SAS Institute, Cary, NC, USA) was used for all statistical analyses.

Study III: New biomarkers and NSN metastases

Patients

This study was designed as a case-control study. We consecutively included all breast cancer patients with micrometastases in the sentinel node operated on at the Department of Breast Surgery, Herlev Hospital, between 2001 and 2007. The Department of Breast Surgery, Herlev Hospital has since 2001 registered all sentinel node operations in the department. Until 2007, 257 patients had been registered having only micrometastases in the sentinel node. Micrometastases had been identified by multilevel sections and IHC cytokeratin staining of the sentinel node and classified according to the AJCC(31) in combination with cell counts as described. NSN had been examined by bisectioning and HE staining. All patients had FFPE tumor tissue stored. Data on tumor size, hormone receptor status, histology type, malignancy grade, age at diagnosis, number of removed lymph nodes and presence of NSN metastases from these patients were reviewed using original pathology files, discharge summaries, medical records and the DBCG database(129). The following 54 patients were excluded: males, patients registered as having only carcinoma in situ, patients with bilateral tumors, patients not registered in the DBCG database and patients without additional ALND. The remaining 203 patients were eligible for the study. On average 20 lymph nodes had been removed in these patients ranging from 7 to 40 lymph nodes. In 26 patients (13%) NSN metastases had been identified. These patients were considered as cases. For each case two matched controls without NSN metastases were found among the remaining 177 patients. Patients were matched by the following criteria: tumor size (≤ 2 cm, > 2 cm), hormone receptor status, age at diagnosis (+/- 5 years) and malignancy grade (grade I, grade II-III), if possible. One patient was excluded because no suitable match was found, leaving 25 cases and 50 matched controls for further analyses.

TIMP-1 analyses

Blinded IHC analyses of TIMP-1 on existing FFPE blocks of the primary tumors were performed. In brief, 3µm full sections were deparaffinized in xylene and rehydrated in graded concentrations of ethanol. For antigen retrieval, the sections were microwave treated in citrate buffer pH=6 and endogen peroxidase activity was blocked by hydrogen peroxide(133).

IHC staining for TIMP-1 used the mouse monoclonal antibody, clone VT7, raised against recombinant human TIMP-1 in concentration 0.25 µg/ml(134). This antibody has previously been found optimal for IHC detection of TIMP-1 on FFPE tissue sections(135). Sections were stained with primary antibody overnight at 4˚C. The antibody was detected with Advance HRP (Code No. K4068), and the reaction was visualized with DAB+ (Code No. K3468). All sections were counterstained with Mayer's haematoxylin. An irrelevant monoclonal antibody (Anti-TNP), raised against tri-nitro-phenol hapten, was used as a negative control. A human mammary carcinoma known to contain the investigated antigens was included as a positive control.

Two independent observers assessed the sections semiquantitatively by light microscopy. In case of discrepancies, agreement was reached by looking at the slides together.

Tumor sections were considered as TIMP-1 positive if any degree of staining was seen. In addition, TIMP-1 antigen immunoreactivity in the tumor cells was graded from 0 to 3 according to intensity and extensity of cytoplasmatic staining, respectively. The extensity score was graded as 0 if no tumor cells were stained, 1 if > 0% and < 25% were stained, 2 if ≥ 25% and ≤ 50% were stained and 3 if more than 50% of the tumor cells were stained (Fig. 7A and 7B). Intensity score was based on the average intensity of staining and graded as 0 if the staining was absent, 1 for weak staining, 2 for moderate staining and 3 for intense staining. Finally, a common score was made for each patient by multiplying the grades. TIMP-1 antigen immunoreactivity in stromal cells of the tumors was characterized as negative if no staining was seen and as positive if any degree of staining was observed (Fig. 7C).

Ki67 analyses

Blinded IHC analyses of Ki67 on existing FFPE tissue blocks of the primary tumors were performed. In brief, 3µm full sections were deparaffinized in xylene and rehydrated in graded concentrations of ethanol. For antigen retrieval, the sections were microwave treated in citrate buffer pH=6 and endogen peroxidase activity was blocked by hydrogen peroxide. IHC staining for Ki67 was performed by using the monoclonal mouse anti-human Ki67, clone MIB-1 (Code No. M7240) (from Dako, Glostrup, Denmark) in a concentration of 1.6 mg/ml. Sections were stained with primary antibody for 30 min. at room temperature. The antibody was detected with Advance HRP (Code No. K4068), and the reaction was visualized with DAB+ (Code No. K3468). All sections were counterstained with Mayer's haematoxylin. An irrelevant monoclonal antibody (Anti-TNP), raised against tri-nitro-phenol hapten, was used as a negative control. A human mammary carcinoma known to contain the investigated antigens was included as a positive control.

Two independent observers assessed the sections semiquantitatively by light microscopy. In case of discrepancies, agreement was reached by looking at the slides together.

Ki67 antigen immunoreactivity in tumor cells was determined as percentage of stained tumor cells present in the invasive front of the tumor. The Ki67 staining was considered as positive if more than 14% of the tumor cells stained for Ki67 (Fig. 7E and 7F)(98).

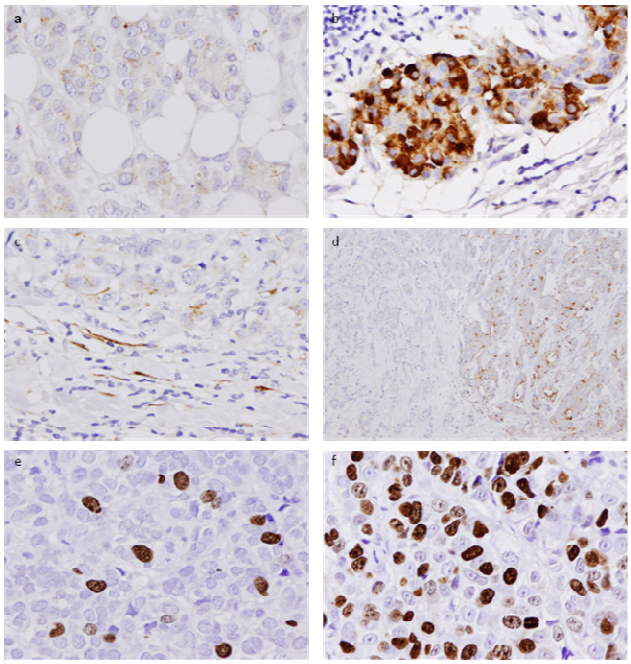

Figure 7(a-f): TIMP-1 and Ki67 IHC. (a) Tumor with weak TIMP-1 staining, considered as intensity grade 1. (b) Tumor with intense TIMP-1 staining, considered as intensity grade 3. (c) TIMP-1 staining stromal cells. (d) TIMP-1 stained section with changing intensity. (e) Ki67 stained tumor with less than 14% positive tumor cells, considered as Ki67 negative. (f) Ki67 stained tumor with more than 14% positive tumor cells, considered as Ki67 positive.

HER2 analyses

27 patients had a known HER2 status registered in the DBCG database. For these patients HER2 status had been determined previously according to international recommendations(136), by using the HercepTest (DakoCytomation, Glostrup, Denmark) for IHC analysis according to the manufactures' manual, where 1+ was considered as negative, 3+ was considered as positive and 2+ was considered as equivocal. In case of 2+ a supplementary fluorescence in situ hybridization (FISH) test for gene amplification had been performed to determine the final HER2 status.

For the remaining 48 patients HER2 status was unknown. In these patients HER2 status was determined retrospectively by using the HER2 FISH pharmDX Kit (Dako, Glostrup, Denmark) for gene amplification on whole sections of existing FFPE tissue blocks from the primary tumors(137). The tumors were considered as HER2 positive if the ratio of gene amplification was > 2.2(138).

Statistical analyses

The exploratory character of this study did not allow precise power analyses, but the sample size of 25 cases and 50 controls would give sufficient power to detect a medium or large difference in the proportions between groups (139) with a power on 90% and $\alpha = 0.05$ (Lenth, R. V. (2006-9), Java Applets for Power and Sample Size [Computer software], retrieved 10th of Oct 2010 from http://www.stat.uiowa.edu/~rlenth/Power).

Associations between the presence of NSN metastases and TIMP-1 positive tumor cells, HER2 positive tumor cells, Ki67 positive tumor cells or TIMP-1 positive stromal cells were analysed by Cochrane-Mantel-Haenszel test excluding unknowns. The differences in TIMP-1 common score and in percentage of cells with Ki67 staining between cases and their matched controls were found normally distributed and analysed by a Paired Student's t-test. For the two matched controls in each pair an average common score of TIMP-1 and an average percentage of Ki67 stained tumor cells was calculated for this analysis. Paraffin specimens from three controls did not contain sufficient tumor tissue for analyses and the remaining control in these three pairs was used alone. Associations between proliferation rate and HER2 status and TIMP-1 status respectively, were analysed by a Student's t-test. Two-tailed p-values were applied and the level of significance was set to 5%. All statistical analyses were done using SAS 9.1 (SAS Institute, Cary, NC, USA).

Ethical aspects

The study was approved by the Ethical Committees of the Capital Region, protocol nr. H-4-2009-087 and by the Danish Data Protection Agency (J.nr. 2009-41-3703).

RESULTS

Study I: Stage Migration

A total number of 24,051 patients were included in study I; 10,231 patients operated between 1993 and 1996, and 13,820 patients operated between 2005 and 2008. Patient and tumor characteristics are shown in table 4. In 2005–2008 we identified 307 patients having only ITC in their lymph nodes, corresponding to about 2% of patients in that period. Patients with ITC are considered as node negative when staged according to the AJCC(31). Thus, these 307 patients were included in the group of node negative patients. No patients with only ITC in the lymph nodes were registered in the first period. The distribution of nodal status, age at diagnosis, tumor size, hormone receptor status, histology type and malignancy grade changed significantly over time. From the first to the second period we found an increasing age at diagnosis (P<0.0001), increasing malignancy grade (P<0.0001), increasing proportion of patients having ductal carcinomas (P<0.0001), increasing proportion of patients having hormone receptor positive tumors (P<0.0001) and decreasing tumor size (P<0.0001).

The overall number of node positive patients increased significantly from 45.6% before to 49.7% after introduction of SLND; the proportion of patients with micrometastases increased from 5.1% to 9.0% (P<0.0001), whereas the proportion of patients having macrometastases was unchanged (Fig 8).

In a univariate analysis the risk of being node positive was significantly increased after introduction of SLND compared to before (OR 1.18; CI 1.12-1.24, P<0.0001). Furthermore, the risk of being node positive was significantly associated with histology type and grade, increasing tumor size and younger age at diagnosis. There was no significant difference in the risk of having lymph node metastases between patients with positive or negative hormone receptor status (Table 5).

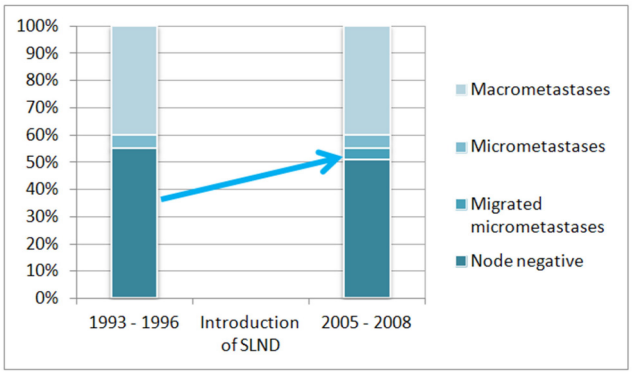

Figure 8: Stage migration after introduction of SLND in breast cancer treatment in Denmark

In a multivariate analysis, adjusting for changes in tumor size, age at diagnosis, hormone receptor status, histology type and grade, the risk of being node positive when operated in the last period compared to the first remained significantly increased (OR 1.20; CI 1.14-1.28, P<0.0001) (Table 5). When specifying this analysis according to the risk of having either macrometastases or micrometastases, we found an even more increased risk for having micrometastases after introduction of SLND compared to before (OR 1.85; CI 1.65-2.07, P<0.0001) while the risk of having macrometastases was unchanged (OR 1.01; CI 0.95-1.07, P=0.77). In the multivariate analysis younger age, increasing tumor size and histology grade remained significantly associated with node positive disease (P<0.0001), but in contrast to the results of the univariate analysis, negative hormone receptor status turned out to be significantly associated with negative nodal status (OR 0.83; CI 0.77-0.90, P<0.0001). Patients with unknown hormone receptor status were found to be significantly associated with negative nodal status as well (OR 0.81; CI 0.71-0.92; P<0.0001). However, this group represented only 5% of all patients and negative hormone receptor status remained significantly associated with negative nodal status when compared to the common group of patients with either positive or unknown hormone receptor status (OR 0.85; CI 0.79-0.91; P<0.0001).

To examine whether different departments of pathology in Denmark contributed equally to the increase in the amount of node positive patients after introduction of SLND, sub-analyses were made for single departments of pathology. Nine departments were no longer part of a breast unit in the last period because of centralization of breast cancer treatment in Denmark. For the remaining 16 departments multivariate analyses adjusting for changes in tumor size, age at diagnosis, hormone receptor status, histology type and malignancy grade were made to investigate interactions between department and period. A total number of 21,276 patients were included in these sub-analyses; 7,478 operated in 1993-1996, and 13,798 in 2005-2008. Odds ratios for being node positive in 2005-2008 compared to 1993-1996 did not vary significantly between the single departments of pathology (P=0.11).

Finally, we estimated the impact of the increased proportion of node positive patients on the proportion offered adjuvant systemic treatment. Patients from the two periods were divided into risk groups according to the modified St. Gallen risk criteria as described (Table 3). By doing this, we estimated that 71% of the patients in the first period and 73% of the patients in the second period would have been high-risk patients according to the risk-criteria of today (Table 4), and out of those only 788 patients (150 with micrometastases, 638 with macrometastases) in the first period, corresponding to 7.8% of the patients, and 1,217 patients (361 with micrometastases, 856 with macrometastases) in the last period, corresponding to 8.8% of the patients, became high-risk patients because of positive nodal

Table 4: Patient and tumor characteristics by period of diagnosis among 24,051 Danish breast cancer patients included in study I.

Period of diagnosis	1993-1996		2005-2008	
	No.	%	No.	%
Number of patients	10,231	100	13,820	100
Removed LN by ALND				
4 – 9 removed LN	3,302	32.3	510	6.9
>10 removed LN	6,929	67.7	6,893	93.1
Nodal status				
Node negative	5,565	54.4	6,952*	50.3
Node positive				
Macrometastases	4,144	40.5	5,630	40.7
Micrometastases	522	5.1	1,238	9.0
Age, years				
≤ 34	193	1.9	217	1.6
35-39	441	4.3	501	3.6
40-49	1,933	18.9	1,885	13.6
50-59	2,761	27.0	3,539	25.6
60-69	2,729	26.7	4,433	32.1
≥ 70	2,174	21.2	3,245	23.5
Tumor size, mm				
1 – 10	1,521	14.9	2,223	16.1
11-20	4,000	39.1	5,701	41.3
21-50	3,935	38.5	5,278	38.2
≥ 51	519	5.1	512	3.7
Unknown	256	2.5	106	0.8
Histology type and grade				
Ductal grade I	2,746	26.8	3,281	23.7
Ductal grade II	3,378	33.0	4,914	35.6
Ductal grade III	1,651	16.1	2,963	21.4
Ductal grade unknown	287	2.8	219	1.6
Lobular grade I-III	1,232	12.0	1,391	10.1
Other	937	9.2	1,052	7.6
Hormone receptor status				
Positive	6,820	66.7	11,375	82.3
Negative	2,260	22.1	2,376	17.2
Unknown	1,151	11.3	69	0.5
Risk allocation				
High-risk	7,276	71.1	10,058	72.8
Low risk	2,802	27.4	3,731	27.0
Allocation not possible	153	1.5	31	0.2

Abbreviations: LN, lymph nodes; ALND, axillary lymph node dissection.
*The number includes 307 patients with only ITC in the lymph nodes

status as the only high-risk criterion. The majority became high-risk patients regardless of nodal status but due to the existence of other high-risk criteria. The minor increase in high-risk patients caused by nodal status, from 7.8% to 8.8%, was however significant (P=0.006). In the last period, 75% of the included patients (10,433 patients) underwent SLND. If we used all available risk-criteria defined at the 10th St. Gallen International Expert Consensus Meeting 2007(131), including HER2 status, lymphovascular invasion and histology grading of lobular carcinomas, the

proportion of high-risk patients increased even further to 80% of the patients (8,334 patients) and still only a minor proportion, 7.9% (820 patients), had nodal status as the only high-risk criterion.

Table 5: Results of study I: Probability of positive axillary lymph nodes (macro- or micrometastases) among 24,051 Danish breast cancer patients treated in 1993-1996 or 2005-2008.

	Univariate analysis			Multivariate analysis		
	OR	95% CI	P-value	OR	95% CI	P-value
Period of diagnosis			< 0.0001			< 0.0001
1993-1996	1			1		
2005-2008	1.18	1.12-1.24		1.20	1.14-1.28	
Age at diagnosis, years			< 0.0001			< 0.0001
≤ 34	1.46	1.20-1.79		1.34	1.08-1.66	
35-39	1.31	1.14-1.50		1.26	1.09-1.46	
40-49	1.28	1.19-1.39		1.26	1.16-1.37	
50-59	1.18	1.10-1.27		1.21	1.13-1.30	
60-69	1			1		
≥ 70	1.15	1.08-1.24		0.96	0.89-1.04	
Tumor size, mm			< 0.0001			< 0.0001
1 - 10	0.41	0.38-0.45		0.43	0.39-0.47	
11-20	1			1		
21-50	2.29	2.16-2.43		2.31	2.18-2.46	
≥ 51	6.57	5.57-7.74		6.91	5.84-8.17	
Unknown	1.38	1.12-1.70		1.81	1.45-2.25	
Histology type and grade			< 0.0001			< 0.0001
Ductal grade I	0.57	0.53-0.61		0.73	0.68-0.78	
Ductal grade II	1			1		
Ductal grade III	1.14	1.06-1.23		0.98	0.90-1.06	
Ductal grade unknown	0.55	0.46-0.66		0.69	0.57-0.84	
Lobular grade I-III	0.84	0.77-0.91		0.75	0.69-0.83	
Other	0.36	0.33-0.40		0.39	0.35-0.43	
Hormone receptor status			< 0.0001			< 0.0001
Positive	1			1		
Negative	1.05	0.99-1.12		0.83	0.77-0.90	
Unknown	0.72	0.64-0.82		0.81	0.71-0.92	

Abbreviations: OR, Odds ratio; CI, Confidence interval

Study II: Predictive model for NSN-metastases

Patient, tumor and sentinel node characteristics according to NSN status of 1577 patients with micrometastases and 304 patients with ITC included in study II are shown in table 6. NSN metastases were found in 28 out of 304 patients with ITC in the sentinel node, corresponding to 9%, and 283 out of 1577 patients with micrometastases in the sentinel node, corresponding to 18%. An average number of 16.4 lymph nodes (Range 7 – 40) were removed.

Isolated tumor cells

In patients with ITC, NSN metastases was significantly associated with younger age at diagnosis (<40 vs. 40+), increasing tumor size (>2 cm vs. ≤2 cm) and increasing proportion of positive sentinel nodes (100% vs. <100%) in the univariate analyses (Table 6). All three variables remained significantly associated with NSN metastases in the multivariate analysis. The adjusted OR's are shown in table 7. Especially, tumor size was a good predictor of NSN metastases (Table 6). No patients with tumor size less than 1 cm (representing 12% of patients with ITC) had NSN metastases. A simple model was constructed based on the three risk factors from table 7. In the model (Table 8), patients ≥ 40 years with tumor size ≤2 cm as well as one or more negative sentinel nodes had a very low risk, about 2%, of having NSN metastases. This group represented 32% of patients with ITC. Only 4 patients had all three risk factors present. 3 out of these four patients (75%) had NSN metastases.

Micrometastases

In patients with micrometastases, NSN metastases was, in the univariate analyses, significantly associated with increasing tumor size, lymphovascular invasion, negative hormone receptor status, multifocality, location of tumor in the upper lateral quadrant, number of removed sentinel nodes and increasing proportion of positive sentinel nodes (Table 6).

In the multivariate analysis, the risk of NSN metastases remained significantly associated with increasing tumor size (trend cm), lymphovascular invasion, negative hormone receptor status, location of tumor in the upper lateral quadrant and increasing proportion of positive sentinel nodes (100% vs. (0-25%), (25-33%), (33-100%)). Number of removed sentinel nodes (P=0.26) and multifocality (P=0.18) were no longer significantly associated with NSN metastases. A significant interaction between tumor size and lymphovascular invasion was found with lymphovascular invasion as a stronger risk factor in larger tumors. Adjusted OR for the significant variables are shown in table 7.

The five significant variables from the multivariate logistical regression analysis were included in a model to predict NSN metastases. A score was assigned to each patient by adding the relevant β-coefficients. In the model, 5% of the patients were identified with a very high risk of NSN metastases on 37.4%. However, the model was unable to identify a subgroup of patients with a very low risk of NSN metastases; patients with the lowest risk score had a 12% risk for NSN metastasis (Table 9). The AUC for the constructed model was 0.64 (Fig. 9). To make the model suitable for daily clinical use, the risk score was simplified according to the number of risk factors present. 57 patients had at least four risk factors present. 40% of these patients had NSN metastases. 58 patients did not have any of the five identified risk factors, but still 10% of these patients had NSN metastasis (Table 10).

Variables	Isolated tumor cells				Micrometastases			
	NSN metastases				NSN metastases			
	No	Yes	%	P-value	No	Yes	%	P-value
Total	276	28	9.2		1294	283	17.9	
Age, years				0.06*				0.41
<40	16	5	23.8		55	12	17.9	
40 - 49	48	5	9.4		247	40	13.9	
50 - 59	77	9	10.5		386	91	19.1	
60 - 69	95	4	4.0		414	94	18.5	
≥70	40	5	11.1		192	46	19.3	
Tumor size, cm				0.003#				0.006
≤1	37	0	0		212	32	13.1	
>1 - ≤2	124	8	6.1		654	137	17.3	
>2 - ≤3	70	16	18.9		309	75	19.5	
>3	42	4	8.1		99	37	27.2	
Unknown	3	0	0		20	2	9.1	
WHO type				0.59				0.35
Ductal	191	18	8.6		1107	250	18.4	
Lobular	66	9	12.0		125	20	13.8	
Other	17	1	5.6		53	10	15.9	
Unknown	2	0	0		9	3	25.0	
Grade				0.13				0.10
Grade I	73	3	3.9		428	86	16.7	
Grade II	119	16	11.9		592	126	17.5	
Grade III	58	8	12.1		192	57	22.9	
Ungradable	26	1	0.04		82	19	14.6	
Lymphovascular invasion				0.40				0.003
Present	16	3	15.8		124	44	26.2	
Absent	255	25	8.9		1152	233	16.8	
Unknown	5	0	0		18	6	25.0	
Hormone receptor status				0.99				0.03
Positive	233	24	9.3		1137	238	17.3	
Negative	42	4	8.7		144	45	23.8	
Unknown	1	0	0		13	0	0	
HER2 status				0.80				0.50
Positive	41	6	12.8		141	30	17.5	
Negative	132	17	11.4		709	175	19.8	
Unknown	103	5	4.6		444	78	14.9	
Location of tumor in breast				0.21				0.0003
Upper lateral	95	9	8.7		468	136	22.5	
Borderline upper lateral	49	9	15.5		200	32	13.8	
Not upper lateral	113	9	7.4		548	94	14.6	
Unknown	19	1	5.0		78	21	21.2	
Focality of tumor in breast				0.49				0.03
Multifocal	24	1	4.0		63	23	26.7	
Unifocal	247	27	9.9		1212	255	17.4	
Unknown	5	0	0		19	5	20.8	
Location of metastasis in SN				0.81				0.20
Capsula	118	14	10.6		469	85	15.3	
Parenchyma	23	1	4.2		117	34	22.5	
Vessels	4	0	0		15	2	11.8	
Multifocal	10	1	9.1		60	12	16.7	
Unknown	121	12	9.0		633	150	19.2	
Extracapsular extension								0.58
Present					35	6	14.6	
Absent					1259	277	18.0	
Number of removed SN				0.23				0.01
1	72	13	15.3		387	103	21.0	
2	96	7	6.8		441	105	19.2	
3	65	5	7.1		249	43	14.7	
4	30	3	9.1		128	16	11.1	
5	11	0	0		85	14	14.1	
Unknown	2	0	0		4	2	33.3	
Positive SN/Removed SN				0.10¤				0.001
100%	94	16	14.5		459	134	22.6	
>33% , <100%	96	7	6.8		462	90	16.3	
>25; ≤ 33	51	4	7.3		208	35	14.4	
≤25%	33	1	2.9		161	22	12.0	
Unknown	2	0	0		4	2	33.3	

Abbreviations: NSN; non-sentinel nodes, SN; sentinel node

* P=0.03 <40 vs. ≥40, #P=0.002 ≤2 cm vs. >2 cm, ¤P=0.02 100% vs. <100%

Table 6 Patient, tumor and sentinel node characteristics according to the risk of NSN metastases in 304 Danish breast cancer patients with ITC and 1577 patients with micrometastases in the sentinel node operated in 2001 – 2008.

Table 7: Results of study II: Risk factors for NSN metastases in a multivariate analysis of 299 Danish breast cancer patients with ITC and 1521 patients with micrometastases in the sentinel node operated in 2001-2008.

Variable	OR	95% CI	P-value
		ITC	
Tumor size, >2 vs. ≤2 cm	4.21	1.74-10.2	0.001
Age at diagnosis, <40 vs. ≥40 years	3.57	1.11-11.4	0.03
Proportion of pos SN, 100% vs. <100%	2.90	1.27-6.60	0.01
		Micrometastases	
Tumor size, cm, trend	1.22	1.06-1.39	0.005
Proportion of pos SN, 100% vs. <100%	1.69	1.29-2.21	0.0001
Lymphovascular invasion	1.74	1.18-2.55	0.005
Hormone receptor status, neg vs. pos	1.47	1.00-2.16	0.049
Location of tumor in upper lateral quadrant	1.72	1.30-2.26	0.0003
Abbreviations: SN; sentinel node, neg; negative, pos; positive, OR; Odds ratio, CI; confidence Interval			

Figure 9: ROC curve for predicting model for NSN metastases in patients with micrometastases in the sentinel node

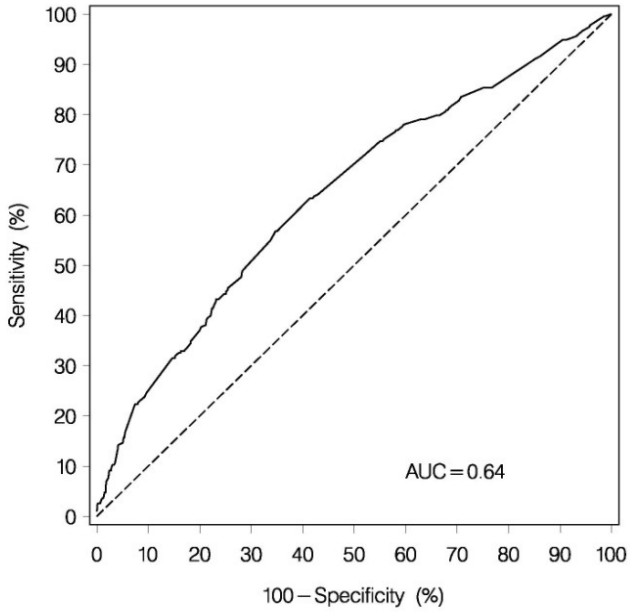

Table 8: Model for predicting the risk of NSN metastases in 299 Danish breast cancer patients with ITC in the sentinel node operated in 2002 – 2008 based on the number of risk factors present

Risk factors	Patients		Patients with NSN metastases	
	No.	%	No.	%
0	95	31.8	2	2.1
1	152	50.8	14	9.2
2	48	16.1	9	18.8
3	4	1.3	3	75.0
Total	299	100	28	9.4
Abbreviations: NSN; non-sentinel node, ITC; isolated tumor cells				

Table 9: Model for predicting the risk of NSN metastases in 1521 Danish breast cancer patients with micrometastases in the sentinel node operated in 2001-2008.

Percentiles dividing patients according to risk scores	Patients		Patients with NSN metastases	
	No.	%	No.	%
0-5	74	4.9	9	12.2
5-10	54	3.6	5	9.3
10-25	247	16.2	31	12.6
25-50	386	25.4	48	12.4
50-75	364	23.9	67	18.4
74-90	241	15.8	63	26.1
90-95	72	4.7	19	26.4
95-100	83	5.5	31	37.4
Total	1521	100	273	17.9
Abbreviations: NSN: non-sentinel node				

Table 10: Simplified model for predicting the risk of NSN metastases in 1521 Danish breast cancer patients with micrometastases in the sentinel node operated in 2002 – 2008 based on number of risk factors present

Risk factors	Patients		Patients with NSN metastases	
	No.	%	No.	%
0	**58**	**3.8**	**6**	**10.3**
1	**455**	**29.9**	**51**	**11.2**
2	**662**	**43.5**	**115**	**17.4**
3	**289**	**19.0**	**78**	**27.0**
4-5	**57**	**3.7**	**23**	**40.4**
Total	**1521**	**100**	**273**	**17.9**
Abbreviations: NSN: non-sentinel node				

Study III: New biomarkers and NSN metastases

Patients included in study III had generally small, low grade tumors and 13% of patients eligible for the study had NSN metastases. Associations between TIMP-1, Ki67 and HER2 expression and NSN status are shown in Table 11. 75% of the patients had TIMP-1 positive tumors, corresponding to 80% of the cases and 72% of the controls. This difference was not significant (P=0.77). A large variation was seen in the number of stained tumor cells and in the intensity of staining between the different specimens (Fig. 7D).

Tabel 11: Results of study III: Associations between TIMP-1, Ki67 and HER2 expression and NSN status in 75 breast cancer patients with micrometastases in the sentinel node with and without additional metastasis in NSN.

Variables	NSN metastases n =25	No NSN metastases n =50	Total n=75	P-value
TIMP-1 positive tumor cells, (%)				P=0.77
Positive	20 (80)	36(72)	56 (75)	
Negative	5(20)	11(22)	16(21)	
Unknown	0 (0)	3 (6)	3 (4)	
TIMP-1 common score, mean (range)	2.88 (0 – 9)	3.81 (0 – 9)	3.49 (0 – 9)	P=0.21
TIMP-1 positive stromal cells, (%)				P=0.93
Positive	11(44)	20 (40)	31 (41)	
Negative	13(52)	18 (36)	31 (41)	
Unknown	1(4)	12 (24)	13 (18)	
Ki67 positive tumor cells, (%)				P=0.93
Positive	11 (44)	22 (44)	33 (44)	
Negative	14 (52)	25 (50)	39 (52)	
Unknown	0 (0)	3 (6)	3 (4)	
% Ki67 positive tumor cells, mean (range)	13.9 (0 – 60)	16,0 (0 – 50)	15.3 (0 – 60)	P=0.54
HER2 status, (%)				P=0.12
Positive	1(4)	6 (12)	7 (9)	
Negative	24(96)	37 (74)	61 (82)	
Unknown	0 (0)	7(14)	7 (9)	

Abbreviations: NSN: Non-sentinel node, TIMP: Tissue inhibitor of Metalloproteinase, HER: Human epidermal growth factor receptor

To reflect this variation a common score including both extensity and intensity of TIMP-1 staining was calculated as described. No significant difference was seen in TIMP-1 common score between patients with and without NSN metastases (P=0.21). Stromal cells were identified in tumors from 62 patients. In half of these patients the stromal cells stained positive for TIMP-1, with an even distribution between cases and controls (P=0.93).

44% of the patients had Ki67 positive tumors, with an even distribution between cases and controls (P=0.93). Furthermore, there was no significant difference in the percentage of Ki67 stained tumor cells in the invasive front of the tumor between patients with and without NSN metastases (P=0.52).

Seven patients (9%) were HER2 positive. Six out of these seven HER2 positive patients did not have NSN metastases (12% of controls). However, this difference in HER2 expression between cases and controls did not reach statistical significance (P=0.12).

In general, the tumor cells of HER2 positive patients showed a significantly higher level of proliferation, measured by immunoreactivity of Ki67, compared to HER2 negative patients (P=0.0016). In contrast, TIMP-1 immunoreactivity in both tumor and stromal cells was not found significantly associated with proliferation rate (P=0.14) or to HER2 overexpression (P=0.17).

GENERAL DISCUSSION

Summary of results

Introduction of the SLND has increased the number of breast cancer patients identified with minimal metastatic disease in the axillary lymph nodes(30;35-37). The optimal surgical treatment of these patients is now under debate worldwide(52). ALND may not be necessary in the majority of the patients (64;65) and efforts must be made to identify subgroups of patients where ALND can safely be omitted.

We have examined the influence of introduction of SLND in breast cancer treatment in Denmark on the proportion of lymph node positive patients, in a nationwide study including more than 24,000 breast cancer patients. Our results provide support for an absolute increase in the proportion of node positive patients of nearly 4% points, and this was exclusively due to more women diagnosed with micrometastases in the sentinel node. However, this stage migration resulted in only 1% absolute increase in the proportion of patients who, according to current guidelines, would be offered adjuvant systemic treatment. In addition, after introduction of SLND metastatic spread as ITC were identified in about 2% of the patients. ITC were not identified in the period before introduction of SLND.

Subsequently we developed two models for these patients identified with micrometastases or ITC in the sentinel node that could predict the risk of NSN metastases. In the model based on patients with ITC in the sentinel node, tumor size ≤ 2 cm, age ≥ 40 years at diagnosis and one or more negative sentinel nodes identified patients with a very low risk of NSN metastases. These patients represented 1/3 of patients with ITC in the sentinel node. If ALND had been omitted in these patients, NSN metastases would have been left in the axilla in only 2% of the patients. In the model based on patients with micrometastases in the sentinel node we identified a subgroup of patients who had a high risk of NSN metastases on nearly 40%. Despite the large data material, the model could not identify a subgroup of patients with a very low risk of NSN metastases when micrometastases were found in the sentinel node.

Finally, we have initiated a search for biomarkers to be used to optimize the model for predicting NSN metastases in patients with micrometastases in the sentinel node. We initially selected three well known prognostic biomarkers; the proteinase inhibitor TIMP-1, the proliferation marker Ki67 and the proto-oncogene

HER2. However, none of the three biomarkers were found useful in predicting NSN metastases and could not be used to support the model.

Discussion of methods

The strengths of the studies on which this thesis is based include its population-based approach. All three studies used information from the DBCG database. This resulted in a very large sample size of more than 24,000 breast cancer patients for the investigation on stage migration and nearly 2300 patients with micrometastasis or ITC in the sentinel node for the development of predictive models for NSN metastases. This makes these studies far the largest studies to date on the subject. Furthermore, the DBCG database allowed us to identify two comparable groups of patients with micrometastases in the sentinel node with and without NSN metastases for a matched case-control study for testing of new biomarkers for NSN metastases. We tested three biomarkers on consecutively included breast cancer patients with micrometastases in the sentinel node, operated at the Department of Breast Surgery, Herlev Hospital, one of the largest centers for breast surgery in Denmark. The period for patient inclusion was from the introduction of the SLND in 2001 to the end of 2007. This resulted in a sample size of 203 eligible patients with micrometastases in the sentinel node. This sample size was adequate for showing a medium or large difference between groups(139). Detection of smaller differences was not considered as clinically relevant.

Data in the DBCG database have been prospectively collected from all Danish women with breast cancer. Earlier studies has shown a more than 95% concordance with the Danish Cancer Register and the National Pathology Register, which are considered close to complete(129;140) . Furthermore, data have been thoroughly validated using original pathology files and specimens. Discrepancies exist, in the proportion of node positive patients and in tumor size between the DBCG database and national databases from other countries(141), but these discrepancies are not likely to be caused by incomplete registration but could rather be explained by differences in population and breast cancer screening policies.

However, basing this thesis on information from a database gives some potential limitations. First, we found a significant drift in risk factors for having lymph node metastases over time which made it difficult compare different periods when estimating the size of stage migration. A decrease in average tumor size over time as well as an increase in estrogen receptor positive tumors and age at diagnosis have been shown earlier(15;142;143), and could be explained by the introduction of mammography screening in some Danish counties(15;143). It may also represent a trend towards an overall biologically less aggressive disease, but at the same time, an increase in malignancy grade was seen, pointing in the opposite direction. These opposite trends resulted in a basically unchanged proportion of high-risk patients (Table 5). Malignancy grading is to some degree a subjective measurement and changes in registrational practice could theoretically explain the trend towards increasing grade. Still, there has been no changes in Danish grading guidelines between the two periods(143) and furthermore, a large intraobserver agreement in malignancy grading has been shown(144). Further studies are needed to evaluate the observed increase in grade.

After adjustment for the described changes, when estimating the size of stage migration, the risk of being node positive after introduction of SLND remained significantly increased compared to the period before. The risk was still only increased in the group of patients with micrometastases. That implies that the increase in micrometastases after introduction of SLND is actually the result of a more extensive lymph node examination and cannot be explained by changes in age at diagnosis, tumor size, hormone receptor status and malignancy grade over time.

In our study of stage migration the number of lymph nodes excised increased from the first to the second period (Table 4). It has previously been shown that the proportion of node positive patients will increase with increasing number of lymph nodes examined(13). In Denmark, removal of at least 10 lymph nodes is today required for sufficient surgery when ALND is performed(129), but this was not the case in the first period of our study where many patients had only between 4–9 lymph nodes removed. It should be noted that this will tend to underestimate the number of node positive patients in the first period and consequently the magnitude of stage migration caused by the introduction of SLND alone will be lower than the estimate found in this study.

A second limitation of the thesis is a possible misclassification of sentinel node metastases. This could arise if the pathologists were not consistent in how they evaluated nodal metastases(145). All Danish departments of pathology report the results of nodal examination prospectively to the DBCG database using a standardized form and classification of metastases has since 2004 been well-defines by measurements of diameter as well as cell count. Still, differences in slicing of the sentinel node can result in different identification rates of metastases(146). To examine whether heterogeneity between departments had any impact on our results we calculated odds ratios for being node positive after introduction of the SLND compared to before, for every single department of pathology. This revealed no significant differences in odds ratios between the departments. Thus, the DBCG data on nodal status are not significantly affected by minor local differences in pathology procedures and can be considered as quite uniform. When comparing results internationally, it should however be noted that until the publication of the 7th edition of the AJCC staging manual in 2010, where the classification of ITC was supplemented by a cell count less than 200 tumor cells, the staging of micrometastases and ITC have internationally only been based on measurements of diameter.

A third limitation is that the biomarker analyses as well as analysis of hormone receptor status were made on primary tumor tissue and not on the corresponding sentinel node metastasis. Analyses on tissue from micrometastases were not technically possible because of limited amounts of metastatic tissue. We cannot be sure that the expression of the tested markers would be the same in the sentinel node metastases and the primary tumor. To underline this problem, a recent study has shown that every third patient change hormone receptor status during tumor progression and 10% change HER2 status(147). However, our study did not concern tumor progression to distant metastases but simultaneous tumor and lymph node metastsases with a possibly larger concordance(148). Previous studies have observed differences in the immunoreactivity of TIMP-1 in primary tumor tissue and the corresponding axillary lymph node metastasis(149). On the other hand, studies have shown a high degree of concordance in the immunoreactivity of Ki67 and in both immunoreactivity and gene amplification of HER2 between primary tumor tissue and corresponding axillary lymph node metastasis(148;150-152), which suggest that even if we could have analyzed the tumor cells in the sentinel node, no major changes in the results would have been observed for these two biomarkers.

Finally, scoring of the biomarkers Ki67 and TIMP-1 is difficult and not yet standardized. The right cut-point for Ki67 is under debate(98;153). We chose a cut-point on 14 % as used in the majority of earlier studies(97;98;154;155). Likewise, no generally accepted scoring system exists for TIMP-1. We used a modified H-score, similar to what have been used in earlier studies154, 155. We found a large variation in the expression of TIMP-1 in the same specimen, changing from areas where all tumor cells were intensively stained, to areas without any staining at all (Fig. 7D). To avoid false negative results, we therefore used analyses of whole sections and not tissue microarrays (TMA), despite the use of TMA becoming more and more widespread. Furthermore, scoring was done manually without the use of image analyzing systems.

Relation to the literature

Since the introduction of the SLND, several studies and reviews have been published internationally concerning the significance and treatment of patients with micrometastases or ITC in the sentinel node and the subject is still under debate(23;41-48;156-160).

Among studies investigating the magnitude of stage migration after introduction of SLND23,41-48, only three studies have been population-based(42;42-44). In the Netherlands van der Heiden-van der Loo et al. have investigated the magnitude of stage migration in 3,665 patients in the central part of the country during the introduction of SLND42. They found that the proportion of node positive patients increased significantly from 28% in 1997 to 38% in 2002 and this increase was mainly caused by micrometastases. In contrast, Maaskant et al. made a similar investigation on 17,100 patients in the south-eastern part of the Netherlands, but included the entire population in the investigated area and the complete period for the introduction of SLND. They found a much lower increase in percentage of patients having micrometastases from 1% in 1994 to 4.3% in 2005(43;44). In Denmark, stage migration after introduction of SLND has been investigated in a smaller study including 2,116 patients from two different counties. An increase in the proportion of node positive patients of 7.3% and 13.3% respectively, was found from 1996–1997 to 2002–200343. These variations in the size of stage migration between different studies may reflect local differences in lymph node examinations but may also be a result of different study sizes and study periods. Our estimated size of stage migration on nearly 4% is similar to the findings of Maaskant et al. which is the only previous study excluding the complete period for implementing SLND in the entire population(44). Our findings of an increase in node positive patients exclusively represented by micrometastases confirm the results of several previous studies(42-44;129;131).

The consequence of identifying these small metastases by the more extensive histopathological examinations might be adjuvant systemic overtreatment. The criteria for offering adjuvant systemic treatment have changed over the years129, 131. By allocating patients from the two periods into risk groups according to the risk criteria of today (Table 3), we showed that introduction of SLND had only minor impact on the number of patients offered adjuvant systemic treatment, with only 1% absolute increase in the proportion of patients with positive nodal status as the only high-risk criterion.

The national criteria for risk allocation used in Denmark is slightly more conservative than the modified St. Gallen criteria used in the study on stage migration (Table 3)(129). Using more conservative high-risk criteria will tend to increase the impact of stage migration on patients offered adjuvant treatment. Conversely, the trend towards inclusion of several new high-risk criteria in the decision for adjuvant systemic treatment will diminish the therapeutic consequences of stage migration due to introduction of SLND. In our study, several high-risk criteria were available in the last period. When using all available criteria we found that nodal status was decisive in risk-allocation of only 10% of high-risk patients offered SLND. This indicates that axillary nodal status is losing its independent significance in the decision for adjuvant systemic treatment.

In contrast to the consequences on adjuvant systemic treatment, the increased identification of patients with micrometastases and ITC will have an important impact on surgical treatment. Today, the majority of Danish breast cancer patients with micrometastases or ITC in the sentinel node is offered an ALND(18;64) , but only a small proportion of these patients will have NSN metastases and benefit from the operation. Metaanalyses have shown that only about 20% of patients with micrometastases64 and 12% of patients with ITC(65) have metastatic spread to NSN. This is in accordance to the results of our studies where 9% of patients with ITC and 18% of patients with micrometastases had NSN metastases. In the case-control study on biomarkers only 13% of patients with micrometastases eligible for the study had NSN metastases. This is however still within the range of earlier studies(64) and may reflect the small study size.

Due to the size of our patient material we were able to test several possible risk factors for NSN metastases in breast cancer patients with micrometastases or ITC in the sentinel node. In accordance to previous studies we confirmed that tumor size and lymphovascular invasion are predictors of NSN metastases in patients with micrometastases in the sentinel node(76;77;79;80;83;84;127). We furthermore identified proportion of positive sentinel nodes, hormone receptor status and age at diagnosis as risk factors. This has not been shown before in a population of patients with micrometastases or ITC in the sentinel node. In contrast to patients with macrometastases(73;91) we did not find an increased risk of NSN metastases in patients with extracapsular extension (Fig. 10). This is in accordance to the results of a previous smaller study on patients with micrometastases(91).

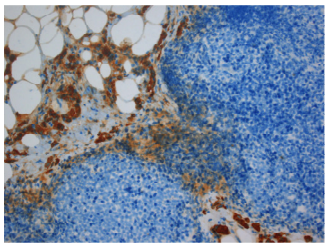

Figure 10: Sentinel node with micrometastasis with extracapsular extension

In addition to these traditional prognostic factors associated with NSN metastases in patients with macrometastases(73;85), we also tested location of the tumor in the breast and location of metastases in the sentinel node in relation to NSN metastases. We confirmed a significant association between NSN metastases and location of the tumor in the upper lateral quadrant, as shown previously85. This can be explained by different lymph draining patterns from different parts of the breast, where tumors in the upper lateral quadrant may be more likely to drain directly to additional lymph nodes in the more apical part of the axilla(82;161;162). Location of metastases in the sentinel node has in previous studies been associated with both NSN metastases82 and worse prognosis(79;83;90;163), but this could not be confirmed in our study.

Size of the micrometastases was only available in 10% of the patients and was not included in our model, despite earlier studies suggesting an association between metastases size and NSN metastases79,83,90.

To identify more possible markers for NSN-metastases we tested three new biomarkers for the prediction of NSN metastases in a case-control study. The three biomarkers selected, TIMP-1, Ki67 and HER2, are all involved in the process of cancer cell dissemination(95;96;98;164) . The proteinase inhibitor, TIMP-1, is a multifactorial protein that in addition to inhibition of matrix metalloproteinasis (MMP) also is involved in inhibition of apoptosis, stimulation of proliferation and regulation of angiogenesis164. TIMP-1 has in preclinical studies, been shown to promote tumor progression(95). Furthermore, overexpression of TIMP-1 has been found to be associated with positive nodal status(99;165) and to high risk of relapse(166-169) in breast cancer patients. The nuclear antigen Ki67 is used as marker for cell proliferation(98). In breast cancer patients Ki67 has been shown to be associated with increased risk of lymph node metastases(101;170) and to poor prognosis(97;98;171). Finally, the proto-oncogene HER2 is a tyrosine kinase receptor involved in the regulation of breast cell growth(96). Overexpression of this receptor has been shown to be associated with both poor prognosis(96) and to positive axillary nodal status(101;172) in breast cancer patients.

Studies on the expression of biomarkers in breast cancer patients with micrometastases in the sentinel node are scarce. Especially the expression of TIMP-1 has never been investigated in relation to micrometastatic disease before. In our case-control study, 75% of all patients had TIMP-1 positive tumors. This level is similar to what has been found in earlier studies on TIMP-1 expression in breast cancer patients(99;133;173;174). We here for the first time investigated the association between TIMP-1 and NSN metastasis. We found no association between the presence of NSN metastases and either qualitative or quantitative levels of TIMP-1 immunoreactivity in tumor cells.

As shown in both the present and previous studies TIMP-1 may also be present in stromal cells of the tumor173,174 and it is possible that the microenvironment around the tumor plays an active role in tumor cell dissemination(92). We therefore tested if TIMP-1 immunoreactivity in stromal cells was associated with the presence of NSN metastases. However, we did not find such an association.

Altogether, we found that TIMP-1 was not significantly associated with NSN status in breast cancer patients with micrometastases in the sentinel node, both according to TIMP-1 immunoreactivity in tumor cell and immunoreactivity in the stromal cells of the tumor.

44% of the patients in the case-control study had Ki67 positive tumors. This is the same level as in other studies using a similar threshold on >14% stained tumor cells as Ki67 positive(97). Four previous studies have tested if immunoreactivity of Ki67 could predict the presence of NSN metastases but the results are conflicting. Most studies including patients with either macro- or micrometastases in the sentinel node found no association between Ki67 immunoreactivity and NSN status(68;76;102). In contrast, Carcoforo et al. found a significantly increased Ki67 immunoreactivity in three out of eight patients with NSN metastases(80), but the study size was small and no adjustment for confounders was made. In our study, where possible confounders were adjusted for by matching, we did not find any association between Ki67 immunoreactivity and the presence of NSN metastases.

9% of patients in the case-control study were HER2 positive compared to 15% of breast cancer patients in general(175). The rate of HER2 positive patients found in our study is similar to what was found in another study only including patients with micrometastases in the sentinel node(80) and to the rate found in the nation-wide population of patients with micrometastases in study II. As a consequence of the low number of HER2 positive patients, our study lacked statistical power to show a significant association between HER2 status and the presence of NSN metastases despite the fact that only one out of seven HER2 positive patients had NSN metastases. HER2 has earlier been tested for the ability to predict NSN metastases in studies including patients with micrometastases in the sentinel node(76;78;80;85;176). None of these studies found HER2 useful in predicting metastatic spread to NSN. The lack of association between HER2 status and NSN metastases in patients with micrometastases in the sentinel node was confirmed in our population-based register study, where HER2 status registered in the DBCG database was found unassociated with NSN status in both patients with micrometastases and ITC in the sentinel node.

Based on all possible risk factors identified using the DBCG database we have constructed two models for the prediction of NSN metastases in patients with minimal metastatic disease in the sentinel node. In the group of patients with ITC in the sentinel node we identified tumor size, age at diagnosis and proportion of positive sentinel nodes as predictors for NSN metastases. A model based on these three predictors could separate patients into substantial risk groups. One third of the patients did not have any of the three risk factors. If ALND had been omitted in this group, NSN metastases would have been missed in only 2 of the 299 patients with ITC. This would be acceptable, bearing in mind that a false negative rate on 5% is accepted for SLND in general. Furthermore, the results of a recent large clinical trial has shown that less than 1% of sentinel node negative patients will have regional recurrence after 8 years of follow-up, despite about 4% being false negative176. This indicates that not all lymph node metastasis will become clinically relevant. Accordingly, omission of ALND in patients with ITC and none of the three identified risk factors might only result in a minimal number of axillary recurrences and ALND may be omitted in this group of patients without deteriorating the safety of the procedure. This is to date the only existing model for predicting NSN metastases based on a population of patients with only ITC in the sentinel node. However, the model needs to be validated in another dataset.

In the group of patients with micrometastases, the model showed no clearly defined low risk group and the AUC was only 0.64. Only two earlier studies has tried to construct a predictive model base on a population of patients with micrometastases or ITC in the sentinel node(76;127). The studies included 909 and 484 patients, respectively, with micrometastases or ITC in the sentinel node. Like us, none of the studies identified a substantial subgroup of patients with a very low risk of NSN metastases and the AUC for the constructed models were only 0.66 and 0.68.

The benefit from ALND in sentinel node positive patient is now under debate(49-52). The recent randomized trial from the American College of Surgeons Oncology Group, could not show any difference in axillary recurrence and survival between sentinel node positive patients with or without ALND59,60. Despite the limitations of the study, described in the background section, this indicates that ALND may safely be omitted in selected patients with metastases in the sentinel node, without impairment of prognosis. Hence, the search for a group of patients with a low risk of NSN metastases will become irrelevant. Instead, a model

will be needed to identify patients with a high risk of relapse who will still need an ALND(51). The presence of NSN metastases can be considered as a surrogate endpoint for axillary recurrence. In contrast to earlier models predicting the risk of NSN metastases(76;127) we also focused our models on patient at the high end of the risk scale. We have identified a group of patients with micrometastases in the sentinel node with a risk of NSN metastases at a level comparable to patients with macrometastases(61;63;72). This group of patients will probably have a high risk of relapse and may still need an ALND in the future.

FINAL CONCLUSIONS AND PERSPECTIVES

Final conclusion

The results of this thesis show that the introduction of SLND has resulted in stage migration, with a 4% absolute increase in the proportion of node positive patients, caused by identification of more patients with micrometastases. In addition, a 2% increase in the proportion of patients with ITC was found. However, this stage migration had only minor impact on the proportion of patients offered adjuvant systemic treatment, because axillary nodal status is losing its significance in risk-allocation, due to introduction of other risk-factors. In contrast, the impact of stage migration on surgical treatment is a reduction in the advantage of SLND, because ALND is generally offered to the majority of patients with micrometastases or ITC in the sentinel node, but may be redundant in most of these patients. The consequence is unnecessary morbidity and economical expenses.

As a tool to identify patients where ALND can be omitted, we have developed two models to predict the risk of NSN metastases in breast cancer patients with micrometastases or ITC in the sentinel node. The model for patients with ITC in the sentinel node showed that omission of ALND may be reasonable in patients over 40 years at diagnosis if tumor size is 2 cm or less in the presence of at least one additional negative sentinel node.

The model for patients with micrometastases was based on 5 risk factors for NSN metastases: tumor size > 2 cm, lymphovascular invasion, negative hormone receptor status, location of tumor in the upper lateral quadrant and absence of negative sentinel nodes. The model was unable to identify a subgroup of patients with a risk of NSN metastases below 10%. However a high risk group with four or more risk factors present was identified with a risk of NSN metastases on 40%. This group of patients will probably have a high risk of relapse and may still need an ALND in the future.

Finally, we have tested if the biomarkers TIMP-1, Ki67 and HER2 could be used to support the model for prediction of NSN metastases in breast cancer patients with micrometastases in the sentinel node. Despite being prognostic markers in breast cancer, these three biomarkers could not predict further spread beyond the sentinel node and cannot support the decision for ALND.

Perspectives

We have developed a model that can safely spare 1/3 of patients with ITC in the sentinel node for an ALND. Implementing our model will optimise the tailored treatment for women with breast cancer and reduce surgical overtreatment. Nevertheless, before taken into clinical use, the accuracy of the model should be validated in another dataset.

It was not possible to identify a substantial subgroup of patients with micrometastases in the sentinel node, where ALND could safely be omitted, despite using a very large and population-based sample size and despite testing of three new biomarkers for prediction NSN-metastases. A further increase in sample size will probably not result in a better model, but it cannot be excluded that the search for new biochemical markers might reveal new clinically significant predictors for NSN metastases(68). Hence, this search must continue. One of the promising new biomarkers is PAI1, which has reached level 1 evidence for clinical markers(177). PAI1 is a proteinase inhibitor, which is involved in the process of cancer cell dissemination(177;178) and is known to be associated with positive axillary nodal status179 and poor prognosis(179) in breast cancer patients. We plan to test expression of PAI1 as a marker for NSN metastases in patients from the case-control study.

It should be noticed that the presence of NSN metastases is only a secondary end point. The overall goal is to prevent relapse and improve prognosis. Results from studies reporting relapse and survival with and without ALND when micrometastases or ITC are found in sentinel node are conflicting and the studies are generally small and with limited follow-up and lack of multivariate analysis(52-57;180). It has been shown, that longer follow-up will reveal a significant amount of recurrences in patient with negative sentinel nodes181, or sentinel nodes with ITC(181) or micrometastases(182), suggesting that smaller metastases require time to grow to a detectable size. Accordingly, larger studies with longer follow-up and multivariate design are needed to investigate the benefit from ALND on the prognosis in patients with micrometastases or ITC in the sentinel node. A large trial from the European International Breast Cancer Study Group, the IBCSG 23-01 trial, included breast cancer patients with tumor less than 5 cm and with micrometastases in sentinel node. Patients were randomized to either ALND or observation(51;183). However, the trial recruited less than half of the patients originally planned, and results are not yet available51. In Denmark, the DBCG database gives us a unique opportunity to investigate the impact of ALND on prognosis in breast cancer patients with micrometastases or ITC in the sentinel node in a large and nationwide data material. Such a study is planned, where the prognosis of more than 250 patients with micrometastases or ITC in the sentinel node, but without ALND, will be investigated.

If this study, together with the previous studies, shows that ALND can safely be omitted in node positive patients without deterioration of prognosis, a tool is needed to identify patients with a high risk of relapse. Our model for patients with micrometastases can be used as such a tool, but first the model needs to be validated in another dataset.

Finally, as a sidefinding in study I, we showed that negative nodal status was significantly associated with negative hormone receptor status, as an independent factor, despite the fact that hormone receptor negative tumors generally are considered being biologically more aggressive(142;184;185). Reports on hormone receptor status in relation to lymph node metastases are conflicting185,186. One of the largest studies on the subject included 18,025 patients and showed the same relation between node negative disease and negative hormone receptor status as we do, indicating that this is a true observation(186;187). The group of hormone receptor negative patients will include all patients with triple negative breast cancers, being estrogen, progesterone and HER2 receptor negative. It is previously shown that these cancers are more likely to develop distant metastases(187) and it has been suggested this is because they tend to spread haematogenously rather than lymphogenously188. This could explain why we find a negative relation between lymph node metastases and negative hormone receptor status. Identify-

ing groups of breast cancers with haematogenously rather than lymphogenously spread could be important in the initial staging and search for metastases. Hormone receptor status might be a marker for haematogenous spread, but our results are based on patients from two different time periods and a study including patients between the two periods is planned to confirm the results.

ABBREVIATIONS

AJCC	**American Joint Committee on Cancer**
ALND	**axillary lymph node dissection**
AUC	**area under the receiver operating characteristic curve**
BCN	**breast cancer nomogram**
CI	**confidence limit**
DBCG	**Danish Breast Cancer Cooperative Group**
FFPE	**formalin fixed paraffin embedded**
FISH	**flourescence in-situ hybridization**
FNAC	**Fine needle aspiration cytology**
HE	**haematoxylin-eosin**
HER2	**human epidermal growth factor receptor 2**
IHC	**immunohistochemistry**
ITC	**isolated tumor cells**
NAC	**neoadjuvant chemotherapy**
NSABP	**National Surgical Adjuvant Bowel and Breast Project**
NSN	**non-sentinel node**
OR	**odds ratio**
ROC	**receiver operating characteristic**
SLND	**sentinel lymph node dissection**
SN	**sentinel node**
TIMP	**tissue inhibitor of metalloproteinasis**
TMA	**tissue micro arrays**

SUMMARY

Today, sentinel lymph node dissection (SLND) has replaced axillary lymph node dissection (ALND) as standard procedure for staging of the axilla in the treatment of breast cancer. SLND can accurately stage the axilla by removing on average only two lymph nodes. Only in case of metastatic spread to sentinel nodes an ALND is offered. Removing fewer nodes has made more extensive histopathological examinations of the lymph nodes possible and as a consequence more metastases are found. This has resulted in stage migration. Based on data from the nationwide Danish Breast Cancer Cooperative Group (DBCG) database we have estimated the magnitude and therapeutic consequences of this stage migration in Denmark by comparing the distribution of lymph node metastases in breast cancer patients operated in 1993–1996 and 2005–2008; before and after introducing SLND. The proportion of patients having macrometastases was not significantly different in the two periods, whereas the proportion of patients with micrometastases increased significantly from 5.1% to 9.0%. However, the proportion of patients offered adjuvant systemic treatment due to positive nodal status as the only high-risk criterion did only increase from 7.8% to 8.8%, when estimated using today's criteria for risk-allocation, because nodal status is now less important in risk-allocation.

In general, only 15-20% of patients with micrometastases and 10-15% of patients with isolated tumor cells (ITC) in sentinel node have further metastatic spread to non-sentinel nodes (NSN). Thus, the majority of these patients does not benefit from additional ALND but still run the risk of arm morbidity. Based on data from the DBCG database, we have developed two models to predict NSN metastases in breast cancer patients with micrometastases or ITC in the sentinel node. A total number of 304 breast cancer patients with ITC and 1577 patients with micrometastases in sentinel node operated in 2001 – 2008 with SLND and subsequent ALND were identified in the database.

In patients with ITC in sentinel node the risk of NSN metastases was significantly associated with younger age at diagnosis, increasing tumor size and increasing proportion of positive sentinel nodes in a multivariate analysis. If patients were ≥ 40 years at diagnosis with tumor size ≤ 2 cm as well as one or more negative sentinel nodes, NSN metastases were found in only 2%. Omission of ALND in this group would spare 1/3 of patients with ITC in sentinel node for an ALND.

In patients with micrometastases in sentinel node the risk of NSN metastases was significantly associated with increasing tumor size, lymphovascular invasion, negative hormone receptor status, location of tumor in the upper lateral quadrant of the breast and increasing proportion of positive sentinel nodes in a multivariate analysis. However, a model based on these traditional prognostic markers could not identify a subgroup of patients with a risk of NSN metastases less than 10%.

We then investigated whether the biochemical prognostic markers TIMP-1, Ki67 and HER2 could support the model. In a matched case-control study 25 cases with micrometastases in sentinel node and additional metastatic spread to NSN were compared to 50 matched controls with micrometastases in sentinel node but without NSN metastases. Despite being prognostic markers in breast cancer, we found no significant differences in the expression of these three biochemical markers between patients with and without NSN metastases.

Not all NSN metastases will become clinically relevant, making ALND redundant in many breast cancer patients. Accordingly, there is a trend towards omission of ALND in breast cancer patients with minimal metastatic disease in sentinel node. As a result, a tool is needed to identify a group of patients with high risk of recurrence, where ALND should still be offered. In our model a small group of patients with micrometastases had a high risk of NSN metastases on nearly 40%, comparable to patients with macrometastases, indicating that ALND may still be recommended in this subgroup in the future.

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