Breast cancer. Quality Assurance and Prognosis.

Grabau, Dorthe

2012

BULLETIN NO 141 FROM DEPARTMENT OF SURGERY, CLINICAL SCIENCES,
LUND UNIVERSITY, SWEDEN. 2012.

Breast cancer

Quality Assurance and Prognosis

DORTHE AAMAND GRABAU

Bulletin No 141 from Department of Surgery, Clinical Sciences, Lund University, Sweden. 2012.

Breast cancer

Quality Assurance and Prognosis

Dorthe Aamand Grabau, MD

LUND UNIVERSITY

Doctoral Dissertation
By due permission of the Faculty of Medicine,
Lund University, Sweden, to be publicly defended in
the Segerfalk Lecture Hall, Sölvegatan 19, BMC, Lund
on Friday the 2th of November 2012, at 1.00 pm.

Faculty Opponent
Professor Jonas Bergh
Section for Oncology, Karolinska Institute
Stockholm, Sweden.

Supervisor
Professor Christian Ingvar
Department of Surgery, Clinical Sciences Lund
Lund University, Sweden.

Co-supervisor
Associate professor Lisa Rydén
Department of Surgery, Clinical Sciences Lund
Lund University, Sweden.

Co-supervisor
Professor Mårten Fernö
Department of Oncology, Clinical Sciences Lund
Lund University, Sweden.

Organization
LUND UNIVERSITY

Document name
DOCTORAL DISSERTATION

Date of issue
2012 11 02

Author(s)
Dorthe Aamand Grabau

Sponsoring organization

Title and subtitle

Breast cancer. Quality Assurance and Prognosis.

Abstract

Background. The Swedish national cancer strategy programme published in 2009 emphasises the patient perspective and focuses on the patient process. Over the years the different modalities in breast cancer treatment are changing position, making accurate diagnosis and quality assurance in breast pathology even more important than before.

Aims. The aim of paper I was to examine overall survival in women with micrometastases in relation to node-negative women. In paper II four different routine methods for the pathological work-up of frozen section negative sentinel nodes (SN) were compared to find the method showing the largest fraction of patients with small deposits in SNs, in order to achieve the highest possible confidence in the negative status. In paper III the aim was to determine whether screening status influences the proportion of patients with additional positive nodes in the axillary lymph node dissection (ALND) specimen after the SNs have been diagnosed with micrometastases. Paper IV deals with immunohistochemistry with the aim of comparing the prevalence of oestrogen receptor (ER)-positive patients when the ER status was determined by three different antibodies and heat-induced epitope retrieval (HIER) methods in premenopausal stage II patients.

Material. In paper I the study cohort consisted of 6,959 women with T1-T3, N0-N1, M0 primary breast cancer aged below 75 years and registered in the Danish Breast Cancer Database from 1 January 1990 to 31 October 1994. The study cohort in paper II was a consecutive series of 1,576 women with a first primary operable breast cancer treated at the University Hospital of Lund from 1 January 2001 to 31 December 2009, of whom 1,098 had sentinel node biopsy (SNB). In paper III the study cohort was 1,993 consecutive women with first primary unilateral breast cancer, of whom 1,458 had SNB, treated at Skåne University Hospital, Lund between 2001 and 2011. In paper IV ER status was assessed on tissue microarrays, with three different ER antibodies and HIER methods: 1D5 in citrate pH 6, SP1 in Tris pH 9 (n=390) and PharmDx in citrate pH 6 (n=361).

Results. Paper I showed in a multivariate analysis that women with micrometastases had a significantly higher risk of death than did node-negative women (adjusted relative risk = 1.49, 95% CI: 1.18–1.90) ($p<0.01$). The result of paper II was that a combination of teamwork and the addition of intensive IHC for cytokeratin (CK) at fixed levels resulted in 13% more patients with isolated tumour cells and micrometastases than if a method with step sections at fixed intervals were used. In paper III the results of a logistic regression analysis showed 5 times higher odds for further metastases in the ALND in patients with micrometastases in SNs when symptomatic presentation was compared with screen-detected breast cancer. The findings in paper IV were that the prevalence of ER-positivity was higher with SP1 (75% and 72%) compared with 1D5 (68% and 66%) and PharmDx (66% and 62%) at cut-offs of 1% and 10%, respectively. The repeatability was good for all antibodies and cut-offs with overall agreement ≥93%.

Conclusion. Patients with micrometastases detected in ALND have an inferior 10-year overall survival compared with node-negative patients. SN examination with step sections at fixed levels including CK at each level is important in ensuring that the node-negative group really is node-negative. Screen-detected breast cancer patients with micrometastases have 5-time lesser odds for additional metastases in the completion ALND compared with symptomatic patients, and are thereby candidates for the omission of completion ALND. The prevalence of ER-positive breast cancer patients is dependent on the antibody and HIER method.

Key words
Breast cancer, micrometastases, overall survival, oestrogen receptor, immunohistochemistry.

Classification system and/or index terms (if any)

Supplementary bibliographical information

Language
English

ISSN and key title
1652-8220

ISBN
978-91-87189-32-6

Recipient's notes

Number of pages
45

Price

Security classification

Distribution by (name and address)

I, the undersigned, being the copyright owner of the abstract of the above-mentioned dissertation, hereby grant to all reference sources permission to publish and disseminate the abstract of the above-mentioned dissertation.

Signature Dorthe Grabau. Date 2012.09.14

Breast cancer

Quality Assurance and Prognosis

LUND
UNIVERSITY

Dorthe Aamand Grabau, MD

Department of Surgery, Clinical Sciences, Faculty of Medicine, Lund University, Sweden. 2012
Boullitin No. 141

Dorthe.Grabau@skane.se

ISBN 1652-8220
ISSN 978-91-87189-32-6
Lund University, Faculty of Medicine, Doctoral Dissertation Series
2012:69

Printed by Media-Tryck, Lund University, Lund, Sweden, 2012

Contents

Abbreviations

ALND	Axillary lymph node dissection
CI	Confidence interval
CK	Cytokeratin
ER	Oestrogen receptor
FFPE	Formalin-fixed paraffin-embedded
FNA	Fine-needle aspiration
HE	Haematoxylin and eosin
HER2	Human epidermal growth factor receptor 2
HIER	Heat-induced epitope retrieval
HR	Hazard ratio
IHC	Immunohistochemistry
PR	Progesterone receptor
RFS	Recurrence-free survival
RR	Relative risk
SN	Sentinel node
SNB	Sentinel node biopsy

Definitions

Due to there being different definitions of node-negative, isolated tumour cells, micrometastases and macrometastases these terms are not abbreviated but written in full throughout the text. The definitions vary in different editions of the AJCC and UICC classifications; the definitions used in various part of the text should be clear from the context.

In brief:

Before 2002[1]

Node-negative	No metastatic tumour deposits in lymph nodes
Micrometastases	Metastasis measuring >0 and ≤2 mm
Macrometastases	Metastasis >2 mm

AJCC and UICC 6th edition[2, 3], 2002

Node-negative	Negative nodes clinically including isolated tumour cells
Isolated tumour cells	Epithelial deposits ≤0.2 mm in lymph nodes
Micrometastases	Metastasis >0.2 mm and ≤2 mm
Macrometastases	Metastasis >2 mm

AJCC and UICC 7th edition[4, 5], 2010 and 2009

Node-negative	Negative nodes clinically including isolated tumour cells
Isolated tumour cells	Epithelial deposits ≤0.2 mm/<200 cells in lymph nodes
Micrometastases	Metastasis >0.2 mm/>200 cells and ≤2 mm
Macrometastases	Metastasis >2 mm

Papers

This thesis is based on the following papers, which will be referred to by their Roman numerals.

I Grabau D, Jensen MB, Rank F, Blichert-Toft M.
Axillary lymph node micrometastases in invasive breast cancer: national figures on incidence and overall survival. APMIS 2007 Jul;115(7):828-837

II Grabau D, Ryden L, Fernö M, Ingvar C.
Analysis of sentinel node biopsy – a single-institution experience supporting the use of serial sectioning and immunohistochemistry for detection of micrometastases by comparing four different histopathological laboratory protocols. Histopathology. 2011 Jul;59(1):129-38.

III Grabau D, Dihge L, Fernö M. Ingvar C, Ryden L.
Completion axillary dissection can safely be omitted in screening detected breast cancer patients with micrometastases. Results from a decade from a single institution. (Submitted)

IV Grabau D, Bendahl P-O, Rydén L, Stål O, Fernö M. For the South and South-East Breast Cancer Groups.
The prevalence of immunohistochemicallt determined oestrogen receptor positivity in primary breast cancer is dependent on the choice of antibody and method of heat-induced epitope retrieval – prognostic implications? (Submitted)

Abstract

Background. The Swedish national cancer strategy programme published in 2009 emphasises the patient perspective and focuses on the patient process. Over the years the different modalities in breast cancer treatment are changing position, making accurate diagnosis and quality assurance in breast pathology even more important than before.

Aims. The aim of paper I was to examine overall survival in women with micrometastases in relation to node-negative women. In paper II four different routine methods for the pathological work-up of frozen section negative sentinel nodes (SN) were compared to find the method showing the largest fraction of patients with small deposits in SNs, in order to achieve the highest possible confidence in the negative status. In paper III the aim was to determine whether screening status influences the proportion of patients with additional positive nodes in the axillary lymph node dissection (ALND) specimen after the SNs have been diagnosed with micrometastases. Paper IV deals with immunohistochemistry with the aim of comparing the prevalence of oestrogen receptor (ER)-positive patients when the ER status was determined by three different antibodies and heat-induced epitope retrieval (HIER) methods in premenopausal stage II patients.

Material. In paper I the study cohort consisted of 6,959 women with T1-T3, N0-N1, M0 primary breast cancer aged below 75 years and registered in the Danish Breast Cancer Database from 1 January 1990 to 31 October 1994. The study cohort in paper II was a consecutive series of 1,576 women with a first primary operable breast cancer treated at the University Hospital of Lund from 1 January 2001 to 31 December 2009, of whom 1,098 had sentinel node biopsy (SNB). In paper III the study cohort was 1,993 consecutive women with first primary unilateral breast cancer, of whom 1,458 had SNB, treated at Skåne University Hospital, Lund between 2001 and 2011. In paper IV ER status was assessed on tissue microarrays, with three different ER antibodies and HIER methods: 1D5 in citrate pH 6, SP1 in Tris pH 9 (n=390) and PharmDx in citrate pH 6 (n=361).

Results. Paper I showed in a multivariate analysis that women with micrometastases had a significantly higher risk of death than did node-negative women (adjusted relative risk = 1.49, 95% CI: 1.18–1.90) ($p<0.01$). The result of paper II was that a combination of teamwork and

the addition of intensive IHC for cytokeratin (CK) at fixed levels resulted in 13% more patients with isolated tumour cells and micrometastases than if a method with step sections at fixed intervals were used. In paper III the results of a logistic regression analysis showed 5 times higher odds for further metastases in the ALND in patients with micrometastases in SNs when symptomatic presentation was compared with screen-detected breast cancer. The findings in paper IV were that the prevalence of ER-positivity was higher with SP1 (75% and 72%) compared with 1D5 (68% and 66%) and PharmDx (66% and 62%) at cut-offs of 1% and 10%, respectively. The repeatability was good for all antibodies and cut-offs with overall agreement ≥93%.

Conclusion. Patients with micrometastases detected in ALND have an inferior 10-year overall survival compared with node-negative patients. SN examination with step sections at fixed levels including CK at each level is important in ensuring that the node-negative group really is node-negative. Screen-detected breast cancer patients with micrometastases have 5-time lesser odds for additional metastases in the completion ALND compared with symptomatic patients, and are thereby candidates for the omission of completion ALND. The prevalence of ER-positive breast cancer patients is dependent on the antibody and HIER method.

Background

Nearly 8000 patients are diagnosed with a new breast cancer each year in Sweden[6]. The Swedish breast cancer group maintains the national guidelines[7], and local applications of the guidelines are produced[8].

Breast cancer treatment

1. Screening and diagnosis

Sweden was the first country to offer a public mammography screening programme to all women in the target population, which in recent years has been extended from 50-70 years to 40-74 years. Inspired by the beneficial results of the randomised Swedish screening studies[9], pilot projects started in Denmark in 1990[10] and in Norway in 1996. Public mammography screening programmes have been running nationwide in Sweden since 1997, in Norway since 2005 and in Denmark since 2007. In screening, there is a calculated risk of overdiagnosis due to the lead time, and in a population-based study from Malmö overdiagnosis was of the order of 10%[11]. Much debate is ongoing concerning the effect of public screening programmes for breast cancer[12, 13]. The relative survival of women with breast cancer in the Nordic countries is steadily increasing[14] and screening might contribute about one third of the reduction in the rate of death. Improvements in diagnosis and treatment might account for the rest[15].

Women with suspicious lesions on the screen mammogram (2-view) and symptomatic patients are referred to clinical mammography (3-view) including clinical investigation and, when necessary, biopsied, either by core needle biopsy or fine-needle aspiration (FNA)[16]. At the same time, ultrasound examination of axillary nodes is performed and, if the nodes are suspicious, a FNA follows. The result of this triple diagnostic procedure decides the women's subsequent treatment. Benign cases are referred back to the screening programme without further treatment. Where there is doubt or a malignant diagnosis, the patient is discussed at the multidisciplinary conference.

2. Surgery

In patients with a diagnosis of invasive breast cancer, partial mastectomy[17] or mastectomy[18], depending on cosmetic concerns and concomitant conditions, is the standard treatment except for patients with local advanced cancer who are offered neoadjuvant therapy before surgery. Axillary staging in ultrasound and clinically negative patients is performed by sentinel node biopsy (SNB)[19]. It is still standard today for sentinel node (SN)-positive patients to bee offered completion axillary lymph node dissection (ALND).

3. Systemic therapy

In addition to the TNM (T = tumour, N = node and M = metastases) classification and age, the administration of adjuvant systemic medical therapy is based on the immunohistochemical translation of the results of gene expression profiles[20] allocating patients to molecular subtypes even though these groups are not completely equivalent[21]. The luminal A group, oestrogen receptor (ER)/progesterone receptor (PR)-positive, human epidermal growth factor receptor 2 (HER2)-normal and with a low Ki67 index, constitutes about 50% of breast cancer patients, and if risk factors are found these patients are offered adjuvant endocrine therapy. Patients that are ER-positive, PR-positive or negative with a high Ki67 are allocated to the luminal B group, and these patients receive chemotherapy and endocrine therapy if they have risk factors. The HER2 group comprises patients with amplified HER2, and patients in this group are recommended chemotherapy and trastuzumab if they have tumours more than 5 mm in size or are node-positive. The triple-negative group (ER- and PR-negative with normal HER2) is a heterogeneous group of patients[22] including among others metaplastic invasive ductal carcinomas with poor prognosis, and the adenoid cystic carcinomas that rarely metastasize when located in the breast. In Sweden these triple-negative patients as a group are offered chemotherapy if they have risk factors. Only patients with negative nodes or ITC in the SN and a tumour size of 10 mm or less (for HER2-amplified tumours 5 mm or less) are not offered any systemic medical adjuvant therapy. Patients with four or more positive nodes receive screening for distant metastases and, if positive, they are offered systemic therapy. Adjuvant radiation therapy of the breast is offered to

all patients treated by partial mastectomy and, where there are two or more positive axillary nodes, also to the axilla.

4. Introduction

In 2009, the Swedish national cancer strategy programme was published[23]. The programme emphasises the patient perspective and focuses on the patient process. This approach offers new challenges to the multidisciplinary patient team by reorganising the cancer patient processes, which focus on delivering the results of the diagnostic procedure in time, when the patient needs it, instead of the old focus on each department's production. This organisation also offers new options for breast pathology in terms of communication between disciplines. In addition, breast pathologists might play a more active role in multidisciplinary teams in order to provide the best fundamentals for treatment plans. Quality assurance (QA) of the breast cancer patient process is paramount, yet today there is no agreed QA programme involving the total patient process. The requirements of a specialist breast unit have been agreed by EUSOMA[24]. Traditionally biomarker QA in pathology laboratories is performed in two steps: the internal QA at the laboratory, covering the implementation of optimal protocols and the control of day-to-day variation, and the external QA achieved by participation in QA programmes executed by, for example Nordiqc[25]or UK Nequas[26]. In Sweden pathology laboratories engaged in breast pathology can achieve accreditation of breast diagnoses by SWEDAC[27], and most laboratories have done so today.

The survival of breast cancer patients today is dependent on adequate surgery and the administration of systemic adjuvant therapy to risk patients. Sweden has one of the best 5-year survival rates for breast cancer in the world, and many more women resident in Sweden live with breast cancer than do women dying from the disease. The drawback of adjuvant systemic therapy is that in fact many patients do not need it, and only experience the side effects. This overtreatment can only be dealt with by the more precise characterisation of patients that will benefit from a specific therapy.

In this context several aspects of breast pathology are important. The widespread use of large sections in Sweden might contribute to better local control, especially in women treated by partial mastectomy,

because this technique examines a larger proportion of the resection margin compared with sampling with conventional small sections[28]. In addition to the QA of biomarkers, uniform and intensive examination of SN is important in order to assure a negative status in patients allocated to the node-negative group. This is especially important because those women that can be cured by surgery alone, and who do not benefit from further adjuvant systemic therapy, come from the node-negative group.

The importance of metastases to the ipsilateral axillary lymph nodes was first shown by Adair in 1933[29]. In 1971 Huvos et al.[30] were the first authors to report on micrometastases finding no difference in the recurrence-free survival (RFS) or OS of 40 patients with micrometastases compared with 62 node-negative and 125 with macrometastases with 8 years of follow-up. In a cohort of 565 patients with a maximum of 4 years of follow-up, Fisher et al.[31] showed that the RFS or OS of patients with micrometastases did not differ from those of node-negative patients. Fisher et al.[31], in a multivariate analysis, also showed that the number of involved nodes was more important than micro- or macrometastases with respect to the patients' prognoses. Correspondingly, Rosen et al.[32] showed that in a cohort of 147 patients, of whom 73 were $T_1N_1M_0$ patients with up to 12 years of follow-up, that the OS of patients with a single metastasis did not differ from that of the node-negative patients up to the 6th year. After 12 years of observation, the OS of $T_1N_1M_0$ patients with a single micro- or a single macrometastasis were alike and significantly worse than those of node-negative patients. A review from the late nineties[33] emphasised that the first studies on fever patients did not show any prognostic difference between patients with micrometastases and node-negative patients. However, the later larger studies show a worse outcome for patients with micrometastases than for node-negative patients. The Ludwig group[34] has shown that the outcome for patients with occult micrometastases who did not receive chemotherapy was worse than that for patients with occult micrometastases receiving chemotherapy. Recently, similar results were reported for women with micrometastases or isolated tumour cells in SNs where the group receiving adjuvant systemic therapy had superior disease-free survival compared with those without systemic therapy[35].

In the area of ALND there has been much debate consigning definitions of micrometastases including size, location in the lymph nodes and

method of detection (one haematoxylin and eosin (HE)-stained section *vs.* step sections with or without immunohistochemistry (IHC) for cytokeratin (CK)). In the SN area, some of the debate has continued and methods of detection have been expanded with various polymerase chain reaction-methods, among others. Despite this dispute and continuous refinements to the definition, a micrometastasis has been defined for the last 20 years of so as a metastatic deposit of 2 mm or less and for the last 10 years a lower limit of 0.2 mm has described isolated tumour cells. Due to the patient perspective in the Nordic countries, it is crucial that prognoses of patients with micrometastases be evaluated. Furthermore, the best protocol for routine use and the impact of micrometastases in screen-detected patients are important. Finally, from a QA perspective knowledge of the prevalence of different ER antibodies is essential.

Aims of the specific studies

I To examine the overall survival in women with micrometastases in relation to node-negative women and to women with 1-3 positive axillary nodes in a population-based patient series.

II To determine which of four different routine methods for the pathological work-up of frozen section negative SNs shows the largest fraction of patients with small deposits in SNs, in order to achieve the highest possible confidence in the negative status.

III To determine whether screening status influences the proportion of patients with additional positive nodes in the axillary dissection specimen after the SNs have been diagnosed with micrometastases.

IV To compare the prevalence of ER-positive patients when ER status is determined by three different antibodies and heat-induced antigen retrieval methods in premenopausal stage II patients originally participating in a randomised trial designed to compare the effect of two years adjuvant tamoxifen *vs.* no tamoxifen.

Material and methods

Paper I
The study cohort was population-based and consisted of 6,959 women with T1-T3, N0-N1, M0 primary breast cancer aged below 75 years and registered in the Danish Breast Cancer Database (DBCG) from 1 January 1990 to 31 October 1994. Women with four or more positive nodes were excluded. All patients were treated systematically according to approved Danish national guidelines and treatment protocols and women with micrometastases received adjuvant systemic treatment.

The study was a register study with the end point overall survival. The Cox proportional hazards regression model was applied to assess the adjusted relative risk (RR) of death according to axillary status.

Paper II
The study cohort was department-based and consisted of a consecutive series of 1,576 women with a first primary operable breast cancer treated at the University Hospital of Lund from 1 January 2001 to 31 December 2009. Of patients included in the study, 70% (1,098) had SNB.

SNs were bisected through the longitudinal axis and a frozen section from each section was analysed. Then the SNs were formalin-fixed and paraffin-embedded (FFPE). A review of all slides from SN-positive patients was undertaken and the 7th edition of AJCC and UICC classification was applied. Four different methods for the definitive SN work-up of FFPE material for frozen section negative cases were compared:

Method 1: One HE and one IHC from each section.
Method 2: HE at step sectioning at three randomly chosen levels, and IHC at the first level
Method 3: HE at step sections at three fixed levels of 0.2 mm. IHC only in suspicious cases.
Method 4: HE and IHC at step sections at three fixed levels of 0.2 mm and teamwork.

Paper III

The study cohort was department-based and consisted of 1,993 consecutive women with first primary unilateral breast cancer of whom 1,458 had SNB treated at Lund University Hospital between 2001 and 2011.

Nearly all patients with micro- and macrometastases had axillary dissection, and the proportion of positive nodes in the completion axillary dissection specimen was examined according to screen-detection *vs.* clinical presentation by logistic regression analysis.

Paper IV

The study cohort consisted of 564 premenopausal stage II patients with primary breast cancer enrolled in a clinical trial between 1986 and 1991. The study is registered as ”SBII:2-premenopausal”. The randomised trial was designed to compare the effect of two years of adjuvant tamoxifen *vs.* no adjuvant systemic treatment.

ER status was assessed on tissue microarrays, initially available for 500 patients, with three different ER antibodies and heat-induced epitope retrieval (HIER) methods: 1D5 in citrate pH 6 (n=390), SP1 in Tris pH 9 (n=390) and PharmDx in citrate pH 6 (n=361). The prevalence and reproducibility at different levels of cut-offs were compared.

Results

Paper I

Of the 6,959 patients included in the study, 4,757 were node negative, 1,765 had macrometastases and 427 had micrometastases. The median observation time was 10 years and 2 months. To evaluate the impact of the adjusted RR of axillary status on overall survival, a multivariate analysis was performed including axillary lymph node status, tumour size, number of examined nodes, number of positive nodes, type of surgery and menopausal status. Due to the lack of proportional hazard rates, the analysis was stratified for histological grade, histological type and hormone receptor status. In this model, women with micrometastases had a significantly higher risk of death than did node-negative women (adjusted RR=1.49, 95% confidence interval (CI): 1.18–1.90) ($p<0.01$), which was also true for women with macrometastases in the nodes (adjusted RR=1.88, 95% CI: 1.55–2.20) ($p<0.01$).

The adjusted RR increased significantly with increasing tumour size. Patients who had breast-conserving surgery had a significantly smaller risk than did patients who underwent mastectomy, and postmenopausal women experienced a significantly higher risk than did premenopausal women. The interaction between axillary status and histological grade showed that the adjusted RR increased with increasing grade, and for women with micrometastases and histological grade 3 even more than for node-negative patients and women with macrometastases ($p=0.02$)

Paper II

The frequencies of patients treated by SNB biopsy were 63%, 68%, 69%, and 86% in the four periods with different methods of SN examination. With a cut-off of zero for classifying SNs with negative status, significantly more patients were node-positive in the last period, both among women treated by SNB and among all women ($p=0.01$). When a cut-off of 0.2 mm /<200 cells was applied for negative status, the difference was only borderline significant ($p=0.07$). IHC was used in 72%, 74%, 21% and 92% of the cases treated by SNB biopsy. The median number of analysed SNs per patient was two, and did not differ between the periods.

The proportion of patients with isolated tumour cells and micrometastases was 9% higher when Method 4 was used than with the use of Methods 1 and 2 with either only one HE section followed by IHC or one HE section and IHC followed by random step sections. Teamwork and the addition of intensive IHC at fixed levels in Method 4 resulted in 13% more patients with isolated tumour cells and micrometastases than with Method 3 where step sections at fixed intervals were used. The frequency of women with isolated tumour cells and micrometastases compared with those with macrometastases did not differ significantly between the study periods, indicating that the isolated tumour cells and micrometastases were found in patients previously classified as node-negative.

Paper III

Micrometastases of >0.2 mm/>200 cells and ≤2.0 mm were detected in 8% (62/575) of screen-detected patients compared with 12% (81/701) of symptomatic patients. Only 5% (3/61) of screen-detected patients with micrometastases, all with tumour size >15 mm (range 18-39 mm), had metastases in the completion ALND whereas this was found in 23% (18/79) of the symptomatic patients with micrometastases (p=0.013), (tumour size range 10-30 mm).

The number of symptomatic patients with micrometastases and further metastases in the completion ALND increased with increasing tumour size, except for tumours larger than 30 mm. Logistic regression analysis adjusted for method of detection, tumour size, histological grade and type of surgery showed 5 times higher odds for further metastases in the ALND in patients with symptomatic presentation *vs.* screen-detected breast cancer. All other variables remained insignificant.

Paper IV

With a cut-off of 1% the prevalence of ER-positivity was higher with SP1 (75%; 292/390) compared with 1D5 (68%; 266/390) and PharmDx (66%; 211/321). The corresponding figures for cut-off 10% were 72%, 66% and 62%, and for cut-off 50% they were 67%, 55% and 41%.

The repeatability of each antibody was excellent with overall agreement between 93% and 100% for 1D5, SP1 and PharmDx, respectively at cut-offs 1% and 10%. The positive agreement (≥93%) and negative agreement (≥92%) were also acceptable. The agreement between

antibodies showed an overall agreement of 1D5 and SP1 at cut-off 1% of 92%, a negative agreement of 77% and a positive agreement of 99%. The corresponding figures for cut-off 10% were 94%, 82% and 100%.

The overwhelming majority of discordant patients went from negative with 1D5 to positive with SP1 at cut-offs of both 1% and 10%. RFS showed that discordant patients had outcomes intermediate to the double-positive and double-negative patients, though this was not statistically significant. An equvivalent pattern appeared on comparing PharmDx at 10% cut-off and the Allred score where the addition of intensity caused the discordant cases to show an intermediate RFS. The addition of intensity by the Allred score to the PharmDx was not superior to the PharmDx alone.

Discussion

During the last ten years, the management of breast cancer patients has changed in a variety of ways. SNB is now the gold standard of axillary staging in clinically node-negative patients instead of upfront axillary dissection[36]. Until resently, axillary dissection has been the choice in the case of a positive SNB, but the need for additional axillary surgery in all these patients is currently being discussed intensively.

1. Micrometastases

The present studies show that patients with micrometastases staged by axillary dissection have a worse overall survival than do node-negative patients. In patients with micrometastases, there are a 5 times higher odds for having positive nodes in the completion ALND if the patients have a symptomatic breast cancer compared with a screen-detected breast cancer. Furthermore, examination of SNs at several fixed levels including IHC with CK is necessary to detect isolated tumour cells (ITC) and micrometastases.

Studies designed to examine a possible prognostic effect of micrometastases[35, 37-45] in SN are prone to be biased in several ways:

First, the outcome for micrometastatic patients is compared with those of the node-negative group, but is this group really node-negative? A potential metastasis in an axillary lymph node can be located in the cut face presented in the microscope, be located deeper in the FFPE block or can be left behind in the patient. In the area of ALND the estimated fraction of node-positive patients allocated to the node-negative group was as high as up to 33%[33, 46]. The false-negative rate of axillary dissection, whose magnitude is unknown, should be added. The SNB procedure has a surgical false-negative rate of the order of 5-8% compared with SNB and ALND[47, 48]. The further allocation of node-positive patients to the positive group is dependent on the intensity of examination of the SNs at the pathology laboratory. Study II shows that the method with step sections at fixed levels and CK at all levels assigned most patients to the node-positive group and thereby carries the highest probability that patients allocated to the node-negative group are in fact “true” node-negative.

Second, so far only a few studies have been population-based and a recommendation for SNB might be modified during the study period with a smaller fraction of the patients eligible in the beginning. In study II running from 2001 to 2009, 63% of all patients had SNB at the start of the period increasing to 86% at the end. This makes the "SNB" group somewhat heterogeneous influencing the frequency of micrometastases. Several large studies have shown that breast cancer patients receiving ALND where the specimen contains micrometastases have a worse outcome than do node-negative patients[49]. The frequency of patients with micrometastases in the combined group of node-negative and micrometastatic patients varies between 4% and 8%. In selected patients treated by SNB where patients with micrometastases do less well compared with node-negative patients[42, 44, 45] the frequency mentioned above is 8-11%, whereas in studies without prognostic effect the frequency is higher at 11-22%[37-40]. At first glance, this difference in micrometastatic frequency is most probably due to selection bias of patients and not by ensuring a "true" node-negative group.

Third, the criteria of axillary lymph node diagnosis have changed during the study period. Before 2002 only the node-negative, the groups with micrometastases and those with macrometastases existed. The 6th edition of AJCC and UICC[2, 3] introduced the concept of ITC. The reason for this change in classification was the documentation of the passive transportation of tissue and epithelial cells to lymph nodes, irrespective of malignant potential. Furthermore, the technique of SNB might increase the problem because the more intense examination introduces stage migration compared with ALND[50]. The mechanism behind passive transportation was known from needle tract seeding[51] though the technical details behind it differ. In addition, the criteria for classification from the 6th edition were different in AJCC and UICC. Neither could be reproduced by pathologists[52, 53], but it seems the UICC classification was better at discriminating which low-volume patients harboured additional metastases in the ALND[54]. In 2010 a clarification of the 6th classification was included in the 7th edition[4, 5] but unfortunately the inferior reproducibility remained unchanged[55]. This was underlined in a central review from the Netherlands, where 24% of studied patients changed classification[43]. Moreover, several reports on the prognostic impact of SN micrometastases have included patients from more than one period of classification, and most have done no

review to ensure that all patients are allocated to the correct axillary staging group by the same classification.

Fourth, the use of adjuvant systemic therapy has not always been reported in these studies, which might also influence the prognosis in patients with minimal spread to axillary lymph nodes[35]. Moreover, in some studies the axillary lymph node classification used in survival analysis is based on the status of SNs, though patients might have had completion ALND, disregarding positive nodes in the latter[39].

Finally, most studies describe protocols for handling the SN but the level of adherence to the protocols is often unknown.

Despite these drawbacks, the Swedish nationwide cohort study of SNB[42] (n=3,369) showed an inferior 5-year cause-specific and event-free survival rate for patients with micrometastases compared with node-negative patients at 5 years of follow-up (94.1% *vs.* 96.9% and 79.6% *vs.* 87.1%, respectively). In Cox proportional hazard regression analysis adjusted for age, tumour size, histological grade, ALND and adjuvant treatment, patients with micrometastases had inferior outcome compared with the node-negative group (HR: 3.04 (95% CI:1.19-7.77) and HR: 1.71 (95% CI: 1.05-2.80)). The inclusion period (2000 to 2004) was before the indication for SNB was expanded, and SNs were assessed according to a specific study protocol. A review was undertaken to re-classify patients according to the 6th edition of AJCC. Of the patients with micrometastases, 22% received chemotherapy and 81% were given endocrine therapy.

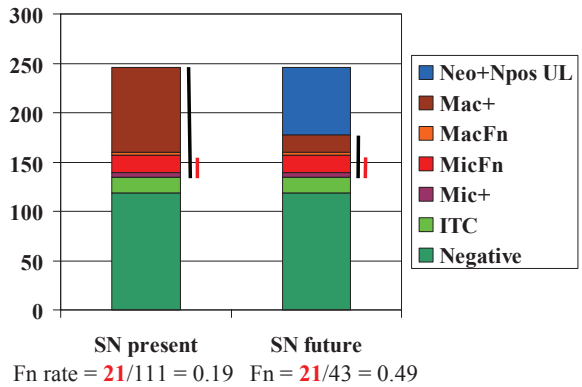
Summing up, in Sweden and Denmark breast cancer patients with micrometastases have a worse prognosis than do node-negative patients, irrespective of axillary staging with ALND or SNB primarily, confirmed in multivariate analyses. Though some authors argue that a significant difference of 3% in 10-years overall survival between node-negative patients and those with micrometastases is too small to consider[56], proper axillary staging might be one of the many reasons why Sweden has the world's best 5-years survival at 87%[57]. Similar argument was used in a resent study of occult metastases[58]. The study used the negative SNs (n=3,887) from The National Surgical Adjuvant Breast and Bowel Project (NSABP) trial B-32[59] that was designed to evaluate whether SNB alone was equivalent to complete axillary

dissection. Originally these SNs were examined by HE without step sections. Occult metastases were found in 16% after step sections and examination with CK in addition to HE. With a median follow-up of 7.9 years the adjusted hazard ratio (HR) for death for patients with occult metastases were 1.40 (95% CI: 1.05-1.85) compared with node-negative patients. The 5-years overall survival of patients with occult metastases were 94.6% as compared with node-negative of 95.8%. Read from the figure 2.a[58] the difference at 10 years is around 3 percentage points. Even though small metastases recognisable in HE stained sections are expelled from the study, this figure is in line with the results of paper I. Despite these facts, the authors argue that the statistical significant difference in 5-year survival of 1.2 percentage points is too small to consider and SNs should not be examined by step sections and CK.

Whether patients with micrometastases in the SNB require a completion ALND is quite a different question. Recently Giuliano and colleges showed no difference between SNB-positive patients with or without completion ALND in a randomised trial[60]. The trial included a subgroup of early breast cancer patients treated by breast conservation where the radiation field included the lower part of the axilla, and nearly all patients received adjuvant systemic therapy. Despite considerable discussion and comments[61-65], the study has prompted surgeons to make a rapid shift in axillary surgical treatment without further evidence. Study III describes a group of patients who will be good candidates for SNB only, i.e. screen-detected breast cancer patients with micrometastases, because they have 5 times lower odds of harbouring additional metastases in the completion ALND compared with symptomatic patients.
Teamwork around SN frozen sections of breast cancer patients is necessary to achieve high quality and at the same time keep the false-negative rate acceptable, as described in paper II.

Figure 1. Present and future scenario of sentinel node frozen sections

246 primary unilateral breast cancer patients offered sentinel node biopsy
Present SN = 246 frozen sections. 21 Fn SN and 90 with SN+ALND in one session = 37% of all
Future SN = 178 frozen sections. 21 Fn SN and 22 with SN+ALND in one session = 12% of all



Fn rate = **21**/111 = 0.19 Fn = **21**/43 = 0.49

In the future more node-positive patients will be detected by UL and 68 patients will switch from SN positive to Neo+Npos UL

SN = sentinel node. Fn = false negative frozen sevtion. ALND = axillary lymph node dissection. Neo = neoadjuvant, Npos UL = positive sentinel nodes diagnosed by ultrasound, Mac+ = node-positive with macrometastases. MacFn = false neative frozen section with macrometastases. MicFn = false neative frozen section with micrometastases. Mic+ = node-positive with micrometastases. ITC = isolated tumour cells.

The majority of frozen-section false-negative cases are found among patients with micrometastases[66] as a consequence of the technique itself for frozen sections of SNs, because lymph nodes are three-dimensional and only the central plane is presented in the frozen section. The false-negative rate for frozen sections is calculated with all FFPE-positive patients in the denominator as: False-negative rate = false-negative / (false-negative + true-positive). The false negative rate will therefore rise if the node-positive patients are left out of the SNB population (Figure 1). In this situation, patients with micrometastases with an unchanged false-negative rate will make up a larger part of the SNB-positive patients. The same conclusion can be extrapolated from the numbers in a recent meta-analysis of false-negative frozen sections[67]. Due to better ultrasound and FNA of possible axillary metastases prior to surgery[68] and the allocation of more patients to neoadjuvant protocols, the rise in the false-negative rate will soon exceed the benefit of frozen sections, and this procedure will become obsolete. Furthermore, due to

the three dimensions of a lymph node, allocation of a patient to the micrometastatic group is only possible after step section with permanent sections.

Step sections with IHC with CK are most important in the node-negative group in order to ensure that this group is as true-negative as possible. Patients with a low risk of disseminated breast cancer who can be cured by surgery alone, avoiding adjuvant systemic therapy, require the highest possible degree of certainty in their allocation to the node-negative group. Moreover, the addition of IHC with CK facilitates work for pathologists. The step section technique leaves just two possibilities for IHC: do it at all levels or not at all. The second option implies due to step sections that tissue between the levels is lost, thereby making CK at specific levels in doubtful cases unfeasible. Some histological subtypes of breast cancer (e.g. the lobular type) progress with lymph node metastases that are very difficult to diagnose in ordinary HE-stained sections. These patients must be examined by IHC with CK, and if the standard method only includes HE-stained sections, they might easily be missed because the histological diagnosis is not always known when the SNs are processed. Performing IHC with CK from the outset ensures all patients are examined equally, and benefits mostly the node-negative patients.

Results in paper I show that patients with grade 3 cancers and micrometastases have a worse outcome than do the corresponding patients with macrometastases; this might reflect different biological subtypes among the grade 3 cancers. Grade 3 cancers typically have high proliferation. The growth rate of cancer is an equilibrium between proliferation and apoptosis, taking into account the time spent in the mitosis of the proliferating nuclei. So far, IHC for apoptotic markers has largely failed in breast cancer[69]. The results of gene expression analysis for individual breast cancers might shed light on which intercellular pathways are operating in which types of breasts cancer[70, 71].

2. ER analysis

Paper IV shows that the proportion of ER-positive patients is dependent on the antibody and the HIER method. The most sensitive ER antibody in the study is SP1, pH 9, which increases the ER-positive proportion by 7% at a cut-off of 1% compared with 1D5, pH 6. The corresponding

figure at a cut-off of 10% (which is used in Sweden) is 6%. At present the most widely used antibody in Sweden is SP1 though, in line with the Swedish experience, the antibody 6F11 is even more sensitive[72, 73].

During the 1980s, IHC was introduced in pathology laboratories with the aim mainly of improving diagnoses of tumours. The biggest impact was markers that could distinguish between embryonic lineages. The result of an IHC assay is dependent on pre-analytical factors (e.g. cold ischemic time and fixation), analytical factors (e.g. antibody, HIER and visualisation system), and interpretation. In the past, every IHC analysis was optimised internally in the pathology laboratories, most commonly by adjusting the analytic factors. ER-receptor analysis by IHC was introduced in the early 1990s, and the cut-off of 10% emerged by comparison with the ligand-binding methods with, overall, approximately 85% concordance[73, 74]. Recently the possibility of false-negative ER test has giving rise to much concern, mainly because in large parts of the world patients with a false-negative ER result will be denied potentially beneficial antihormonal treatment. On the other hand, in Sweden a false-positive ER test might result in the recommendation of antihormonal treatment instead of chemotherapy.

Despite universal agreement on the importance of correct ER results in offering patients the right therapy, the IHC ER test has not been standardised. Only one test has recently obtained an FDA approval (ER/PR PharmDx™). Instead, guidelines[75-77] with a meticulous description of special steps in the pre-analytical, analytical, and interpretation settings of IHC analysis have emerged. However, the guideline[75] still recommends among other things the antibodies examined in study IV and the antibody 6F11 despite the difference in the prevalence of ER-positive patients tested by these antibodies. Before the introduction of a new ER-antibody and HIER, a comparison with a clinically validated assay must show positive agreement of ≥90% and negative agreement of ≥95% with positive regarded as immunoreactivity in ≥1% of cells. The negative agreement in study IV of SP1 compared with 1D5 was much lower, at 77% and 82% at cut-offs 1% and 10%, respectively. Despite these facts, 1D5 is rarely used in clinical practice today. Furthermore, the guidelines[75, 76] argue that the intensity of ER IHC staining should be reported and used in a combined score of the percentage of ER-positive cells and intensity, despite the fact that intensity might be fixation-and HIER-dependent. Formalin

fixation of tissue stabilises proteins via sulphate cross-bridges. HIER counteracts this to some extent by loosening these bridges again, thereby enabling the antigens to be exposed to antibodies[78]. These processes are still not fully understood but imply that the protein content by weight cannot be determined.

Another huge drawback of the IHC test is that new IHC machines function with HIER-standardised industrial buffers whose contents are proprietary to the company, and the exact contents of HIER buffers are now unknown to pathologists. Some IHC machines function only with a pH 9 buffer, which means all antibodies including the biomarkers must be shifted to HIER, pH 9. Moreover, some biomarkers are now delivered as specific kits that function with a specific IHC machine, so the pathology laboratory has little influence on the results of biomarker analysis today[79]. Simultaneously, the production of IHC analysis is becoming more labour-insensitive. In biomarker analysis using the now "old" bulk staining method[80], running negative and positive control slides in each batch sufficed alongside the positive controls provided by normal breast tissue on the slides. The new single-slide staining machines demand external positive control tissue on every slide. These positive controls are typically cut and mounted on the slide some time before the actual IHC analysis is performed, and if stored at room temperature for too long the controls might fade[81].

Future perspectives

1. Screening

The targeted screening of subgroups according to risk has been proposed[82], and in the future might replace public mammography screening. Candidates for targeted screening include, among others, patients with a risk of hereditary breast cancer.

2. Surgery

As mentioned in the discussion, axillary surgery is at present being vigorously debated. It is hoped that in the near future new protocols on SN findings will be introduced in Sweden, allocating all patients with micrometastases to a cohort study with follow-up, and randomise patients with macrometastsis to ALND or no ALND. Patients eligible for randomisation are those positive patients left after proper ultrasound of the axilla combined with FNA has detected node-positive patients, some of whom will receive ALND directly and others will be offered neoadjuvant therapy. At the other end of the spectrum are patients with early breast cancer with so little risk of nodal involvement that they perhaps do not need axillary staging at all. A randomised trial of these patients is planned[83]. The results of a large Swedish cohort of patients with early breast cancer without axillary staging with tumour size <10 mm and of histological grade 1 and 2 are soon to be published (personal communication). In the future, new modalities of minimal local invasive surgery (heat, laser[84] etc.) will perhaps replace traditional surgery in selected patients with small, often screen-detected, tumours.

3. Low-risk patients

Due to the high 5-year survival in Sweden and the nationwide public mammography screening programme running for the last 15 years, Swedish breast cancer researchers have a unique opportunity to more precisely define node-negative patients with luminal A breast cancer that can be cured by minimal surgery alone. Much more focus on this group may encompass overdiagnosis by public mammography screening and overtreatment due to the limited knowledge of the benefits from systemic therapy at the patient level. Moreover, 70% of the cost of breast cancer in Sweden is indirect costs[85]. From the Swedish

patients' perspective, living actively with children, grandchildren, family, job, careers, and a social life, the specific side effects of surgery and adjuvant therapy are more or less tolerable. Modern research for example with gene expression profiles, should focus on this problem because it is so important to offer sufficient therapy to those that can benefit and to avoid overtreatment and unnecessary side effects for those that cannot, to enable patients to continue their social lives[86-91].

4. Neoadjuvant therapy

In the future, neoadjuvant therapy will be used more extensively than today, and a new proposal is being discussed that will present studies recommending neoadjuvant therapy to all patients with tumours larger than 20 mm. This offers the possibility of assessing the response to systemic therapy "in vivo", i.e. whether this specific patient responds to the specific therapy. The therapy known to be efficient is then used adjuvantly after surgery. In the future, surgery for a subset of patients with complete regression of the cancer might even be unnecessary, or perhaps a limited procedure with some sort of heating of the residual scarring tissue will suffice. A system to evaluate the effect of neoadjuvant therapy should be developed in a collaboration between radiologists and pathologists. For example, a biopsy technique using large-core needle biopsies at fixed systematically chosen intervals over the tumour area might offer a good change of diagnosing the residual infiltration of the cancer and hence referring the patient for complementary surgery, or total regression requiring no or a limited intervention. A similar strategy combined with SNB before the start of neoadjuvant therapy might be used to target the axilla.

5. Subgross pathology

A breast cancer often combines an in situ and an invasive component. The relative amounts of each differ, but the biological meaning of the different growth patterns has been hard to understand and has therefore been neglected until recently. Using subgross pathology and pathologic/radiologic correlation the new concepts of lesion distribution, unifocal (about 40%), multifocal (40%) and diffuse breast cancers (20%), are described using the extent (largest distance of two cancer foci being in situ or invasive) to combine the in situ and invasive components. Unifocal lesions and lesions with limited extents have the

best outcome[92]. Furthermore, unifocal lesions are more often node-negative[93]. The combined procedure of minimal surgery for small tumours and expansion of the indication for neoadjuvant therapy unfortunately limits opportunities to exploit further subgross pathology and pathological/radiological correlation.

6. Histopathology

For pathologists the approach involving expansion of the indication for neoadjuvant therapy carries several challenges. First biomarkers have to be assessed on core needle biopsies. There is a need for standardization of the size of needle used and the quantity of tissue that must be represented for the report of the biomarkers. One core totally infiltrated with carcinoma has been suggested, but it seems more relevant to define an area, for example a cumulative area of 10-20 mm^2 containing infiltration of carcinoma. In addition, because chemotherapy has side effects that can be lethal, core biopsies must be stained with and reported negative for markers of myoepithelial cells even though the skilled pathologist is convinced that the diagnosis is breast cancer. This staining is also required when scoring HER2 and Ki67 and reporting the result only for the invasive component, because both HER2 and Ki67 tend to be expressed to a greater degree in the in situ component than in the invasive component. In large parts of the world, biomarker analysis is carried out only on core needle biopsies. Studies comparing results of biomarkers assessed on core needle biopsies with results from tumours in the surgical specimen have shown comparable results for ER[94, 95], but probably due to heterogeneity and perhaps differences in the cold ischemic time Ki67 results might be a little lower[96]. Due to progress in IHC methods, the prevalence of ER-positive patients is steadily increasing over the years[97] and the shift in HIER from citrate pH 6 to Tris pH 9 contributes substantially to the increase as described in paper IV.

ER has largely bimodal expression and the results are easy to reproduce, but with Ki67 there are many more problems. A recent review reporting the prognostic effect of Ki67 included 45 studies published between 1995 and 2004. The majority of the studies used the antibody MIB1, the method of Ki67 scoring was not reported, and the mean cut-off for Ki67 was 13.5% (range 0-30%)[98]. The cut-off for allocating patients to the luminal A or B group has been reported to be 13.25%[99, 100]. The last

authors used the antibody SP6, pH 9 and made no count but, "Ki67 was visually scored for percentage of tumour cell nuclei with positive immunostaining above the background level by two pathologists", i.e. they read the slides according to what the pathologist judged by eye to be the result. In Sweden, the cut-off was determined as the percentage of Ki67-positive nuclei at the 66th percentile using MIB1 in HIER with citrate pH 6. The scoring method agreed was counting 200 cells in hot spots and reporting the positive percentage[101]. This approach gives a cut-off of 20%. With the method used in Sweden, the 95% CI (exact binominal) is in fact overlapping: 20% (14-26%) and 14% (8-20%), so the Swedish method does not distinguish between 14% and 20%. Reports of a lack of reproducibility of Ki67 are now emerging[102]. The results of Ki67 are typically reported in large research studies that often use some sort of counting, and it seems that the researchers' solution is merely to count more cells[103]. This approach narrows the 95% CI but it is much too time-consuming to be implemented in everyday routine.

Due to the varying intensity of Ki67, the discussion of reproducibility often focuses on determining when a nucleus is positive or negative. Moreover, Ki67 in breast cancer is a continuous variable, which implies that irrespective of cut-off a substantial number of tumours will have values near the cut-off. Accompanying the more intensive HIER methods used today is unfortunately also a decrease in morphology of the negative elements of an IHC staining. Often it is difficult to decide whether a negative nucleus is a cancer nucleus, an endothelial cell or a fibroblast. The decision on what to count as negative cancer nuclei has a paramount influence on the positive percentage of Ki67: the denominator matters. The revival of Ki67 in breast cancer is inspired by gene expression profiles that independent of study consistently selects genes for proliferation[21]. The results of Ki67 are especially important in patients with histological grade 2 because Ki67 can split this group into one with a superior prognosis and another with an inferior outcome[104]. One alternative for ascheiving better Ki67 results could be to do the reading with inspiration from mitotic count in histological grade: i.e. counting positive nuclei per area instead of in relation to negative nuclei[105]. This method is fast and can easily be implemented in the routine. In some tumours, Ki67 is expressed very homogeneously all over the tumour area; at the other extreme are tumours with a few hot spots with very high expression at the periphery of the carcinoma.

Another approach to Ki67 evaluation might be to examine whether breast cancers can be divided into tumours with homogeneous expression and tumours with hot spots. This approach carries the drawback intrinsic to much pathology, i.e. taking a continuum of expression patterns and forcing them into boxes; allowing for a middle group of uncertainty is therefore mandatory. Subsequently it should be evaluated whether this division carries prognostic information comparable with low and high Ki67 expression. This way pathologists can perform pattern recognition, at which they excel instead of counting cells based on intensity, a difficult task for the human eye.

In the metastatic setting, pathologists also are involved in the biomarker assessment of metastases. Because ER[106] and HER2[107] in particular are known to convert in a subset of patients, re-assessment of biomarkers is important in deciding on further systemic therapy. Quality assurance and validation of analysis are also paramount in this setting[108].

7. Quality assurance

Quality assurance of the breast cancer patient process is paramount, but today there is no agreed QA programme involving the total patient process. From the pathologists' perspective, inspiration comes from industry which has long used ISO certifications. QA in industry avoids double-checking of everything. Instead there is a system with team organisation, standardisation with definitions, checklists and audit in the form of small random sample checking. Transferred to a pathology laboratory, this implies specifications of the training and workload of breast pathologists. According to EUSOMA[24] a breast pathologist responsible to the multidisciplinary team should, among other things, work in a breast unit treating at least 150 new breast cancer patients yearly, be the first author of at least one article or co-author of at least five articles dealing with breast cancer, attend an international course in breast pathology, know how to interpret IHC and FISH and know the epidemiology of breast cancer. All other pathologists participating in breast pathology must have primary responsibility for at least 50 new patients yearly.

8. Teamwork

Teamwork including all levels in a pathology laboratory is also important because everyone contributes to the outcome of the patient process. Laboratory technicians must be allowed to specialise and to have the opportunity to do the work frequently to keep up with the best standards, especially in frozen section technique, large section handling, and IHC. Secretaries are also partly responsible for the consistency in the communication of the pathology reports and partly for the internal QA at the department. The patient perspective offers the opportunity for breast pathologists to become each patient's pathologist. An important aspect of QA in breast cancer is that information is lost every time a specimen from a specific patient changes hands. No element in gross with correlation to radiology and microscopy stands alone; the final diagnosis is a combination of all of it. To maintain quality and make the time spent working with a specimen most useful, ideally the same pathologists should do both. In addition, unnecessary mistakes can be avoided if biomarkers are analysed and signed out by the pathologist responsible for the specimen. In this way, much disagreement can be dealt with directly and corrected before the pathology report reaches the patient and clinician.

9. Standardisation

The standardisation of referral forms and pathology reports is perhaps the single issue with the greatest impact on most patients. Standardization enables the pathologist to be confident of having the necessary information at hand when working with a specific patient. More importantly, the standardisation of pathology reports ensures that the message is easy to use by the clinicians, who in turn benefit the patient by ensuring the best treatment possible. The languish used in the pathology report should also be concise and easy for patients to understand. Unnecessarily long descriptions not directly pertinent to patient treatment, and abbreviations, should be avoided. The standardisation of pathology reports also offers the opportunity to monitor quality by referral of information to regional and national databases[109]. EUSOMA suggests a data manager be on the staff of the patient process, and these employees are urgently needed.

10. Audit

A second opinion is increasingly demanded in pathology. The problem with QA in breast pathology is first and foremost that the absolute "truth" does not exist. Breast pathologists have the responsibility to do their job with similar quality and care as would another skilled breast pathologist. Nevertheless, skilled pathologists can disagree and no one owns the right answer. The inspiration from industry includes a check of a small random sample. Instead of measurements as used by industry, adherence to a protocol for how a patient specimen should be handled throughout the pathology laboratory can be checked in a small random sample. The audit must include specified items taking place from the moment the specimen enters the laboratory to when the final pathology report is signed out, including the quality of biomarkers. To get the most of such a system it is paramount that all members of staff participate in the audit group.

11. Turnover time

The turnover time in the pathology laboratory is extremely important assuring the patient that the pathology report will be ready the moment she needs it, which will typically be at the planned multidisciplinary conference. New tissue-procession techniques including fixation assisted by microwaves are now available. A study comparing biomarker results with the results of conventional tissue processing is ongoing (personal communication), and the results look promising. On the assumption that the surgical department and pathology laboratory can cooperate on planning the time schedule for operations on breast cancer patients, if fully implemented this technique might reduce the turnover time in the pathology laboratory to three or four days. This time schedule for the pathological report on surgical breast cancer specimens will also contain the results of large sections and the final results of all biomarkers including in situ hybridisation of HER2.

In conclusion, fascinating perspectives of breast cancer treatment lie in the near future, and breast pathologists will play a more important role than ever, especially in ensuring the quality of the pathology part of the breast cancer patient process and in communication with clinicians.

Future studies

1. A biopsy technique using large-core needle biopsies at fixed systematically chosen intervals over the tumour area might offer a good change of diagnosing residual infiltration of cancer after neoadjuvant therapy. The system should be developed in collaboration with radiologists. The first step could be to evaluate the system with surgical specimens from patients receiving neoadjuvant therapy today followed by a step with in vivo evaluation where the radiologists do the biopsies.

2. Ki67-positivity by counting by area compared with counting by negative nuclei. A pilot study[105] of 100 patients is nearly finished with comparable results. Ideally, the study should be coupled with the effect of chemotherapy and therefore conducted on needle core biopsies from patients receiving neoadjuvant therapy. This way it may be possibly to establish a cut-off for the effect of chemotherapy.

3. To examine whether breast cancers can be divided into tumours with homogeneous Ki67 expression and those with hot spots. The first analysis should determine the frequencies in each group including a group of uncertainty. Subsequently it should be evaluated whether this separation carries prognostic or predictive information comparable with low and high Ki67 expression. A combination with histological grade might be necessary.

4. A pilot study for an audit of breast cancer pathology files in parts of Skåne is planned this autumn. Criteria to be evaluated have been specified and the intake period is currently running.

5. Comparison of biomarker analysis on conventionally treated cancer tissue and tissue fixed with the assistance of microwaves and quickly dehydrated. A study is ongoing with ER (SP1), PR (1E2), Ki67 (MIB1) and HER2 (4B5) in 94 tumours of which FISH and SISH had been performed on 20.

Conclusions

Breast cancer patients with micrometastases in lymph nodes detected by ALND have an inferior 10-year overall survival compared with node-negative patients.

SN examination with step sections at fixed levels including CK at each level is important in ensuring that the node-negative group is “true” node-negative.

Screen-detected breast cancer patients with micrometastases have 5-time lower odds for additional metastases in the completion ALND compared with symptomatic patients, and are thereby candidates for the omission of completion ALND.

The prevalence of ER-positive results is dependent on the specific antibody and HIER method used.

Quality-assured breast pathology will be in even greater demand in the future especially when molecular profiling develops and subgroups of cancers are defined for targeted treatments.

Acknowledgements

Thanks to the BMA in the breast team in the Pathology Department, Lund. At present, the team consists of Magdalena Nagi, Iren Atakhan, Nina Preis, Camilla Ståhl, Anna Nilsson and Vida Abbasi. Previous members were Nadine Olsson, Lina Karlsson, Liv Gröntoft and Dalton Baumann. Due to their enthusiastic work and open attitude, the processing of different breast cancer specimens is progressing. Thanks also to Helen Svensson in the immunohistochemical department, to Kristina Anderson for performing staining with 1D5 and SP1 and to Kristina Lövgren for performing the staining with PharmDx. Thanks to my colleges and especially to Alexander Prazic for performing highly professional work in the team. Thanks to the Pathology Department in Lund for making this work possible.

Thanks to my supervisor Professor Christian Ingvar and co-supervisors Associate Professor Lisa Rydén and Professor Mårten Fernö for continuous support during the processes of data collection and writing manuscripts.

Thanks to all team members in the breast cancer patient process in Lund for excellent cooperation and interesting discussions, and to all the breast cancer patients for whom, wee all care. Thanks to Mogens Blichert-Toft and Maj-Britt Jensen for the support that made possible the manuscript of the Danish micrometastases article. Thanks to the Unilabs Mammography Units for providing data about referral status (screening or clinical) and to Pär-Ola Bendahl for statistical assistance in Paper III.

Thanks to the participating departments of the South and South-East Swedish Breast Cancer Groups for providing samples and clinical follow-up for study IV. Thanks to the Swedish Breast Cancer Organisation (BRO), the Swedish Cancer Society, Swedish Research Council, the Gunnar B Nilsson Cancer Foundation, the Mrs. Berta Kamprad Foundation, Anna and Edwin Bergers foundation, Skåne University Hospital Research Foundation, Skåne County Council's Research and Development Foundation and Governmental Funding of Clinical Research within the National Health Service (ALF) for supporting the studies with funds.

Sammanfattning på svenska

Den svenska nationella cancerstategin från 2009 understryker patientperspektivet och fokus ligger på själva patientprocessen. Modern bröstcancerbehandling omfattar en mer individualiserad behandling med bröstbevarande kirurgi, diagnostik av portvaktskörteln, ett ökat användande av medicinsk terapi med cellgift, antihormon samt antikroppar i såväl förebyggande syfte som vid konstaterad spridning. En individuell behandling kräver dock en noggrann stadieindelning och klassning av tumören så att inte onödigtvis många patienter får behandling de inte kan ha nytta av. Ju fler som behandlas adjuvant desto fler får biverkningar där alla inte är lindriga eller försumbara.
Denna avhandling berör frågor kring bröstcancerns prognos, spridning till lymfkörtlar samt hur arbetet på patologen skall bedrivas för säkra och snabba svar på alla de parametrar som krävs för rekommendation av rätt behandling.
Fynden i avhandlingen visar att även en minimal spridning till lymfkörtlar i armhålan påverkar prognosen negativt. Sättet på vilket portvakten undersöks är väsentligt och arbete II visar att relativt täta snitt genom hela körteln krävs och att man inte bara färgar snitten med rutinfärger utan även använder antikroppsfärgningar. I samband med snabbundersökning vid operation av portvaktskörteln upptäcks inte alltid spridningen och för närvarande diskuteras om dessa patienter (med falskt negativt svar) rutinmässigt måste genomgå ytterligare en operation, för risk att fler körtlar är angripna. Arbete III visar att screeningsupptäckta cancrar med minimal spridning till lymfkörtlarna nästan aldrig har fler sjuka körtlar än portvakten varför dessa bör kunna slippa en onödig operation. I sista arbetet visas att det finns en viss skillnad mellan de kommersiella produkter för bestämning av hormonreceptorstatus (östrogen). Detta kan påverka bedömningen av vilken terapi patienten rekommenderas. Bröstcancer patientprocessen ställer stora krav på patologen för snabb och säker diagnostik vilket driver fram en omorganisation internt för kvalitetssäkring med standardisering, specialisering, inte bara med bröstpatologer utan även de biomedicinska analytikerna. Man arbetar numer i team och alla ingående kan därmed få ett bättre helhetsperspektiv från det att provet anländer tills att svaret avgår.

References

1. Danish Breast Cancer Cooperative Group. DBCG-89. Program for behandling og kontrol af patienter med primär, operabel cancer mammae. P. 74. DBCG-Sekretariatet; 1989.http://www.dbcg.dk/PDF%20Filer/DBCG%2089%20protokol.pdf.
2. AJCC Cancer Staging Handbook - TNM Classification of Malignant Tumors. 6th ed. Greene FL, Page DL, Fleming ID, Fritz A, Balch CM, Haller DG, Morrow M, editors. New York: Springer Verlag; 2002.
3. UICC TNM Classification of Malignant Tumours. 6th ed. Sobin LH, Wittekind C, editors. New York: John Wiley and Sons Inc.; 2002.
4. AJCC Cancer Staging Manual. Edge SB, Byrd DR, Compton CC, Fritz AG, Greene FL, Trotti A, eds. Springer Verlag; 2010.
5. UICC International Union Against Cancer. TNM Classification of Malignant Tumours. L H Sobin, M K Gospodarowicz, C Wittekind, eds. 7th ed.. Wiley-Blackwell; 2009.
6. Åberg A, Ericsson J, Holmberg L, Rozell B.L., Ayoubi S, Khan S, lint Å. Cancer Incidence in Sweden 2010. 2011.www.socialstyrelsen.se.
7. Nationella riktlinjer för behandling av bröstcancer. Emdin S, Fernö M, Bergh J, eds. 2012.http://www.swebcg.se/index.asp?P=NatRikt.
8. Lathund SOK. Alkner S, Borgquist S, Ridderheim M, eds. 2012.http://www.skane.se/sv/Webbplatser/tumorregistret/Lathund-Onkologiska-behandlingsprinciper-for-Skanes-onkologiska-klinik/.
9. Andersson I, Aspegren K, Janzon L, Landberg T, Lindholm K, Linell F, Ljungberg O, Ranstam J, Sigfusson B. Mammographic screening and mortality from breast cancer: the Malmo mammographic screening trial. BMJ. 1988;297(6654):943-948.
10. Dyreborg U, Axelsson C, Bak M, Bech M, Bellstrøm T, Christensen S, Grabau D Aa, Gyrd-Hansen D, Jensen A B, Rose C, Schwartz W, Jørgensen T, Bakketeig L. Mammografiscreeningen i Fyns Amt 1993-97. En medicinsk teknologivurdering. Sundhedsstyrelsen, Center for Evaluering og Medicinsk Teknologvurdering; 2004.http://www.sst.dk/publ/Publ2004/CEMTV_mammo_fyn.pdf.
11. Zackrisson S, Andersson I, Janzon L, Manjer J, Garne JP. Rate of over-diagnosis of breast cancer 15 years after end of Malmo mammographic screening trial: follow-up study. BMJ. 2006;332(7543):689-692.
12. Autier P, Koechlin A, Smans M, Vatten L, Boniol M. Mammography Screening and Breast Cancer Mortality in Sweden. J Natl. Cancer Inst. 2012.
13. Segnan N, Rosso S, Ponti A. Is the breast cancer mortality decrease in sweden due to screening or treatment? Not the right question. J Natl. Cancer Inst. 2012;104(14):1040-1041.
14. Tryggvadottir L, Gislum M, Bray F, Klint A, Hakulinen T, Storm HH, Engholm G. Trends in the survival of patients diagnosed with breast cancer in the Nordic countries 1964-2003 followed up to the end of 2006. Acta Oncol. 2010;49(5):624-631.

15. Kalager M, Zelen M, Langmark F, Adami HO. Effect of screening mammography on breast-cancer mortality in Norway. N. Engl. J Med. 2010;363(13):1203-1210.
16. Willems SM, van Deurzen CH, van Diest PJ. Diagnosis of breast lesions: fine-needle aspiration cytology or core needle biopsy? A review. J Clin Pathol. 2012;65(4):287-292.
17. Blichert-Toft M, Nielsen M, During M, Moller S, Rank F, Overgaard M, Mouridsen HT. Long-term results of breast conserving surgery vs. mastectomy for early stage invasive breast cancer: 20-year follow-up of the Danish randomized DBCG-82TM protocol. Acta Oncol. 2008;47(4):672-681.
18. Cady B. Total mastectomy and partial axillary dissection. Surg Clin North Am. 1973;53:313-318.
19. Veronesi U, Viale G, Paganelli G, Zurrida S, Luini A, Galimberti V, Veronesi P, Intra M, Maisonneuve P, Zucca F, Gatti G, Mazzarol G, De CC, Vezzoli D. Sentinel lymph node biopsy in breast cancer: ten-year results of a randomized controlled study. Ann Surg. 2010;251(4):595-600.
20. Perou CM, Sorlie T, Eisen MB, van de RM, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lonning PE, Borresen-Dale AL, Brown PO, Botstein D. Molecular portraits of human breast tumours. Nature. 2000;406(6797):747-752.
21. WHO Classification of Tumours of the Breast. Page 31. Lakhani S.R., Ellis I.O., Schnitt S.J., Tan P.H., van de Vijver M.J., eds.Lyon: International Agency for Research on Cancer (IARC); 2012.
22. Eiermann W, Bergh J, Cardoso F, Conte P, Crown J, Curtin NJ, Gligorov J, Gusterson B, Joensuu H, Linderholm BK, Martin M, Penault-Llorca F, Pestalozzi BC, Razis E, Sotiriou C, Tjulandin S, Viale G. Triple negative breast cancer: proposals for a pragmatic definition and implications for patient management and trial design. Breast. 2012;21(1):20-26.
23. Wigzell K, Halle C, Hållén J, Högberg M, Jonsson PM, Lindblom B. En nationell cancerstrategi för framtiden. 2009.http://www.regeringen.se/content/1/c6/12/09/76/9b6cd326.pdf.
24. The requirements of a specialist breast unit. This is the 2010 updated version of the revised version published in the 4th Edition of the European guidelines for quality assurance in breast cancer screening and diagnosis, supported and printed by the European Commission. Audretsch W, Bartelink H, Cataliotti L, Cuzick J, Decker T, Goldhirsch A, Marotti L, Ponti A, Rosselli Del Turco M, Rutgers E, Senn HJ, van Asperen C, van Limbergen E, Wells C, Wengstrom Y, Wilson R, eds. 2010.http://www.eusoma.org/Engx/Guidelines/Guideline.aspx?cont=breast.
25. NordiQC. 2012.http://www.nordiqc.org/.
26. UK NEQAS. 2012.http://www.ukneqas.org.uk/content/Pageserver.asp.
27. SWEDAC. 2012.http://www.swedac.se/.
28. Tot T. Cost-benefit analysis of using large-format histology sections in routine diagnostic breast care. Breast. 2010;19(4):284-288.
29. Adair FE. Clinical manifestations of early cancer of the breast. With a discussion on the subject of biopsy. N. Engl. J. Med. 1933;208:1250-1255.
30. Huvos AG, Hutter RVP, Berg JW. Significance of axillary macrometatstases and micrometastases in mammary cancer. Ann. Surg. 1971;173:44-46.

31. Fisher ER, Palekar A, Rockette H, Redmond C, Fisher B. Pathologic findings from the National Surgical Adjuvant Breast Project (Protocol No. 4). V. Significance of axillary nodal micro- and macrometastases. Cancer. 1978;42:2032-2038.
32. Rosen PP, Saigo PE, Braun DW, Weathers E, Fracchia AA, Kinne DW. Axillary micro- and macrometastases in breast cancer: prognostic significance of tumor size. Ann. Surg. 1981;194:585-591.
33. Dowlatshahi K, Fan M, Snider HC, Habib FA. Lymph node micrometastases from breast carcinoma: reviewing the dilemma. Cancer. 1997;80(7):1188-1197.
34. International (Ludwig) Breast Cancer Study Group. Prognostic importance of occult axillary lymph node micrometastases from breast cancers. Lancet. 1990;335:1565-1568.
35. de Boer M., van Deurzen CH, van Dijck JA, Borm GF, van Diest PJ, Adang EM, Nortier JW, Rutgers EJ, Seynaeve C, Menke-Pluymers MB, Bult P, Tjan-Heijnen VC. Micrometastases or isolated tumor cells and the outcome of breast cancer. N. Engl. J Med. 2009;361(7):653-663.
36. Grabau DA, Jensen MB, Blichert-Toft M, Andersen JA, Dyreborg U, Carstensen B, Al-Suliman NN, Graversen HP, Rose C. The importance of surgery and accurate axillary staging for survival in breast cancer. Eur J Surg Oncol. 1998;24(6):499-507.
37. Hansen NM, Grube B, Ye X, Turner RR, Brenner RJ, Sim MS, Giuliano AE. Impact of micrometastases in the sentinel node of patients with invasive breast cancer. J Clin Oncol. 2009;27(28):4679-4684.
38. Gobardhan PD, Elias SG, Madsen EV, Bongers V, Ruitenberg HJ, Perre CI, van DT. Prognostic value of micrometastases in sentinel lymph nodes of patients with breast carcinoma: a cohort study. Ann Oncol. 2009;20(1):41-48.
39. Maaskant-Braat AJ, van de Poll-Franse LV, Voogd AC, Coebergh JW, Roumen RM, Nolthenius-Puylaert MC, Nieuwenhuijzen GA. Sentinel node micrometastases in breast cancer do not affect prognosis: a population-based study. Breast Cancer Res Treat. 2011;127(1):195-203.
40. Gobardhan PD, Elias SG, Madsen EV, van WB, van den WF, Theunissen EB, Ernst MF, Kokke MC, van der PC, Borel R, I, Wijsman JH, Bongers V, van GJ, van DT. Prognostic value of lymph node micrometastases in breast cancer: a multicenter cohort study. Ann Surg Oncol. 2011;18(6):1657-1664.
41. Onishi T, Jinno H, Takahashi M, Hayashida T, Sakata M, Nakahara T, Shigematsu N, Mukai M, Kitagawa Y. Non-sentinel lymph node status and prognosis of breast cancer patients with micrometastatic sentinel lymph nodes. Eur Surg Res. 2010;45(3-4):344-349.
42. Andersson Y, Frisell J, Sylvan M, de Boniface J., Bergkvist L. Breast cancer survival in relation to the metastatic tumor burden in axillary lymph nodes. J Clin Oncol. 2010;28(17):2868-2873.
43. Vestjens JH, Pepels MJ, de BM, Borm GF, van Deurzen CH, van Diest PJ, van Dijck JA, Adang EM, Nortier JW, Rutgers EJ, Seynaeve C, Menke-Pluymers MB, Bult P, Tjan-Heijnen VC. Relevant impact of central pathology review on nodal classification in individual breast cancer patients. Ann Oncol. 2012.
44. Cox CE, Kiluk JV, Riker AI, Cox JM, Allred N, Ramos DC, Dupont EL, Vrcel V, Diaz N, Boulware D. Significance of sentinel lymph node micrometastases in human breast cancer. J Am Coll Surg. 2008;206(2):261-268.

45. Reed J, Rosman M, Verbanac KM, Mannie A, Cheng Z, Tafra L. Prognostic implications of isolated tumor cells and micrometastases in sentinel nodes of patients with invasive breast cancer: 10-year analysis of patients enrolled in the prospective East Carolina University/Anne Arundel Medical Center Sentinel Node Multicenter Study. J Am Coll Surg. 2009;208(3):333-340.
46. Tan LK, Giri D, Hummer AJ, Panageas KS, Brogi E, Norton L, Hudis C, Borgen PI, Cody HS, III. Occult axillary node metastases in breast cancer are prognostically significant: results in 368 node-negative patients with 20-year follow-up. J Clin Oncol. 2008;26(11):1803-1809.
47. Bergkvist L, Frisell J. Multicentre validation study of sentinel node biopsy for staging in breast cancer
26. Br. J Surg. 2005;92(10):1221-1224.
48. Gill G. Sentinel-lymph-node-based management or routine axillary clearance? One-year outcomes of sentinel node biopsy versus axillary clearance (SNAC): a randomized controlled surgical trial. Ann Surg Oncol. 2009;16(2):266-275.
49. Grabau D. Breast cancer patients with micrometastases only: is a basis provided for tailored treatment? Surg Oncol. 2008;17(3):211-217.
50. Cserni G. Minimal disease in sentinel nodes. Pathol Oncol Res. 2008;14(2):117-121.
51. Grabau DA, Andersen JA, Graversen HP, Dyreborg U. Needle biopsy of breast cancer. Appearance of tumour cells along the needle track. Eur J Surg Oncol. 1993;19(2):192-194.
52. Cserni G, Bianchi S, Boecker W, Decker T, Lacerda M, Rank F, Wells CA. Improving the reproducibility of diagnosing micrometastases and isolated tumor cells
18. Cancer. 2005;103(2):358-367.
53. Turner RR, Weaver DL, Cserni G, Lester SC, Hirsch K, Elashoff DA, Fitzgibbons PL, Viale G, Mazzarol G, Ibarra JA, Schnitt SJ, Giuliano AE. Nodal stage classification for breast carcinoma: improving interobserver reproducibility through standardized histologic criteria and image-based training
52. J. Clin. Oncol. 2008;26(2):258-263.
54. Cserni G, Bianchi S, Vezzosi V, van DP, van DC, Sejben I, Regitnig P, Asslaber M, Foschini MP, Sapino A, Castellano I, Callagy G, Arkoumani E, Kulka J, Wells CA. Variations in sentinel node isolated tumour cells/micrometastasis and non-sentinel node involvement rates according to different interpretations of the TNM definitions. Eur J Cancer. 2008;44(15):2185-2191.
55. Cserni G, Amendoeira I, Bianchi S, Chmielik E, Degaetano J, Faverly D, Figueiredo P, Foschini MP, Grabau D, Jacquemier J, Kaya H, Kulka J, Lacerda M, Liepniece-Karele I, Penuela JM, Quinn C, Regitnig P, Reiner-Concin A, Sapino A, van Diest PJ, Varga Z, Vezzosi V, Wesseling J, Zolota V, Zozaya E, Wells CA. Distinction of isolated tumour cells and micrometastasis in lymph nodes of breast cancer patients according to the new Tumour Node Metastasis (TNM) definitions. Eur J Cancer. 2011;47(6):887-894.
56. WHO Classification of Tumours of the Breast. Page 21. Lakhani S.R., Ellis I.O., Schnitt S.J., Tan P.H., van de Vijver M.J., eds.Lyon: International Agency for Research on Cancer (IARC); 2012.

57. Öppna jämförelser av cancersjukvårdens kvalitet och effektivitet. Jämförelser mellan landsting 2011. Heurgren M, ed. 2011.http://www.socialstyrelsen.se/publikationer2011-2011-8-1.
58. Weaver DL, Ashikaga T, Krag DN, Skelly JM, Anderson SJ, Harlow SP, Julian TB, Mamounas EP, Wolmark N. Effect of occult metastases on survival in node-negative breast cancer. N. Engl. J Med. 2011;364(5):412-421.
59. Krag DN, Anderson SJ, Julian TB, Brown AM, Harlow SP, Costantino JP, Ashikaga T, Weaver DL, Mamounas EP, Jalovec LM, Frazier TG, Noyes RD, Robidoux A, Scarth HM, Wolmark N. Sentinel-lymph-node resection compared with conventional axillary-lymph-node dissection in clinically node-negative patients with breast cancer: overall survival findings from the NSABP B-32 randomised phase 3 trial. Lancet Oncol. 2010;11(10):927-933.
60. Giuliano AE, Hunt KK, Ballman KV, Beitsch PD, Whitworth PW, Blumencranz PW, Leitch AM, Saha S, McCall LM, Morrow M. Axillary dissection vs no axillary dissection in women with invasive breast cancer and sentinel node metastasis: a randomized clinical trial. JAMA. 2011;305(6):569-575.
61. Carlson GW, Wood WC. Management of axillary lymph node metastasis in breast cancer: making progress. JAMA. 2011;305(6):606-607.
62. Barry M, Kell MR. Breast cancer: can axillary lymph node dissection be avoided? Eur J Surg Oncol. 2012;38(1):6-7.
63. Williams RT, Winchester DP, Yao K, Winchester DJ. Who should have or not have an axillary node dissection with breast cancer? Adv. Surg. 2012;46:1-18.
64. Guth U, Myrick ME, Viehl CT, Schmid SM, Obermann EC, Weber WP. The post ACOSOG Z0011 era: Does our new understanding of breast cancer really change clinical practice? Eur J Surg Oncol. 2012;38(8):645-650.
65. Kumar A, Puri R, Gadgil PV, Jatoi I. Sentinel lymph node biopsy in primary breast cancer: window to management of the axilla. World J Surg. 2012;36(7):1453-1459.
66. Grabau DA, Rank F, Friis E. Intraoperative frozen section examination of axillary sentinel lymph nodes in breast cancer. APMIS. 2005;113(1):7-12.
67. Liu LC, Lang JE, Lu Y, Roe D, Hwang SE, Ewing CA, Esserman LJ, Morita E, Treseler P, Leong SP. Intraoperative frozen section analysis of sentinel lymph nodes in breast cancer patients: a meta-analysis and single-institution experience. Cancer. 2011;117(2):250-258.
68. Yang WT. Staging of breast cancer with ultrasound. Semin. Ultrasound CT MR. 2011;32(4):331-341.
69. Sirvent JJ, Aguilar MC, Olona M, Pelegri A, Blazquez S, Gutierrez C. Prognostic value of apoptosis in breast cancer (pT1-pT2). A TUNEL, p53, bcl-2, bag-1 and Bax immunohistochemical study. Histol. Histopathol. 2004;19(3):759-770.
70. Scan-B. 2010.http://scan.bmc.lu.se/index.php/South_Sweden_Cancerome_Analysis_Network_-_Breast_(sv).
71. Loi S, Haibe-Kains B, Desmedt C, Lallemand F, Tutt AM, Gillet C, Ellis P, Harris A, Bergh J, Foekens JA, Klijn JG, Larsimont D, Buyse M, Bontempi G, Delorenzi M, Piccart MJ, Sotiriou C. Definition of clinically distinct molecular

subtypes in estrogen receptor-positive breast carcinomas through genomic grade. J Clin Oncol. 2007;25(10):1239-1246.
72. SweQA. Ferno M, ed. 2012.http://www.swebcg.se/index.asp?P=SweQA.
73. Dowsett M. Estrogen receptor: methodology matters. J Clin Oncol. 2006;24(36):5626-5628.
74. Grabau DA, Thorpe SM, Knoop A, Vach W, Schroder HD, Blichert-Toft M, Al-Suliman NN, Graversen HP, Rose C. Immunohistochemical assessment of oestrogen and progesterone receptors: correlations with the DCC method and clinical outcome in primary breast cancer patients. Breast. 2000;9(4):208-217.
75. Hammond ME, Hayes DF, Dowsett M, Allred DC, Hagerty KL, Badve S, Fitzgibbons PL, Francis G, Goldstein NS, Hayes M, Hicks DG, Lester S, Love R, Mangu PB, McShane L, Miller K, Osborne CK, Paik S, Perlmutter J, Rhodes A, Sasano H, Schwartz JN, Sweep FC, Taube S, Torlakovic EE, Valenstein P, Viale G, Visscher D, Wheeler T, Williams RB, Wittliff JL, Wolff AC. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer (unabridged version). Arch Pathol Lab Med. 2010;134(7):e48-e72.
76. Fitzgibbons PL, Murphy DA, Hammond ME, Allred DC, Valenstein PN. Recommendations for validating estrogen and progesterone receptor immunohistochemistry assays. Arch Pathol Lab Med. 2010;134(6):930-935.
77. Yaziji H, Taylor CR, Goldstein NS, Dabbs DJ, Hammond EH, Hewlett B, Floyd AD, Barry TS, Martin AW, Badve S, Baehner F, Cartun RW, Eisen RN, Swanson PE, Hewitt SM, Vyberg M, Hicks DG. Consensus recommendations on estrogen receptor testing in breast cancer by immunohistochemistry. Appl. Immunohistochem. Mol. Morphol. 2008;16(6):513-520.
78. Shi SR, Shi Y, Taylor CR. Antigen retrieval immunohistochemistry: review and future prospects in research and diagnosis over two decades. J Histochem. Cytochem. 2011;59(1):13-32.
79. CLSI. Quality Assurance for Design Control and Implementation of Immunohistochemistry assays; Approved Guidelines - Second Edition. CLSI document I/LA28-A2. ed. Hewitt S.M., Robinowitz M, Bogen SA, Gown A.M., Kalra KL, Otis CN, Spaulding B, Taylor CR, editors. Wayne, PA: Clinical and Laboratory Standards Institute: 2011.
80. Pfeiffer P, Grabau DA, Nielsen O, Clausen PP. Immunohistochemical bulk staining of slides using a rack peroxidase-labeled streptavidin-biotin techinque. Appl. Immunohistochem. 1996;4:135-138.
81. Grabau DA, Nielsen O, Hansen S, Nielsen MM, Lænkholm A-V, Knoop A, Pfeiffer P. Influence of storage temperature and high-temperature antigen retrieval buffers on results of immunohistochemical staining in sections stored for long periods. Appl. Immunohistochem. 1998;6:209-213.
82. Vannier MW. Screening Mammography: What Good Is It and How Can We Know If It Works? J Natl. Cancer Inst. 2012.
83. Gentilini O, Veronesi U. Abandoning sentinel lymph node biopsy in early breast cancer: A new trial in progress at the European Institute of Oncology of Milan (SOUND: Sentinel node vs Observation after axillary UltraSouND). Breast. 2012.

84. Haraldsdottir KH, Ivarsson K, Gotberg S, Ingvar C, Stenram U, Tranberg KG. Interstitial laser thermotherapy (ILT) of breast cancer. Eur J Surg Oncol. 2008;34(7):739-745.
85. Lidgren M, Wilking N, Jonsson B. Cost of breast cancer in Sweden in 2002. Eur J Health Econ. 2007;8(1):5-15.
86. Hoyer M, Nordin K, Ahlgren J, Bergkvist L, Lambe M, Johansson B, Lampic C. Change in Working Time in a Population-Based Cohort of Patients With Breast Cancer. J Clin Oncol. 2012.
87. Eaker S, Halmin M, Bellocco R, Bergkvist L, Ahlgren J, Holmberg L, Lambe M. Social differences in breast cancer survival in relation to patient management within a National Health Care System (Sweden). Int. J Cancer. 2009;124(1):180-187.
88. Shah C, Wilkinson JB, Baschnagel A, Ghilezan M, Riutta J, Dekhne N, Balaraman S, Mitchell C, Wallace M, Vicini F. Factors associated with the development of breast cancer-related lymphedema after whole-breast irradiation. Int. J Radiat. Oncol Biol. Phys. 2012;83(4):1095-1100.
89. Sjovall K, Attner B, Englund M, Lithman T, Noreen D, Gunnars B, Thome B, Olsson H, Petersson IF. Sickness absence among cancer patients in the pre-diagnostic and the post-diagnostic phases of five common forms of cancer. Support. Care Cancer. 2012;20(4):741-747.
90. Lundstedt D, Gustafsson M, Steineck G, Malmstrom P, Alsadius D, Sundberg A, Wilderang U, Holmberg E, Johansson KA, Karlsson P. Risk factors of developing long-lasting breast pain after breast cancer radiotherapy. Int. J Radiat. Oncol Biol. Phys. 2012;83(1):71-78.
91. Montazeri A. Health-related quality of life in breast cancer patients: a bibliographic review of the literature from 1974 to 2007. J Exp. Clin Cancer Res. 2008;27:32.
92. Tot T, Gere M, Pekar G, Tarjan M, Hofmeyer S, Hellberg D, Lindquist D, Chen TH, Yen AM, Chiu SY, Tabar L. Breast cancer multifocality, disease extent, and survival. Hum. Pathol. 2011;42(11):1761-1769.
93. Tot T. Axillary Lymph Node Status in Unifocal, Multifocal, and Diffuse Breast Carcinomas: Differences Are Related to Macrometastatic Disease. Ann Surg Oncol. 2012.
94. Arnedos M, Nerurkar A, Osin P, A'Hern R, Smith IE, Dowsett M. Discordance between core needle biopsy (CNB) and excisional biopsy (EB) for estrogen receptor (ER), progesterone receptor (PgR) and HER2 status in early breast cancer (EBC). Ann Oncol. 2009;20(12):1948-1952.
95. Nofech-Mozes S, Vella ET, Dhesy-Thind S, Hagerty KL, Mangu PB, Temin S, Hanna WM. Systematic review on hormone receptor testing in breast cancer. Appl. Immunohistochem. Mol. Morphol. 2012;20(3):214-263.
96. Romero Q, Bendahl PO, Klintman M, Loman N, Ingvar C, Ryden L, Rose C, Grabau D, Borgquist S. Ki67 proliferation in core biopsies versus surgical samples - a model for neo-adjuvant breast cancer studies. BMC. Cancer. 2011;11:341.
97. Grabau DAa. A population-based analysis of breast cancers in a region at Funen, 1980 - 1990. Relevance of histological, biological, and clinical prognostic factors. Ph. d thesis. 1995;57-68.

98. Stuart-Harris R, Caldas C, Pinder SE, Pharoah P. Proliferation markers and survival in early breast cancer: a systematic review and meta-analysis of 85 studies in 32,825 patients. Breast. 2008;17(4):323-334.
99. Goldhirsch A, Wood WC, Coates AS, Gelber RD, Thurlimann B, Senn HJ. Strategies for subtypes--dealing with the diversity of breast cancer: highlights of the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011
23. Ann Oncol. 2011;22(8):1736-1747.
100. Cheang MC, Chia SK, Voduc D, Gao D, Leung S, Snider J, Watson M, Davies S, Bernard PS, Parker JS, Perou CM, Ellis MJ, Nielsen TO. Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer. J Natl. Cancer Inst. 2009;101(10):736-750.
101. Grabau D, Kovács A, Tolockiene E, Sylvan M, Tot T, Stemme S. KVAST dokument brösttumörer. 2011.http://www.svfp.se/node/214.
102. Varga Z, Diebold J, Dommann-Scherrer C, Frick H, Kaup D, Noske A, Obermann E, Ohlschlegel C, Padberg B, Rakozy C, Sancho OS, Schobinger-Clement S, Schreiber-Facklam H, Singer G, Tapia C, Wagner U, Mastropasqua MG, Viale G, Lehr HA. How reliable is Ki-67 immunohistochemistry in grade 2 breast carcinomas? A QA study of the Swiss Working Group of Breast- and Gynecopathologists. PLoS. One. 2012;7(5):e37379.
103. Dowsett M, Nielsen TO, A'Hern R, Bartlett J, Coombes RC, Cuzick J, Ellis M, Henry NL, Hugh JC, Lively T, McShane L, Paik S, Penault-Llorca F, Prudkin L, Regan M, Salter J, Sotiriou C, Smith IE, Viale G, Zujewski JA, Hayes DF. Assessment of Ki67 in breast cancer: recommendations from the International Ki67 in Breast Cancer working group. J Natl. Cancer Inst. 2011;103(22):1656-1664.
104. Klintman M, Bendahl PO, Grabau D, Lovgren K, Malmstrom P, Ferno M. The prognostic value of Ki67 is dependent on estrogen receptor status and histological grade in premenopausal patients with node-negative breast cancer. Mod. Pathol. 2010;23(2):251-259.
105. Gaspar V, Bendahl P-O, Fernö M, Grabau D. A fast and easy way to determine Ki-67 in breast cancer patients. Unpublished. Breast. 2012.
106. Bernsdorf M, Balslev E, Lykkesfeldt AE, Kroman N, Harder E, von der MH, Jakobsen EH, Grabau D, Ejlertsen B. Value of post-operative reassessment of estrogen receptor alpha expression following neoadjuvant chemotherapy with or without gefitinib for estrogen receptor negative breast cancer. Breast Cancer Res Treat. 2011;128(1):165-170.
107. Lindstrom LS, Karlsson E, Wilking UM, Johansson U, Hartman J, Lidbrink EK, Hatschek T, Skoog L, Bergh J. Clinically used breast cancer markers such as estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 are unstable throughout tumor progression. J Clin Oncol. 2012;30(21):2601-2608.
108. Domanski AM, Monsef N, Domanski HA, Grabau D, Ferno M. Comparison of the oestrogen and progesterone receptor status in primary breast carcinomas as evaluated by immunohistochemistry and immunocytochemistry: a consecutive series of 267 patients. Cytopathology. 2012.
109. INCA.
2012.http://www.cancercentrum.se/sv/INCA/kvalitetsregister/Brostcancer1/.

Paper I

APMIS 115: 828–37, 2007
Printed in Denmark . All rights reserved

ISSN 0903-4641

Axillary lymph node micrometastases in invasive breast cancer: national figures on incidence and overall survival

D. GRABAU, M. B. JENSEN, F. RANK and M. BLICHERT-TOFT

DBCG Secretariat, Copenhagen University Hospital, Copenhagen, Denmark

Grabau D, Jensen MB, Rank F, Blichert-Toft M. Axillary lymph node micrometastases in invasive breast cancer: national figures on incidence and overall survival. APMIS 2007;115:828–37.

The purpose of this study was to estimate the incidence and prognostic value of axillary lymph node micrometastases (Nmic) of 2 mm or less in breast carcinomas. Results are based on data from the Danish Breast Cancer Cooperative Group (DBCG). The study was carried out as a nationwide, population-based trial with a study series consisting of 6,959 women under 75 years of age registered in the national DBCG data base from 1 January 1990 to 31 October 1994. All patients had contracted operable primary breast carcinoma, stage I–III, classified according to the TNM system as T1-T3, N0-N1, M0. Women with four or more metastatic axillary lymph nodes were excluded. All patients were treated systematically according to approved national guidelines and treatment protocols. Metastases were recognized microscopically on haematoxylin and eosin-stained sections. In case of doubt immunohistochemical staining for cytokeratin was performed. There was no serial sectioning. Micrometastases were tumour deposits of 2 mm or smaller, and accordingly included deposits of 0.2 mm and smaller. With a median observation time of 10 years and 2 months, women with Nmic (N=427) experienced a significantly worse overall survival (OS) compared with node-negative (Nneg) women (N=4,767) (relative risk (RR)=1.20, 95% CI: 1.01–1.43), irrespective of menopausal status. Women with macrometastases (Nmac) (N=1,765) had significantly worse final outcome than women with Nmic (RR=1.54, 95% CI: 1.29–1.85), irrespective of menopausal status. Multivariate analysis adjusted for patient-, histopathologic-, and loco-regional therapeutic variables showed that cases with Nmic had a significantly higher risk of death relative to Nneg cases (adjusted RR=1.49, 95% CI: 1.18–1.90). Interaction analysis showed that the number of nodes examined had a significant impact on adjusted relative risk of death according to axillary status. Furthermore, the number of nodes involved significantly influenced adjusted risk of death in the Nmic compared to the Nmac series. In conclusion, the results of the present study revealed worse final outcome in women with Nmic compared with Nneg, where all Nmic cases received adjuvant systemic treatment. Interaction analysis showed that the number of retrieved axillary nodes and the number of affected nodes had a different influence on survival related to axillary status. The different risk pattern in Nmic *vs* Nmac patients indicates that Nmic cases do not show the traditional risk pattern as revealed by the Nmac cases, in which increasing number of positive nodes is associated with an orderly increasing adjusted RR.

Key words: Breast carcinoma; axillary micrometastases; incidence; overall survival.

Dorthe Grabau, Dept. of Pathology and Cytology, Lund University Hospital, 221 85 Lund, Sweden. e-mail: dorthe.grabau@skane.se

Axillary lymph node status generally constitutes the most powerful prognostic factor in breast carcinoma. Parallel with the implementation of more dedicated methods of breast cancer diagnosis and treatment with respect to early detection, limited surgery, sentinel lymph node biopsy, full axillary dissection in node-positive (Npos) patients, and adjuvant therapy in risk groups, more attention has been directed towards lymph node status, especially focusing on the size of metastases. The WHO TNM classi-

Received 8 February 2006.
Accepted 15 March 2007.

fication of carcinomas of the breast (1) and the American Joint Committee on Cancer Staging Manual, sixth edition (2), classify cases with metastases of 0.2 mm or smaller, including isolated tumour cells, as node negative (Nneg), while cases with micrometastasis between 0.2 and 2 mm are classified as Npos. Before taking the clinical consequences of such a distinction it is important to know more about the prognostic value of micrometastases.

The aim of this study was to estimate the incidence and prognostic significance of non-sentinel axillary lymph node micrometastases of 2 mm or smaller in a large series of unselected breast cancer patients registered in the national data base. Routine methods were applied in the handling of the axillary specimen, i.e. no serial sectioning of lymph nodes. The routine set up also implies that all women with axillary micrometastases independent of size were classified as Npos and for that reason received postoperative adjuvant systemic therapy.

MATERIALS AND METHODS

Patients

The study population consisted of women registered in the national Danish Breast Cancer Cooperative Group (DBCG) data base according to approved guidelines. Entry took place from 1 January 1990 to 31 October 1994. The patients were under 75 years of age and defined as axillary Nneg or Npos with ≤ 3 metastatic nodes irrespective of size of the metastases. Patients with four or more positive nodes were excluded. All patients had contracted operable primary breast carcinoma of stage I–III classified in the TNM system as T1–T3, N0–N1, M0. Patients with *in situ* lesions were excluded. If axillary status initially was unknown the original pathology files were re-examined. In the Copenhagen area, mammography screening started in 1991, with the result that less than 10% of the study population aged 50 to 69 years had undergone prevalence screening (1st round). Composition of the study material appears in Table 1. The final study population comprised 6,959 women. The study was completed by October 2003, and the median observation time was 10 years and 2 months. Patients entered the DBCG protocols following nationwide inclusion criteria, and exclusions were according to general protocol directions (3).

Therapeutic design (Fig. 1)

The basic surgical procedure included total mastectomy and lower axillary dissection (level I-II) or breast-conserving surgery and axillary dissection (level I-II) followed by radiotherapy of the residual breast. Sentinel lymph node biopsy was not used at the time. Menopausal status was defined according to DBCG criteria, and adjuvant systemic therapy was administered systematically according to DBCG national guidelines. Patients were divided into a low-risk group without adjuvant systemic therapy and a high-risk group. Low-risk patients had tumour size of 50 mm or less and no lymph node metastases, and premenopausal patients with ductal carcinomas had histological malignancy grade I. High-risk patients had tumour size of more than 50 mm and/or lymph node metastases. Premenopausal Nneg patients with ductal carcinomas of histological malignancy grade II and III were also assigned to the high-risk group (Fig. 1). Receptor status was not a determinant for risk group allocation. No patients in the study group received radiotherapy of the axilla. Patients were followed regularly according to protocol recommendations. Case report forms ran for 10 years. Patient records in the DBCG registry were linked with records in the Danish Civil Registration System Registry to obtain complete information on vital status.

Pathology

Tumour size was measured in millimetres by the pathologist as the maximum diameter of the invasive component. Histological malignancy grading was performed using a modified version of the grading system of Scaff and Bloom & Richardson (4). The axillary specimens were examined by the pathologist either on fresh tissue or tissue fixed in formaldehyde. All retrieved lymph nodes were identified and paraffin embedded. All lymph nodes were totally embedded. Nodes of 5 mm or more were isolated and bisected, and both halves were embedded. Smaller nodes were not bisected. One section from each block was examined. Metastases were recognized microscopically on haematoxylin and eosin-stained sections. In case of doubt, immunohistochemical staining for cytokeratin was used. Serial sectioning was not applied. Micrometastasis was defined as tumour deposits of 2 mm or smaller. Deposits of 0.2 mm or less including isolated tumour cells were not subclassified in the present study.

Statistics

Association between axillary status and other characteristics was analysed by chi-square test in contingency tables. Survival time included time between the date of primary operation and death of all causes or 1 October 2003, whichever occurred first. Survival rates were estimated by the Kaplan-Meier method. Log-rank test was used for univariate comparison. The Cox proportional hazards regression model was applied to assess the relative risk and adjusted relative risk of death according to axillary status. Factors included in the multivariate analysis were axillary

TABLE 1. *Composition of the study population*

Study material	
Women in DBCG protocols operated on between 1 Jan 1990 and 31 Oct 1994	8,629
Excluded were patients with:	
>3 positive nodes	1,436
Treated outside protocols:	
Premenopausal grade II–III who are Nneg and have tumour size ≤50 mm from one oncology centre	117
Receptor negative and ≤70 years who are Npos or have tumour size >50 mm	34
Unknown micrometastatic status of axillary lymph nodes	83
Total	6,959

lymph node status, tumour size, histological type, histological malignancy grade, number of examined nodes, number of positive nodes, type of surgery, menopausal status, and hormone receptor status, all being of statistical significance in both univariate and multivariate analysis. All were included categorically as presented in Table 3. Interactions were investigated by including the Cox model parameters representing interactions between axillary lymph node status and other histopathological variables. The assumptions of proportional hazards were checked by log(-log)S plots and by including a time-dependent component in the model. The hazard rates of histological type, histological malignancy grade and hormone receptor status were not proportional, and therefore stratification was used. The level of statistical significance was set at 5%. All the estimated p-values were two-tailed. SAS 8.2 was used for statistical analysis.

RESULTS

In the study group including women with up to three metastatic axillary lymph nodes, micrometastases (Nmic) only occurred in 6% of all women and in 8% of the combined series of Nneg and Nmic patients. The median tumour size reached 19 mm (range 1–200 mm). Median age was 56 years (range 24–74 years). Carcinomas of ductal type occurred in 80% of the cases, and hereof the frequency of histological malignancy grade I, II, and III amounted to 41%, 39% and 16% (unknown 4%), respectively. 32% of the women were classified as premenopausal and 68% as postmenopausal. Surgical procedures consisted of mastectomy in 75% of cases and breast-conserving therapy in 25%. Median number of examined axillary nodes reached nine nodes (range 1–37).

Associations (Table 2)

Compared with Nneg women, Nmic women were significantly more often premenopausal and of younger age. Women with Nmic had larger tumour size compared with Nneg women, albeit smaller tumour size if compared with Nmac women. A histological malignancy grade II more often appeared among Nmic than Nneg cases. Compared with Nmac cases, a histological malignancy grade I appeared more frequently among Nmic cases. It is noteworthy that a higher proportion of Nmic cases had 11 or more lymph nodes retrieved compared with both Nneg and Nmac cases. Women with Nmic more often underwent mastectomy compared with Nneg women ($p=0.04$), whereas when compared with women with Nmac breast conservation prevailed ($p=0.03$).

Univariate analysis (Fig. 2)

Women with Nmic had a significantly lower OS than Nneg women (relative risk (RR)=1.20, 95% CI: 1.01–1.43). This was found overall ($p=0.04$), in premenopausal ($p=0.04$) and in postmenopausal women ($p=0.03$). Women with Nmac experienced significantly inferior OS than women with Nmic among all women (RR=1.54, 95% CI: 1.29–1.85) ($p<0.01$), premenopausal ($p<0.01$) and postmenopausal women ($p<0.01$).

Multivariate analysis (Table 3)

To evaluate the adjusted RR of axillary status on survival we performed a multivariate analysis that included axillary lymph node status, tumour size, histological type, histological malignancy grade, number of examined nodes, number of

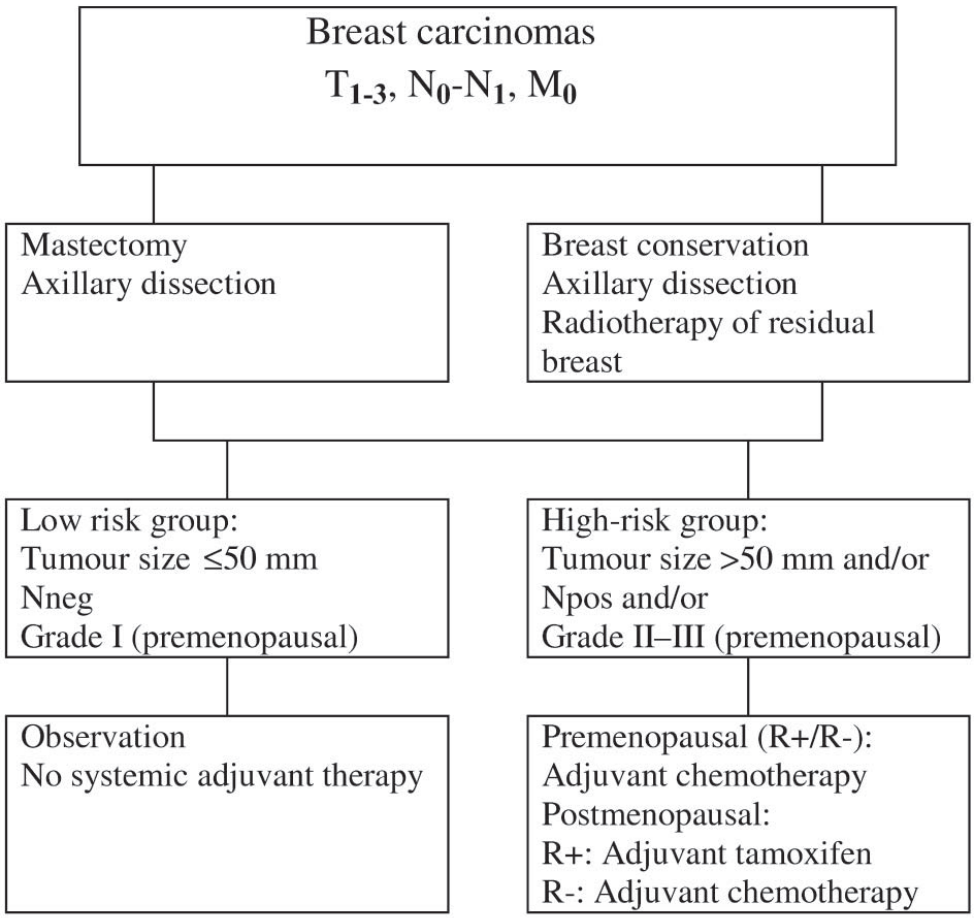


Fig. 1. Risk groups and primary therapy.

positive nodes, type of surgery, menopausal status, and hormone receptor status. In this model, women with Nmic had a significantly higher risk of death than women who were Nneg (adjusted RR =1.49, 95% CI: 1.18–1.90) ($p<0.01$). As for Nmac women, the risk of death was significantly higher than for the Nneg women (adjusted RR= 1.88, 95% CI: 1.55–2.20) ($p<0.01$). The adjusted RR increased significantly with increasing tumour size. Cases with few lymph nodes examined had a higher RR than cases with more lymph nodes removed. The RR increased significantly with increasing number of positive lymph nodes. Patients who had breast-conserving surgery had a significantly smaller risk than patients who underwent mastectomy. Postmenopausal women experienced a significantly higher risk than premenopausal women.

Interactions (Table 4)

To evaluate the adjusted RR of death according to axillary status in relation to other prog-

TABLE 2. *Patient and tumour characteristics* vs *nodal status*

	Nneg		Nmic≤2 mm		Nmac> 2 mm		p	p
	N	%	N	%	N	%	Nneg-Nmic	Nmic-Nmac
Total	4767		427		1765			
Tumour size							<0.01	<0.01
0–10 mm	990	*21*	57	*13*	148	*8*		
11–20 mm	2,195	*46*	188	*44*	708	*40*		
21–30 mm	981	*21*	112	*26*	494	*28*		
31–50 mm	348	*7*	48	*11*	286	*16*		
>50 mm	62	*1*	13	*3*	91	*5*		
Unknown	191	*4*	9	*2*	38	*2*		
Histological type							<0.01	0.42
Ductal	3,681	*77*	367	*86*	1,481	*84*		
Lobular	553	*12*	42	*10*	184	*10*		
Special types	490	*10*	14	*3*	82	*5*		
Unknown	43	*1*	4	*1*	18	*1*		
Histological grade							<0.01	<0.01
Grade I	1,651	*45*	140	*38*	463	*31*		
Grade II	1,291	*35*	169	*46*	692	*47*		
Grade III	550	*15*	47	*13*	281	*19*		
Unknown	189	*5*	11	*3*	45	*3*		
Non-ductal	1,086		60		284			
Number of examined nodes							<0.01	<0.01
1–6	977	*21*	70	*16*	424	*24*		
7–10	1,913	*40*	136	*32*	689	*39*		
=11	1,877	*39*	221	*52*	652	*37*		
Number of positive nodes								
Negative	4,767	*100*	0	*0*	0	*0*		<0.01
1	0	*0*	296	*69*	838	*47*		
2	0	*0*	101	*24*	564	*32*		
3	0	*0*	30	*7*	363	*21*		
Type of surgery							0.04	0.03
Mastectomy	3,438	*72*	328	*77*	1,438	*81*		
Breast-conserving surgery	1,329	*28*	99	*23*	327	*19*		
Menopausal status							<0.01	0.16
Premenopausal	1,411	*30*	174	*41*	655	*37*		
Postmenopausal	3,356	*70*	253	*59*	1,110	*63*		
Age, years							<0.01	0.44
<40	254	*5*	33	*8*	120	*7*		
40–49	1,116	*23*	130	*30*	503	*28*		
50–59	1,403	*29*	134	*31*	535	*30*		
60–69	1,498	*31*	98	*23*	484	*27*		
70–74	496	*10*	32	*7*	123	*7*		
Risk Group								
Low risk, no systemic therapy	4,254	*89*	0	*0*	0	*0*		
High risk with systemic therapy	513	*11*	427	*100*	1765	*100*		

Nneg=node negative, Nmic=micrometastases, Nmac=macrometastases.

TABLE 3. *Multivariate analysis of overall survival (OS). Stratified for histological malignancy grade*

	Multivariate analysis	
	Adjusted RR* (95% CI)	p
Axillary status		
Nneg	1 (reference)	
Nmic	1.49 (1.18–1.90)	<0.01
Nmac	1.88 (1.55–2.29)	<0.01
Tumour size		
0–10 mm	0.72 (0.62–0.83)	<0.01
11–20 mm	1 (reference)	
21–30 mm	1.22 (1.10–1.35)	<0.01
>30 mm	1.45 (1.28–1.64)	<0.01
Number of examined nodes		
1–6	1.18 (1.06–1.31)	<0.01
7–10	1 (reference)	
>10	0.92 (0.83–1.01)	0.08
Number of positive nodes		
1	1 (reference)	
2	1.29 (1.11–1.51)	<0.01
3	1.64 (1.38–1.94)	<0.01
Type of surgery		
Mastectomy	1 (reference)	
Breast-conserving surgery	0.82 (0.74–0.92)	<0.01
Menopausal status		
Premenopausal	1 (reference)	
Postmenopausal, low risk	2.21 (1.91–2.56)	<0.01
Postmenopausal, high risk	1.44 (1.25–1.66)	<0.01

Nneg=node negative, Nmic=micrometastases, Nmac=macrometastases.
* Adjusted for characteristics listed and stratified for histological type, histological malignancy grade, and hormone receptor status.

nostic variables, we decided to include interactions between axillary lymph node status *vs* number of examined nodes, *vs* number of positive nodes, *vs* tumour size and *vs* histological malignancy grade. The number of examined nodes had a different influence on survival in Nneg compared with the combined Nmic and Nmac group ($p<0.01$). Nmic cases with 1–6 examined nodes had an adjusted RR of 1.60 (95% CI: 1.00–2.55) relative to cases with 7–10 examined nodes. The similar figure for Nmac cases rose to a value of adjusted RR=1.37 (95% CI: 1.15–1.64), whereas the Nneg cases had an adjusted RR=1.04 (95%CI: 0.90–1.20). Consequently, the number of examined nodes is crucial only in involved nodes, marked by the high RR of a few examined nodes. The effect of number of positive nodes differed significantly in Nmic as compared to Nmac cases ($p=0.04$). Women with Nmic and only one positive lymph node had an adjusted RR of 1.12 (95% CI: 0.74–1.69) relative to cases with two affected nodes. In the Nmac group, the analogous value showed an adjusted RR=0.72 (95% CI: 0.61–0.85). The adjusted RR increased with increasing grade, and for Nmic histological malignancy grade III even more than for Nneg and Nmac ($p=0.02$) (data not shown). No interaction between axillary status and tumour size was found ($p=0.63$).

Case load

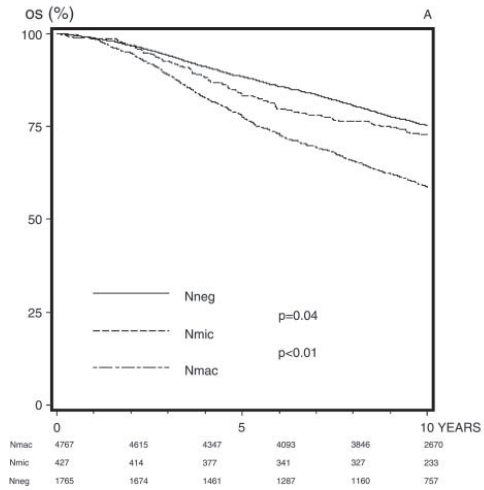
Departments performing a greater number of surgical operations had significantly more cases with many lymph nodes removed compared with departments performing only a few operations ($p<0.01$) (Table 5).

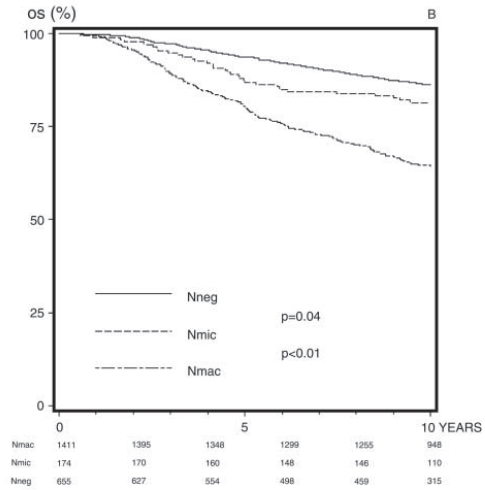
DISCUSSION

The study shows a significantly worse final outcome for women with Nmic compared to women with Nneg, although better than for Nmac. It should be emphasized that Nmic women routinely received adjuvant systemic therapy. To our knowledge this is the largest study to date showing population-based national figures on incidence rates and prognostic value of micrometastases in a multivariate analysis.

The routine set up implies that the lymph nodes are bisected and each half is examined in one single haematoxylin and eosin-stained section. No serial sectioning occurred and therefore possibly some of the micrometastases might in fact have been a macrometastasis tangentially cut. However, the differences in OS between metastatic subgroups (Fig. 2) seem to indicate that there is only minor overlap—if any—between Nmic and Nmac.

Most studies examining micrometastases performed some sort of serial sectioning and/or immunohistochemical stains to detect the occurrence of micrometastases (5). However, a few studies did not carry out serial sectioning. Some studies demonstrated prognostic significance of





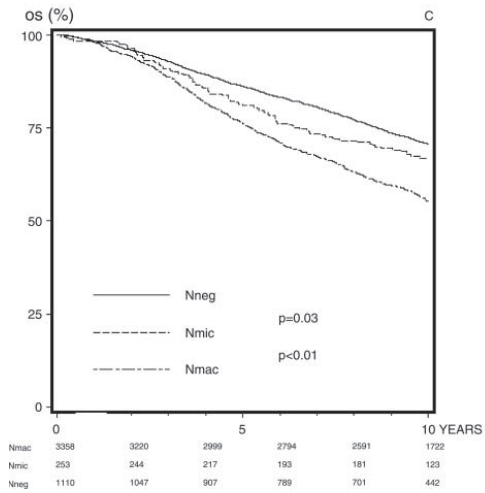


Fig. 2. Overall survival (OS) in years from primary surgery of Nneg, Nmic and Nmac patients. In all women (A), premenopausal women (B), and postmenopausal women (C).

micrometastases (6–10). In 1997, a review (11) concluded that more recent studies with larger patient series and longer follow up tended to show prognostic significance of axillary micrometastases while earlier small studies failed to do so.

A recent study by Kuijt et al. (12) reports significantly inferior survival of women with micrometastases (N=87) compared with Nneg women in a multivariate analysis after excluding 92 Nmic patients receiving adjuvant systemic therapy. On the other hand, if one examines the combined group of Nmic patients (N=179) with or without adjuvant systemic therapy, overall survival did not differ significantly between Nneg *vs* Nmic patients. The patients in the series were treated some 15 calendar years prior to the patients in the present study. Poorer outcome of Nmic patients was also revealed by Colleoni et al. (13). In this recent study a significant difference in disease-free survival and risk of distant metastasis was demonstrated in patients with minimal lymph node involvement of a single node (pN1mi/pN0i+) compared with

TABLE 4. *Adjusted relative risk of death according to axillary status and number of examined nodes, number of positive nodes, and tumour size. Each item is added separately to the model shown in Table 3*

	Axillary status		
	Nneg	Nmic	Nmac
Adj. RR (95% CI)	Adj. RR (95% CI)	Adj. RR (95% CI)	
Number of examined nodes			
1–6	1.04 (0.90–1.20)	1.60 (1.00–2.55)	1.37 (1.15–1.64)
7–10	1 (reference)	1 (reference)	1 (reference)
11+	0.93 (0.82–1.05)	0.98 (0.66–1.45)	0.89 (0.75–1.06)
Number of positive nodes			
1		1.12 (0.74–1.69)	0.72 (0.61–0.85)
2		1 (reference)	1 (reference)
3		1.34 (0.67–2.68)	1.24 (1.03–1.49)
Tumour size			
0–10 mm	0.73 (0.62–0.86)	0.73 (0.38–1.39)	0.66 (0.48–0.92)
11–20 mm	1 (reference)	1 (reference)	1 (reference)
21–30 mm	1.23 (1.07–1.40)	1.54 (1.03–2.31)	1.16 (0.98–1.38)
>30 mm	1.34 (1.11–1.61)	2.04 (1.30–3.20)	1.46 (1.22–1.75)

Nneg=node negative, Nmic=micrometastases, Nmac=macrometastases.

TABLE 5. *Number of removed nodes in relation to case-load of participating surgical departments*

Number of operations per surgical department	Number of removed nodes						
	N 1–6	%	N 7–10	%	N 11+	%	N Total
<100	284	*22*	525	*41*	479	*37*	1,288
100–149	285	*23*	479	*39*	457	*37*	1,221
150–249	445	*19*	935	*41*	913	*40*	2,293
250+	621	*16*	1,343	*35*	1,825	*48*	3,793
Total	1,639		3,282		3,674		8,595

Departments with a case load of fewer than 10 operations have been left out (n=31 operations). Three breast surgeries without axillary dissection are also excluded.

Nneg disease. Patient entry was between 1997 and 2000, resulting in a short follow-up time of only 3.8 years, and therefore overall survival, as expected, did not differ between groups. Furthermore, adjuvant systemic therapy was offered to 210 patients from among 232 patients with minimal involvement of a single axillary lymph node.

Data from the Surveillance, Epidemiology, and End Results Program (SEER) of the National Cancer Institute (14) also show modestly inferior survival among 1,724 patients with micrometastases of 2 mm or less compared with 42,197 node-negative cases. Patient entry took place between 1988 and 2001, and the end point constituted death due to breast cancer. Patients who had unknown number or fewer than 10 axillary lymph nodes examined were excluded, as were cases with tumour size exceeding 2 cm. In multivariate analysis, patients with a solitary lymph node with micrometastasis had a hazard ratio of 1.62 (95% CI 1.26–2.10) and patients with multiple lymph nodes with micrometastases reached a hazard ratio of 1.78 (95% CI 1.19–2.66) relative to node-negative cases.

The frequency of cases with micrometastases differs among studies. In the study of Kuijt et al. (12), the 179 cases with micrometastases made up 4% (179/4,556) of the combined Nneg+Nmic group. In the study of Colleoni et al. (13) this figure amounted to 14% (232/1,632), and in the selected patient material in the SEER study (14) the figure was 4% (1,724/43,921). In our study the figure reached 8% (427/5,194). These differences in incidence of micrometastases in four large studies underline the essen-

tial role of the method applied by the pathologist to detect the metastases. Furthermore, patient selection might be of importance. In the study of Kuitj et al. one single H&E-stained section of each axillary lymph node was used in patients entering the study between 1975 and 1997 (12). In our study with patient entry between 1990 and 1994, each lymph node was examined using one H&E-stained section of each half and immunohistochemistry when required. In the study by Colleoni et al. (13) all women had axillary surgery and 44% underwent sentinel lymph node biopsy. It is important to underline that the pathological work-up of sentinel lymph nodes includes serial sectioning and immunohistochemistry to detect metastases. The high proportion of patients with extensive examination of lymph nodes may explain the high frequency of 14% of women with minimal lymph node involvement. Method of pathological work-up was not commented on in the SEER study (14).

Our study included women with only up to three positive axillary lymph nodes due to the fact that our data base revealed micrometastases only in up to three positive nodes and only in exceptional cases also in patients with four positive nodes. In our study, 69% of Nmic women had metastases in only one lymph node. Therefore, studies including patients with only one affected node might miss around one third of the women with micrometastases.

The present study shows significantly lower OS in Nmic women compared with Nneg women both in premenopausal and postmenopausal women. This finding is to some extent inconsistent with the results of the large study on occult micrometastases by the International Breast Cancer Study Group. In this study, a significantly poorer disease-free survival and overall survival was found in postmenopausal women but not in premenopausal women (15).

No interaction between axillary status and primary tumour size appeared in our study, which indicates that the prognostic influence of micrometastases proves equal in women with small and large tumours. Furthermore, the interactions in the present study signify a worse final outcome for women with positive axillary status and few nodes removed. The finding most probably reflects less than optimal locoregional therapy and to some extent inaccurate axillary classification. This assumption is further supported by the fact that several studies found inferior survival parallel with less successful locoregional tumour control and insufficient axillary dissection (16–19). Deficient surgery may have taken place in our study based on the finding that surgical departments with a low case load retrieved significantly fewer lymph nodes from the axilla than surgical units with a high case load (Table 5).

The different risk pattern in Nmic *vs* Nmac patients indicates that Nmic cases do not show the traditional pattern as revealed by the Nmac cases, in which increasing number of positive nodes reflects an orderly increasing adjusted RR. This is not caused by misallocations, because cases with Nmic had more lymph nodes removed compared to cases with Nmac. This finding implies that the different risk pattern is probably not caused by failure in technical methods but indicates a biological phenomenon of the primary tumour.

Sentinel lymph node examination requires a more thorough histological work-up and seems to be a safe method of axillary staging (20, 21). However, there might be a risk of stage migration if the sentinel node is introduced during the course of the study. In the present study the sentinel node method was not applied and the risk of stage migration was therefore avoided.

The interaction between axillary status and histological malignancy grade shows that the adjusted RR of grade III Nmic cases rose even higher than the adjusted RR of grade III Nmac cases. The results are in line with the findings of Maibenco et al. (14). They reported that the highest impact on 12-year survival disadvantage was associated with Nmic cases belonging to the grade III subset, where grade III cases were defined as cases with poor grade on a three tailored scale.

In conclusion, the results of the present study showed worse final outcome in women with Nmic compared with Nneg, where all Nmic cases received adjuvant systemic treatment. Interaction analysis showed that the number of retrieved axillary nodes and the number of affected nodes had a different influence on survival related to axillary status. The different risk pattern in Nmic *vs* Nmac patients indicates that Nmic cases do not show the traditional risk pattern as revealed by the Nmac cases, in which

increasing number of positive nodes is associated with an orderly increasing adjusted RR.

REFERENCES

1. Sobin LH, Wittenberg J, editors. TNM classification of malignant tumours. 6th ed. New York: John Wiley and Sons, 2002.
2. Greene GL, Page DL, Fletcher C, editors. AJCC cancer staging manual. 6th ed. New York: Springer-Verlag, 2002.
3. DBCG (Danish Breast Cancer Cooperative Group). Protocol collection, management of breast cancer 1977–1997. Copenhagen: DBCG, 1997:1–91.
4. Sobin LH. Histological typing of breast tumours. 2nd ed. Geneva: World Health Organization, 1981.
5. Sakorafas GH, Geraghty J, Pavlakis G. The clinical significance of axillary lymph node micrometastases in breast cancer. Eur J Surg Oncol 2004;30:807–16.
6. Fisher ER, Palekar A, Rockette H, Redmond C, Fisher B. Pathologic findings from the National Surgical Adjuvant Breast Project (Protocol No. 4). V. Significance of axillary nodal micro- and macrometastases. Cancer 1978;42:2032–8.
7. Black RB, Roberts MM, Stewart HJ, Prescott R, Cant ELM, Sumerling MD et al. The search for occult metastases in breast cancer: does it add to established staging methods? Aust N Z J Surg 1980;50:574–9.
8. Rosen PP, Saigo PE, Braun DW, Weathers E, Fracchia AA, Kinne DW. Axillary micro- and macrometastases in breast cancer: prognostic significance of tumor size. Ann Surg 1981;194:585–91.
9. Clayton F, Hopkins CL. Pathologic correlates of prognosis in lymph node-positive breast carcinomas. Cancer 1993;71:1780–90.
10. de Mascarel I, Bonichon F, Coindre JM, Trojani M. Prognostic significance of breast cancer axillary lymph node micrometastases assessed by two special techniques: reevaluation with longer follow-up. Br J Cancer 1992;66:523–7.
11. Dowlatshahi K, Fan M, Snider HC, Habib FA. Lymph node micrometastases from breast carcinoma. Reviewing the dilemma. Cancer 1997;80: 1188–97.
12. Kuijt GP, Voogd AC, van de Poll-France LV, Scheijmans LJ, van Beek MW, Roumen RM. The prognostic significance of axillary lymph-node micrometastases in breast cancer patients. Eur J Surg Oncol 2005;31:500–5.
13. Colleoni M, Rotmensz N, Peruzzotti G, Maisonneuve P, Mazzarol G, Pruneri G et al. Size of breast cancer metastases in axillary lymph nodes: Clinical relevance of minimal lymph node involvement. J Clin Oncol 2005;23:1379–89.
14. Maibenco DC, Dombi GW, Kau TY, Severson RK. Significance of micrometastases on the survival of women with T1 breast cancer. Cancer 2006;107:1234–9.
15. Cote RJ, Peterson HF, Chaiwun B, Gelber RD, Goldhirsch A, Castiglione-Gertsch M, et al. Role of immunohistochemical detection of lymph-node metastases in management of breast cancer. International Breast Cancer Study Group. Lancet 1999;354:896–900.
16. Axelsson CK, Mouridsen HT, Zecchini A, Zedeler K, on behalf of The Danish Breast Cancer Cooperative Group (DBCG). Axillary dissection of level I and II lymph nodes is important in breast cancer classification. Eur J Cancer 1992;28A: 1415–8.
17. Overgaard M, Hansen PS, Overgaard J, Rose C, Andersson M, Bach F, et al. Postoperative radiotherapy in high-risk premenopausal women with breast cancer who receive adjuvant chemotherapy. Danish Breast Cancer Cooperative Group 82b Trial. N Engl J Med 1997;337:949–55.
18. Overgaard M, Jensen MB, Overgaard J, Hansen PS, Rose C, Andersson M et al. Postoperative radiotherapy in high-risk postmenopausal breast-cancer patients given adjuvant tamoxifen: Danish Breast Cancer Cooperative Group DBCG 82c randomised trial. Lancet 1999;353:1641–8.
19. Grabau DA, Jensen MB, Blichert-Toft M, Andersen JA, Dyreborg U, Carstensen B, et al. The importance of surgery and accurate axillary staging for survival in breast cancer. Eur J Surg Oncol 1998;24:499–507.
20. Viale G, Zurrida S, Maiorano E, Mazzarol G, Pruneri G, Paganelli G, et al. Predicting the status of axillary sentinel lymph nodes in 4351 patients with invasive breast carcinoma treated in a single institution. Cancer 2005;103:492–500.
21. Veronesi U, Paganelli G, Viale G, Path FR, Luini A, Zurrida S, et al. A randomized comparison of sentinel-node biopsy with routine axillary dissection in breast cancer. N Engl J Med 2003;349:546–53.

Paper II

Histopathology 2011, **59**, 129–138. DOI: 10.1111/j.1365-2559.2011.03881.x

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

Dorthe Grabau,[1,2] Lisa Ryden,[3,4] Mårten Fernö[5,6] & Christian Ingvar[3,4]

[1]*Department of Pathology, University Hospital of Lund, Lund,* [2]*Department of Pathology, Clinical Science, Lund University, Lund,* [3]*Department of Surgery, University Hospital of Lund, Lund,* [4]*Department of Surgery, Clinical Science, Lund University, Lund,* [5]*Department of Oncology, University Hospital of Lund, Lund, and* [6]*Department of Oncology, Clinical Sciences, Lund University, Lund, Sweden*

Date of submission 17 June 2010
Accepted for publication 4 January 2011

Grabau D, Ryden L, Fernö M & Ingvar C
(2011) *Histopathology* **59**, 129–138

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

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

Methods and results: Four different protocols for the work-up of sentinel lymph nodes (SLNs) after frozen sectioning were applied in the routine diagnostic process from 2001 to 2009. In addition, team-work with a limited number of laboratory technicians and pathologists handling SLNs was introduced in 2008. The present study shows that there were, overall, significantly more node-positive patients in the period when team-work and intensive step sections including immunohistochemistry (IHC) were used ($P = 0.01$). This resulted in 13% more patients being found to have ITCs and micrometastases than in a time period when only step sections were performed. No increase in the number of false-negative frozen sections was seen.

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

Keywords: breast cancer, immunohistochemistry, isolated tumour cells, micrometastases, sentinel node biopsy

Abbreviations: AJCC, American Joint Committee on Cancer; ALND, axillary lymph node dissection; FFPE, formalin-fixed paraffin-embedded; HE, haematoxylin and eosin; IHC, immunohistochemistry; ITCs, isolated tumour cells; SLN, sentinel lymph node

Address for correspondence: D Grabau, Department of Pathology, University Hospital of Lund, 221 85 Lund, Sweden.
e-mail: Dorthe.grabau@skane.se

Introduction

During the last 10 years, sentinel lymph node (SLN) biopsy has gained acceptance as the routine method for staging the ipsilateral axilla in women with early breast cancer. Preoperative procedures, including the injection of radioactive colloidal substances and/or blue dye, scintigraphy and gamma probe handling, and the intraoperative surgical technique, are standardized and largely agreed upon. In the beginning of the 2000s, Swedish guidelines for surgeons included SLN biopsy as the standard procedure after a period of validation with respect to the false-negative rate of axillary staging.[1]

On the other hand, the optimal work-up of sentinel nodes in the laboratory is still under debate. The 6th edition of the pTNM classification introduced the concept of isolated tumour cells (ITCs) in 2002,[2,3] and stated that these should be considered as node-negative for staging and treatment purposes. The 7th edition[4,5] clarifies the concept, and recommends finding all metastases >2 mm by macroscopically slicing the SLN at 2 mm intervals and stating the accuracy of the subsequent formalin-fixed paraffin-embedded (FFPE) protocol in finding micrometastases and ITCs. In 2006, Swedish guidelines for SLN handling were largely adapted from the European guidelines.[6]

The risk of upstaging axillary status by a more thorough examination of SLNs has been considered something to be avoided, because clinical data were based on only one histological slice from each axillary lymph node.[7] Nevertheless, a recent study of women with early-stage breast cancer who underwent sentinel node biopsy reported that ITCs or micrometastases in regional lymph nodes were associated with decreased disease-free survival in women without adjuvant therapy, and that disease-free survival was improved in this patient group if they received adjuvant therapy.[8] Another review concluded that patients with metastases of 2 mm or smaller in axillary lymph nodes examined by use of a single haematoxylin and eosin (HE) section have a worse outcome than node-negative patients.[9]However, for patients treated by SLN biopsy, the data are still inconclusive. A further review of SLN micrometastases concluded that such deposits are likely to represent an incremental detriment to prognosis and an increased risk of non-SLN involvement.[10] In addition, metastases uncovered in formerly negative axillary lymph node dissections (ALNDs) with an SLN protocol including immunohistochemistry (IHC) have prognostic significance in breast cancer patients.[11] The Swedish national figures show a worse outcome for patients with micrometastases in SLNs than in node-negative patients,[12] but other reports have shown no difference in survival between SLN-negative patients and those with micrometastases.[13]

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

Materials and methods

The study was conducted on a consecutive series of 1576 women with a first primary operable breast cancer treated at the University Hospital of Lund from 1 January 2001 to 31 December 2009 (Figure 1). Twenty-five women diagnosed in October 2008 were excluded, because these patients' SLN were analysed using Method 4, but before the laboratory was reorganized into work teams. Patients, diagnosed with the topography code for breast in the SNOMED system, were selected by extraction from the data files of the pathology department. By manual examination of the files, patients with a previous history in the pathology files of invasive carcinoma in the breasts were excluded, as were patients with previous malignancy elsewhere. Men with breast cancer, benign cases and cases with *in-situ* carcinoma were also excluded. A total of 27 women with synchronous bilateral breast cancer were included, by use of the side showing the worse axillary status. The other side was excluded in these women.

The surgical procedure for SLN biopsy was preceded by subdermal injection with ^{99}Tc-labelled nanocolloid followed by a scintigraphy. About 0.5 ml of Patent V Blue was injected intradermally after anaesthesia in the operating room. Any node that was hot, blue or palpable was considered to be an SLN and removed. The surgical procedure, instrument and surgical staff did not differ in the cohort period.

As a general rule, frozen section examination was undertaken of up to four nodes, with subsequent examination of FFPE sections stained with HE and/or

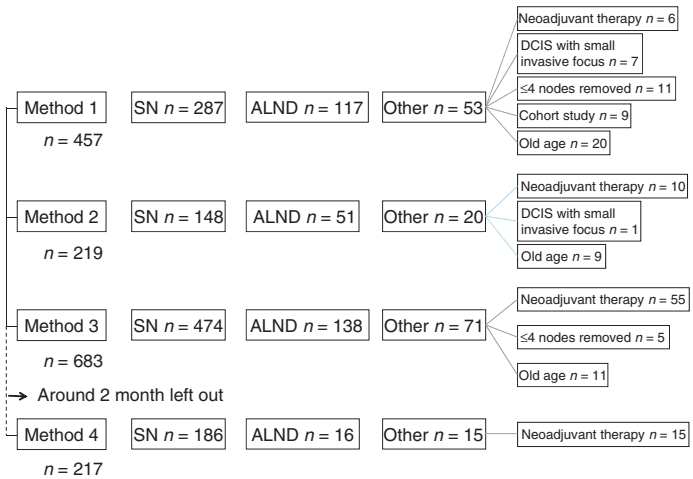


Figure 1. Composition of material. Method 1 was used from January 2001 to September 2003. Method 2 was used from October 2003 to December 2004. Method 3 was used from January 2005 to September 2008. Method 4 was used from November 2008 to December 2009. In the first period, a cohort study included women with early breast cancer with an expected risk of loco regional recurrence of <1% in a protocol without axillary staging (n = 9).

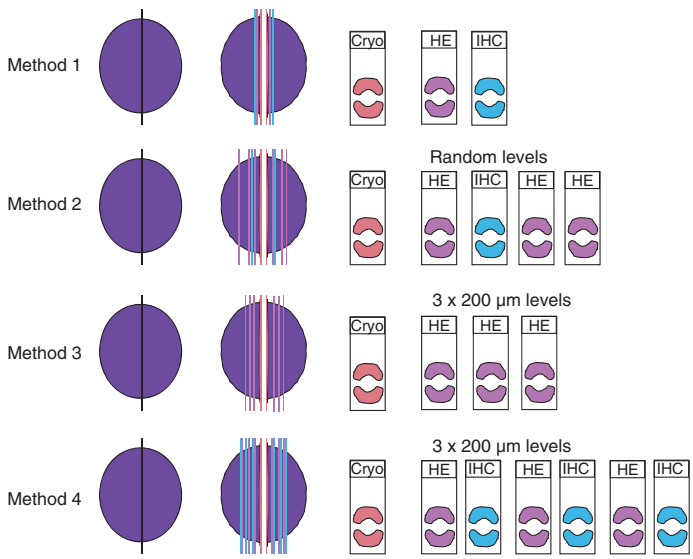


Figure 2. Method of sentinel node work-up in the four different periods.

IHC with pan-anticytokeratins, according to the different protocols. Throughout the study period, four different protocols for routine histopathological work-up of SLNs were used (Figure 2). Generally, SLNs of 4 mm or more were cut through the longitudinal axis, and both halves examined. Smaller nodes were examined *in toto*. All specimens were examined as one or two HE-stained frozen sections, and no immunohistochemical staining was performed intraoperatively. After formalin fixation and paraffin embedding, the protocols differed. During the first period, the sections were examined as one paraffin section and by immunohistochemical analysis with anticytokeratin in frozen section negative cases (n = 287; Method 1). In the

second period, the SLNs were analysed by step sectioning at three randomly chosen levels, and by IHC of the first level in frozen section negative cases (n = 148; Method 2). The third period introduced step sectioning of SLNs at three fixed levels of 0.2 mm and the use of IHC in suspicious cases only (n = 474; Method 3). The last period included step sectioning of SLNs at three fixed levels of 0.2 mm, with IHC at every level in cases that were negative on frozen section examination or had a metastasis of <2 mm on the frozen sections (n = 186; Method 4). During the last period, the laboratory work was performed by fewer, specially trained technicians and fewer pathologists. The number of laboratory technicians was reduced from 12 to five, and the number of pathologists was decreased from about 14 to about three. The laboratory technicians were specially trained in preparing frozen sections on SLNs with fatty tissue around and inside the lymph nodes. The frozen sections were examined microscopically by the pathologist and the laboratory technician who participated in the analysis of the quality of the frozen sections. This method is what we refer to as team-work.

A review of the slides of all cases with SLN positivity was undertaken, and the 7th edition of the American Joint Committee on Cancer (AJCC) and Union for International Cancer Control classification was applied.[4,14] Women without detectable tumour cells in the nodes were designated node-negative, equal to a cut-off of zero. With a cut-off of 0.2 mm, women were staged as negative when they had no metastases, or had ITCs of 0.2 mm or smaller, or had fewer than 200 tumour cells (Figure 3). Patients with micrometastases had metastases >0.2 mm or more than 200 tumour cells, but not measuring more than 2.0 mm. A cut-off of 2.0 mm separated women with metastases larger than 2.0 mm from all those with smaller ones.

The size of the metastatic deposit was determined on the slide where it was largest. In 2004, the 6th edition of the AJCC was implemented, and women with deposits of 0.2 mm or smaller or with ITCs were thereafter not routinely offered axillary dissection. All other women with metastases larger than 0.2 mm in at least one SLN were offered axillary dissection, either at the time of primary surgery for breast cancer because of a positive frozen section, or in a second operative procedure as a result of a positive SLN on FFPE sections following false-negative frozen section of the SLN.

From 2001 to October 2008, lymph nodes from the axillary clearance were handled by isolation of the lymph nodes in formalin-fixed surgical specimens and embedding one slice from each node, often including several nodes in each block. From October 2008 and thereafter, lymph nodes from the axillary clearance were bisected, and macroscopically negative nodes were entirely embedded in separate blocks. One slice sufficed for macroscopically evident metastatic nodes. One HE section from each block was examined, and in difficult cases IHC was applied. Details of the age of the patient, tumour size, histological grade, oestrogen receptor status and progesterone receptor status were recorded from the pathology reports. Histological grade was assessed with the Nottingham modification[15] of the Scharff, Bloom and Richardson grading system.[16] Oestrogen and progesterone receptor status was determined by IHC assay, and carcinomas with >10% stained nuclei were considered to be positive. A mammography screening programme was conducted in the local area from 1989, and women aged 45–74 years were invited every 18–24 months.

STATISTICS

Differences between proportions were examined by chi-square test, and $P < 0.05$ was considered to be significant. Differences between continuous variables were analysed with the Kruskal–Wallis test.

Results

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

Of 1576 patients included in the study 70% had SLN biopsy, 20% were treated by primary ALND, and 10% were allocated to other protocols without adequate determination of axillary status at the time of surgery (Figure 1). The frequencies of patients treated by SLN biopsy were 63%, 68%, 69% and 86% in the four periods with different methods of SLN examination. With a cut-off of zero for classifying SLNs with negative status, significantly more patients were node-positive in the last period, both among women treated by SLN biopsy and among all women (P = 0.01) (Table 2). When a cut off of 0.2 mm/<200 cells was applied for negative status, the difference was only borderline significant (P = 0.07; Table 3). IHC was used in 72%, 74%, 21% and 92% of the cases treated by SLN biopsy. The median number of analysed SLNs per patient was two, and did not differ between the periods.

Table 1. Patients and tumour characteristics of all women

	Method 1 *N* = 457	Method 2 *N* = 219	Method 3 *N* = 683	Method 4 *N* = 217	*P*
Tumour size (mm), median (range)	17 (1–80)	18 (0–90)	16 (0–90)	17.5 (0–80)	0.40
Age (years), median (range)	58 (23–99)	59 (30–91)	61 (27–94)	64 (25–91)	<0.01
Histological grade, *n* (%)					
1	106 (23)	57 (26)	154 (23)	48 (22)	0.003
2	256 (56)	106 (48)	360 (53)	92 (43)	
3	84 (18)	47 (21)	156 (23)	72 (33)	
No grade	11 (2)	9 (4)	13 (2)	5 (2)	
Histological type, *n* (%)					
Ductal	353 (77)	170 (78)	574 (84)	182 (84)	0.04
Lobular	77 (17)	34 (16)	74 (11)	22 (10)	
Other type	27 (6)	15 (7)	35 (5)	13 (6)	
Oestrogen receptor, *n* (%)					
Negative	83 (18)	43 (20)	101 (15)	30 (14)	0.18
Positive	364 (80)	174 (79)	575 (84)	185 (85)	
Unknown	10 (2)	2 (1)	7 (1)	2 (1)	
Progesterone receptor, *n* (%)					
Negative	168 (37)	75 (34)	221 (32)	61 (28)	0.14
Positive	279 (61)	142 (65)	455 (67)	154 (71)	
Unknown	10 (2)	2 (1)	7 (1)	2 (1)	

Postoperative tumour size was zero in a few patients included in a study using laser therapy.[32]

Table 2. Frequency of positive nodes in women primarily treated by sentinel lymph node (SLN) biopsy, axillary lymph node dissection (ALND) or referred to the group 'other' by the different methodologies of SLN handling, with a cut-off for metastases at zero

	Method 1		Method 2		Method 3		Method 4		
Primary surgery	*N*	No. positive (%)	*N*	No. positive (%)	*N*	No. positive (%)	*N*	No. positive (%)	*P*
SLN cut-off 0	287	123 (43)	148	52 (35)	474	168 (35)	186	92 (49)	<0.01
ALND	117	68 (58)	51	28 (55)	138	84 (61)	16	14 (88)	0.12
Other*	53	46 (87)	20	16 (80)	71	57 (80)	15	15 (100)	0.53
Total cut-off 0	457	237 (52)	219	96 (44)	683	309 (45)	217	121 (56)	0.01

*For definition of the group 'Other' please see Figure 1.

With regard to those who were totally negative for SLNs and those with ITCs of ≤0.2 mm/<200 cells or micrometastases of 2 mm or smaller, some striking differences appeared (Table 4). In the last period, using Method 4 with step sections at fixed intervals and IHC at every level, where frozen sections and step sections

Table 3. Frequency of positive nodes in women primarily treated by sentinel lymph node (SLN) biopsy, axillary lymph node dissection (ALND) or referred to the group 'other' by the different methodologies of SLN handling, with a cut-off for metastases at 0.2 mm/200 cells

	Method 1		Method 2		Method 3		Method 4		
Primary surgery	*N*	No. positive (%)	*N*	No. positive (%)	*N*	No. positive (%)	*N*	No. positive (%)	*P*
SLN cut-off 0.2 mm/<200 cells	287	112 (39)	148	45 (30)	474	154 (32)	186	76 (41)	0.06
Total cut-off 0.2 mm/200 cells	457	226 (49)	219	89 (41)	683	295 (43)	217	105 (48)	0.07

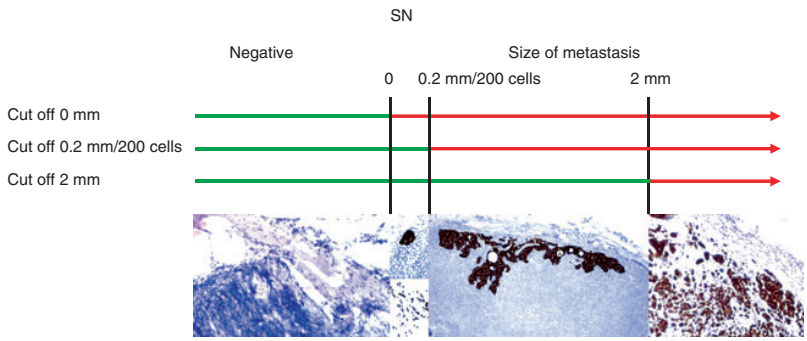


Figure 3. Different cut-offs of metastatic deposits used in sentinel nodes. The status of the sentinel node is determined at the level with most deposits/tumour cells.

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

Table 4. Frequency of isolated tumour cells, micrometastases and macrometastases in the intervals with different methodologies for sentinel lymph node (SLN) handling

were performed by trained laboratory technicians, significantly more patients were diagnosed with ITCs or micrometastases in the SLNs ($P < 0.01$). The proportion of patients with ITCs and micrometastases was 9% higher when Method 4 was used than with the use of methods 1 and 2 with either only one HE section followed by IHC or one HE section and IHC followed by random step sections. Teamwork and intensive IHC in Method 4 resulted in 13% more patients with ITCs and micrometastases than with Method 3 with step sections at fixed intervals. The frequency of women with ITCs and micrometastases as compared with those with macrometastases did not differ significantly between the study periods, indicating that the ITCs and micrometastases were found in patients who were previously classified as node-negative.

Frozen sections were reported to the surgeons as being positive if a metastasis of larger than 0.2 mm

Table 5. False-negative results of frozen sections of sentinel lymph nodes (SLNs) compared with formalin-fixed paraffin-embedded (FFPE) sections, with a cut-off of 0.2 mm

	Method 1	Method 2	Method 3	Method 4
	n (%)	*n* (%)	*n* (%)	*n* (%)
SLN total negative	164 (57)	94 (64)	308 (65)	91 (49)
ITC ≤0.2 mm/200 cells	11 (4)	7 (5)	14 (3)	15 (8)
Positive >0.2 mm/200 cells				
Positive >0.2 mm and ≤2 mm	12 (4)	4 (3)	10 (2)	4 (2)
Positive >2 mm	73 (25)	25 (17)	96 (20)	49 (26)
Frozen false-negative metastases >0.2 mm				
Positive >0.2 mm and ≤2 mm	13 (5)	9 (6)	25 (5)	13 (7)
Positive >2 mm	13 (5)	5 (3)	15 (3)	3 (2)
Frozen false-positive	1 (0)	2 (1)	0	0
No frozen section	0	2 (1)	6 (1)	11 (6)
False-negative rate	0.23	0.33	0.27	0.23
Accuracy	0.91	0.89	0.92	0.91

was encountered. The false-negative rate of the frozen section technique was similar during the period for Method 4 with intensive IHC of step sections in FFPE slides and team work, and during the period for Method 1 with IHC but without step sections (Table 5). When methods 2 and 3 without team work were used, the false-negative rate of frozen sections was higher.

Discussion

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

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

With the application of step sections and/or IHC to negative regional lymph nodes, the use of step sections increases the harvest of small metastases by about 7–13%, and the addition of IHC leads to about 15–30% more cases with small metastases being found.[11,17] Our results and the conclusion of another report[18] imply that smaller metastases will be missed if step sections and IHC of SLNs are not performed. The varying false-negative rate of metastasis obtained with frozen sections among the study periods indicates that small metastases might be missed. The sensitivity of frozen section methodology is within the range found in other studies.[19,20]

From the surgical point of view, discussion is ongoing as to whether patients with micrometastases benefit from subsequent ALND, because the frequency of local recurrence is extremely low.[21,22] However, systemic therapy might improve survival even in patients with tumour deposits as small as ITCs.[8]

A consensus with regard to an optimal pathological work-up of SLNs is hard to achieve, because viewpoints

differ on the objectives of the examination. A more modest work-up favours attempts to avoid stage migration, and acknowledges the possibility of benign transportation of epithelial tissue to the SLNs. Upstaging of breast cancer patients implies that a more thorough method of SLN examination results in more positive cases.[23] The clinical implications of the additional metastases are not determined in the clinical trials where adjuvant systemic therapy is used. Another problem with taking therapeutic action as a consequence of small metastatic deposits that are only detectable with IHC is raised by the possibility of benign transportation of cells to the SLNs[24] after needle diagnostic procedures or manipulation of the breast.[25] Malignant cells might even be displaced to the SLNs without being metastatic in nature. Recently, benign elements have been identified as elements of papillomas from the breast.[26]

Different models for handling of SLNs have been presented,[27,28] and all models show that use of an acceptable number of steps and sections in routine work-up results in missed deposits of small metastases. Furthermore, pathologists do not agree on what constitutes an ITC.[29] Therefore, the 7th AJCC staging recommends slicing the SLN in 2-mm intervals and stating the sensitivity of the step section method used to detect small metastases afterwards. In addition, clarifications of the concept of the ITC and/or fewer than 200 cells have been reported.[5] The lymph node slices of 2 mm are obtained in practice by dividing the SLN along the longitudinal axis, and this will always be an approximation, as lymph nodes are of different sizes. One survey of routine practice in Europe revealed that 73% of participating institutions performed IHC on SLNs.[30] More intensive protocols using IHC reveal more metastases,[28] but Method 4 in this study seems to combine an acceptable level of work with an acceptable cost.

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

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

This study has some drawbacks. As it is a single-institution study design, the different methods compared are based on examination of SLNs in successive time intervals, and comparison of the methods was not randomized during a fixed time interval. Also, the frequency of patients treated by SLN biopsy increased during the study period, and the frequency of positive nodes in the SLN population would therefore be expected to increase. Therefore, data from all breast cancer patients in the institution were considered, and because the increase was general in the entire study population, the increase in node positivity among patients with SLNs is interpreted as being method-related and not a result of selection bias. With the study design reported here, it will never be possible to prove that the increase in ITCs and micrometastases found with Method 4 is not simply a result of more patients having SLN biopsies performed. During the study period, the indication for axillary clearance also changed, so that only patients with a suspicion of, or preoperatively diagnosed with, regional metastases would receive axillary clearance primarily. The protocol for the handling of nodes from axillary clearance was also altered before Method 4 was used. The frequency of screening-detected breast cancer in the population did not change during the study. In patients undergoing SLN biopsy, the increased numbers of ITCs and micrometastases were found in patients who had previously been classified as node-negative, and therefore the increase might be method-related.

To our knowledge, the results of work by a specialized team including laboratory technicians have not been previously studied. Therefore, we cannot state whether the 13% increase in ITCs and micrometastases

that we documented during the time period when Method 4 was used is solely a result of the specialized team-work, or is just the result of more intensive step sectioning and IHC.

If the handling of SLNs is confined to a small, specialized group of laboratory technicians and pathologists, it may be expected that the rate of false-positive frozen sections will decrease. In fact, one of the three false-positive cases in the present series was caused by the presence of a benign nevus in the lymph node capsule, a condition of which not all pathologist are aware.[31]

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

Acknowledgements

The admirable work of the laboratory technicians in the breast team in 2008–2009 (Iren Atakhan, LivGröntoft, Magdalena Nagy, Nadine Nyawakira, Anna Nilsson and Helen Svensson) and the immunohistochemical laboratory is much appreciated. Also, the residents Jacob Elebro and David Nord Gisselsson performed meticulous and highly professional work. This work was funded by Skaane County Council's Research and Development Foundation, and Governmental Funding of Clinical Research within the National Health Service. The authors wish to thank Robert Cameron for linguistic support.

References

1. Bergkvist L, Frisell J. Multicentre validation study of sentinel node biopsy for staging in breast cancer. *Br. J. Surg.* 2005; **92;** 1221–1224.
2. Greene GL, Page DL, Fletcher C (eds). *AJCC cancer staging manual*, 6th edn. New York: Springer-Verlag, 2002.
3. Sobin LH, Wittenberg J (eds). *TNM classification of malignant tumours*, 6th edn. New York: Wiley, 2002.
4. Edge SB, Byrd DR, Compton CC, Fritz AG, Greene FL, Trotti A eds. *AJCC cancer staging manual*, 7th edn. New York: Springer Verlag, 2010.
5. Lester SC, Bose S, Chen YY *et al.* Protocol for the examination of specimens from patients with invasive carcinoma of the breast. *Arch. Pathol. Lab. Med.* 2009; **133;** 1515–1538.
6. Perry N, Broeders M, de Wolf C, Tornberg S, Holland R, von Karsa L. *European guidelines for quality assurance in breast cancer screening and diagnosis*, 4th edn. European Breast Cancer Network (EBCN): Lyon, 2006; 329–331.
7. Layeequr RR, Siegel E, Boneti C *et al.* Stage migration with sentinel node biopsy in breast cancer. *Am. J. Surg.* 2009; **197;** 491–496.
8. de Boer M, van Deurzen CH, van Dijck JA *et al.* Micrometastases or isolated tumor cells and the outcome of breast cancer. *N. Engl. J. Med.* 2009; **361;** 653–663.
9. de Boer M, van Dijck JA, Bult P, Borm GF, Tjan-Heijnen VC. Breast cancer prognosis and occult lymph node metastases, isolated tumor cells, and micrometastases. *J. Natl Cancer Inst.* 2010; **102;** 410–425.
10. Patani N, Mokbel K. The clinical significance of sentinel lymph node micrometastasis in breast cancer. *Breast Cancer Res. Treat.* 2009; **114;** 393–402.
11. Tan LK, Giri D, Hummer AJ *et al.* Occult axillary node metastases in breast cancer are prognostically significant: results in 368 node-negative patients with 20-year follow-up. *J. Clin. Oncol.* 2008; **26;** 1803–1809.
12. Andersson Y, Frisell J, Sylvan M, de Boniface J, Bergkvist L. Breast cancer survival in relation to the metastatic tumor burden in axillary lymph nodes. *J. Clin. Oncol.* 2010; **28;** 2868–2873.
13. Gobardhan PD, Elias SG, Madsen EV *et al.* Prognostic value of micrometastases in sentinel lymph nodes of patients with breast carcinoma: a cohort study. *Ann. Oncol.* 2009; **20;** 41–48.
14. Sobin LH, Gospodarowicz MK, Wittekind C (eds). *UICC International Union against Cancer. TNM classification of malignant tumours*, 7th edn. Oxford: Wiley-Blackwell, 2009.
15. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology* 1991; **19;** 403–410.
16. Bloom HJG, Richardson WW. Histological grading and prognosis in breast cancer. A study of 1409 cases of which 359 have been followed for 15 years. *Br. J. Cancer* 1957; **11;** 359–377.
17. Jaffer S, Nagi C, Bleiweiss IJ. Occult axillary node metastases in breast cancer are prognostically significant: results in 368 node negative patients with 20-year follow-up. *Adv. Anat. Pathol.* 2008; **15;** 370–373.
18. Sahin AA, Guray M, Hunt KK. Identification and biologic significance of micrometastases in axillary lymph nodes in patients with invasive breast cancer. *Arch. Pathol. Lab. Med.* 2009; **133;** 869–878.
19. Celebioglu F, Sylvan M, Perbeck L, Bergkvist L, Frisell J. Intraoperative sentinel lymph node examination by frozen section, immunohistochemistry and imprint cytology during breast surgery – a prospective study. *Eur. J. Cancer* 2006; **42;** 617–620.
20. Ryden L, Chebil G, Sjostrom L, Pawlowski R, Jonsson PE. Determination of sentinel lymph node (SLN) status in primary breast cancer by prospective use of immunohistochemistry increases the rate of micrometastases and isolated tumour cells: analysis of 174 patients after SLN biopsy. *Eur. J. Surg. Oncol.* 2007; **33;** 33–38.
21. Morrow M. Patterns of care with a positive sentinel node: echoes of an opportunity missed. *Ann. Surg. Oncol.* 2009; **16;** 2429–2430.
22. Grabau D. Breast cancer patients with micrometastases only: is a basis provided for tailored treatment? *Surg. Oncol.* 2008; **17;** 211–217.
23. Groen RS, Oosterhuis AW, Boers JE. Pathologic examination of sentinel lymph nodes in breast cancer by a single haematoxylin-eosin slide versus serial sectioning and immunocytokeratin staining: clinical implications. *Breast Cancer Res. Treat.* 2007; **105;** 1–5.

24. Diaz NM, Cox CE, Ebert M *et al.* Benign mechanical transport of breast epithelial cells to sentinel lymph nodes. *Am. J. Surg. Pathol.* 2004; **28;** 1641–1645.
25. Hansen NM, Ye X, Grube BJ, Giuliano AE. Manipulation of the primary breast tumor and the incidence of sentinel node metastases from invasive breast cancer. *Arch. Surg.* 2004; **139;** 634–639.
26. Bleiweiss IJ, Nagi CS, Jaffer S. Axillary sentinel lymph nodes can be falsely positive due to iatrogenic displacement and transport of benign epithelial cells in patients with breast carcinoma. *J. Clin. Oncol.* 2006; **24;** 2013–2018.
27. Cserni G. A model for determining the optimum histology of sentinel lymph nodes in breast cancer. *J. Clin. Pathol.* 2004; **57;** 467–471.
28. Madsen EV, van Dalen J, van Gorp J, Borel Rinkes I, van Dalen T. Strategies for optimizing pathologic staging of sentinel lymph nodes in breast cancer patients. *Virchows Arch.* 2008; **453;** 17–24.
29. Turner RR, Weaver DL, Cserni G *et al.* Nodal stage classification for breast carcinoma: improving interobserver reproducibility through standardized histologic criteria and image-based training. *J. Clin. Oncol.* 2008; **26;** 258–263.
30. Cserni G, Amendoeira I, Apostolikas N *et al.* Pathological work-up of sentinel lymph nodes in breast cancer. Review of current data to be considered for the formulation of guidelines. *Eur. J. Cancer* 2003; **39;** 1654–1667.
31. Pantanowitz L, Upton MP. Benign axillary lymph node inclusions. *Breast J.* 2003; **9;** 56–57.
32. Haraldsdottir KH, Ivarsson K, Gotberg S, Ingvar C, Stenram U, Tranberg KG. Interstitial laser thermotherapy (ILT) of breast cancer. *Eur. J. Surg. Oncol.* 2008; **34;** 739–745.

Paper III

Completion axillary dissection can safely be omitted in screen-detected breast cancer patients with micrometastases. A decade's experience from a single institution.

Dorthe A Grabau[1] MD, PhD, Looket Dihge[2] MD, Mårten Fernö[3] PhD, Christian Ingvar[4] MD, PhD, Lisa Rydén[4] MD, PhD.

1. Department of Pathology, Skåne University Hospital. Clinical Science, Lund University, Lund, Sweden.
2. Department of Surgery and Oncology, Halland Hospital, Halmstad, Sweden
3. Division of Oncology, Department of Clinical Sciences, Lund University, Lund, Sweden.
4. Department of Surgery, Skåne University Hospital. Clinical Science, Lund University, Lund, Sweden.

Corresponding author: Dorthe Grabau, Department of Pathology, Skåne University Hospital, SE-221 85 Lund, Sweden.
Phone number: +46 46 173511
Fax: +46 46 143397
E-mail address: Dorthe.grabau@skane.se

Requests for reprints should be addressed to the corresponding author.

Conflicts of interest and source of funding: No conflicts of interests. The study was supported by funds from the Swedish Breast Cancer Organisation (BRO), the Swedish Cancer Society, Swedish Research Council, the Gunnar Nilsson Cancer Foundation, the Mrs. Berta Kamprad Foundation, Skåne County Council's Research and Development Foundation and Governmental Funding of Clinical Research within the National Health Service (ALF).

Mini abstract

Completion axillary lymph node dissection (ALND) in breast cancer patients with micrometastases in sentinel nodes is controversial. This study shows that patients with screen- detected breast cancer and tumour size of 15 mm or less have little risk of harbouring metastases in the completion ALND specimen.

Abstract

Objective: The aim of this retrospective observational study is to determine if the method of detection of early breast cancer is predictive for additional positive nodes in patients with micrometastases in the sentinel node biopsy (SNB).

Summary Background Data: The need for completion axillary lymph node dissection (ALND) in breast cancer patients with micrometastases in the SNB is controversial.

Material and methods. Between 2001 and 2011 a total of 1,993 women with primary unilateral breast cancer had surgery at Skåne University Hospital, Lund. Of 1,993 patients, 1,458 had an SNB and nearly all patients with micro- and macrometastases had ALND.

Results. Micrometastases defined as >0.2 mm/>200 cells mm and ≤2.0 mm were found in 62 (8%) of 757 screen-detected patients and in 81 (12%) of 701 patients with symptomatic breast cancer. Only 3 (5%) of the screen-detected patients with micrometastases, all with tumour size >15 mm (range 18-39 mm), had metastases in the completion ALND whereas this was found in 18 (23%) of the symptomatic patients with micrometastases (p=0.013), (tumour size range 10-30 mm). Logistic regression analysis adjusted for method of detection, tumour size, histological grade and type of surgery showed 5 times higher odds for further metastases in ALND specimen in patients with symptomatic presentation *vs.* screen-detected breast cancer; all other variables remained insignificant.

Conclusion. Despite the small number of patients with micrometastases in this large cohort of breast cancer patients, these results support the contention that completion ALND can safely be omitted in screen-detected breast cancer patients with micrometastases in the SNBs.

Keywords. Breast cancer, micrometastases, sentinel node biopsy, completion axillary dissection.

Introduction

The axillary nodal stage is still the most important prognostic factor in breast cancer. Treatment of the axilla once the status is known is currently a matter of vigorous debate. Previously surgery was part of the treatment for positive nodes detected by ultrasound and fine needle aspiration or by sentinel node biopsy (SNB). Recently published papers[1,2] suggest that adjuvant therapy may stand alone for some node-positive breast cancer patients and additional surgery may not improve the patients' outcomes. Recently data from the National Cancer Data Base from the United States examined for 2,203 patients with micrometastases treated between 1998 and 2000 showed no significant difference in axillary recurrence or survival for patients treated by SNB alone compared with patients treated by SNB and completion axillary lymph node dissection (ALND)[3]. Equivalent results are reported from the SEER database[4] examining 6,838 patients with micrometastases with a median follow-up of 50 months. Both studies report a time trend towards omission of completion ALND in women with micrometastases especially if they are older. Therefore it is important to define which group of node-positive breast cancer patients that does not benefit from additional axillary surgery once the status of the nodes is known. Here we report on the method of detection (screen or symptomatic) with the aim of determining if the method of detection is predictive for additional positive nodes if the SNB contains micrometastases.

Material and methods

Between January 1, 2001 and November 1, 2011 a total of 1,993 women had surgery for primary unilateral breast cancer at Skåne University Hospital, Lund (Figure 1). Of these 757 were detected by a public mammography screening programme. From 2001 to 2009 women aged 45 to 70 years were invited to the screening programme. During 2010 the target population was enlarged to 40 to 74 years. In 2001 SNB was offered to women with tumours of 30 mm or smaller who were included in the Swedish cohort study[5]. From 2004 SNB was the standard procedure of axillary staging for early breast cancer without any limit on tumour size. Excluded from the study were 354 women offered primary axillary lymph node dissection (ALND), most of them because of involved nodes detected by ultrasound, 60 patients without axillary staging at surgery, most of them elderly, and 121 treated by neoadjuvant therapy (Figure 1). The study population comprised the remaining 1,458 women who had axillary staging by SNB; of these 757 were screen-detected.

Information on the method of detection was supplied by the mammography screening programme.

The surgical procedure of SNB was preceded by subdermal injection with ^{99}Tc-labelled Nanocolloid followed by a scintigraphy until 2009, after which this was only performed in selected patients. Injection of 0.3 ml of Patent V Blue was performed intradermally after anaesthesia in the operating room. Any node that was hot, blue or palpable was considered an SNB and removed.

All SNBs were examined by intraoperative frozen section methodology, but the handling of the paraffin sections varied over the years. In brief, during the first years paraffin sections were analysed by one haematoxylin and eosin (HE) section and one section with immunohistochemistry for cytokeratin (CK). Then random levels were added, and subsequently levels at fixed intervals of 200 μm were introduced. Finally, CK at the fixed levels was added. For details see[6]. A review of the slides of all patients with positive SNB was undertaken and the 7th edition of the AJCC and UICC classification was applied[7,8]. Axillary specimens were initially studied by embedding a single slice from each node followed by examination of the corresponding HE section. After November 2008 the method of investigation was changed to slicing the nodes through the longitudinal axis and examine HE sections from all slices.

Tumour size was the largest diameter of the invasive component[7,8]. Histological grade was applied following the Nottingham modification[9] of the Bloom and Richardson grading scheme[10]. Oestrogen and progesterone receptors were determined by immunohistochemistry and results >10% were considered positive.

Statistics
Differences in proportions between groups of patients were evaluated by the Chi-square test and mean differences for continuous variables by the Mann-Whitney test. Logistic regression was used to evaluate the effect of screening after adjustment for tumour size, histological grade and type of surgery. In all analyses, $p<0.05$ was considered significant. SPSS version 20 was used.

Results
In patients treated by SNB the frequency of screen-detection varied over the years (Figure 2). In 2001 about 35% of patients were screen-detected and this figure increased to about half of the patients in 2008. In 2010 and 2011 more than half the patients with primary unilateral breast cancer and SNB were detected by the public mammography screening programme.
Smaller tumour size, lower histological grade and breast-conserving therapy occurred significantly more often in screen-detected patients compared with patients with clinical presentation (Table 1). The pattern appeared similar among patients with micrometastases (Table 2). Receptor-positive status was more frequent in screen-detected patients but this difference disappeared in patients with micrometastases. No differences appeared in age, tumour type, number of sentinel nodes, number of sentinel nodes with metastases or size of metastases. Screen-detected patients were significantly more often SNB-negative than were the symptomatic patients ($p=0.001$).
Micrometastases of >0.2 mm/>200 cells and ≤2.0 mm were detected in 8% (62/575) of screen-detected patients compared with 12% (81/701) of symptomatic patients (Table 1). Only 5% (3/61) of screen-detected patients with micrometastases had metastases in the ALND specimen compared with 23% (18/79) of patients with symptomatic presentation (Table 3). One of these patients had a familial history of breast cancer and was diagnosed by prevalence screening at the age of 40; she had a tumour of 18 mm. One of the others had two invasive foci and a tumour size of 39 mm, and the last had a tumour size of 35 mm. The number of symptomatic patients with micrometastases and further metastases in the completion ALND specimen increased with increasing tumour size (Table 4) except for tumours larger than 30 mm. For the symptomatic patients with micrometastases and further metastases in the ALND specimen, the range of tumour sizes were 10-30 mm. Logistic regression analysis, adjusting for tumour size, histological grade and type of surgery showed 5 times higher odds for further metastases in ALND specimen in symptomatic patients compared with screen-detected patients (Table 5). Logistic regression with tumour size as a grouped variable and a model also including histological type, oestrogen- and progesterone receptor status, and age showed similar results (data not shown).
Overall 66% (956/1,458) of patients were SNB-negative or had isolated tumour cells in the SNBs. After surgery with completion ALND in SNB-positive patients, overall one node was positive in 66% (330/502). In patients with SNB micrometastases one node was positive in 83% (118/143) in contrast to patients with SNB macrometastases where overall one node was positive in only 59% (212/359) ($p=0.000$). Patients with further metastases in ALND specimen according to the number of positive SNBs are presented in Table 3.
In the symptomatic patients the histopathological method of SNB examination was roughly without influence on the number of patients with micrometastases in the SNB and further metastases in the completion ALND specimen except for the method without immunohistochemistry (method 3) where only 9% had further metastases in the ALND specimen (Table 4). Among the screen-detected patients two patients with micrometastases in the SNB and further metastases in the ALND specimen were examined with method 4 and one with method 2.
The size of axillary metastases was recorded from 2007, and 274 patients with metastases received SNB and ALND. In the screen-detected patients with micrometastases the largest metastasis appeared in the SNB in 92% (34/37) compared with the symptomatic patients where the largest metastasis was found in the SNB in 82% (37/45) of the patients. For patients with macrometastases the corresponding figures were 89% (81/91) and 89% (80/90). Overall the largest metastasis appeared in the SNB in 87% (71/82) of patients with micrometastases and in 89% of patients with macrometastases (161/181).

Discussion

This study shows that completion ALND safely can be omitted in screen-detected patients with primary unilateral breast cancer with micrometastases in the SNB because the frequency of additional metastases is only 5% compared with 23% in patients presenting symptomatically. Due to the side effects of axillary surgery there is much debate about avoiding completion ALND in selected patients with breast cancer with metastases in the SNB[1] and thereby replacing surgery with adjuvant medical therapy and radiation therapy. This study shows that one candidate for substituting surgery with adjuvant therapy is screen-detected patients with micrometastases.

Recently the role of ALND in screen-detected breast cancer has been discussed by Berry and Kell[11]. Their study included 519 women aged 50 to 65 years with screen-detected breast cancer stage T1/T2, of whom 110 (21%) had a macrometastatic SNB. Their results correspond well to the results of the present study. Unfortunately, women with micrometastases were excluded from their study.

Another study[12] included 82 SNB-positive patients and reported that the screen-detected patients had significantly fewer metastases in the ALND specimen (24%) compared with women with symptomatic tumours (52%) (p=0.035). The authors noted that the method of detection was correlated with age because their national breast cancer screening programme primarily targeted women over 50 years of age, and did not therefore include the method of detection in the regression analysis.

Galimberti et al.[13] reported on 377 patients with micrometastases in a single SNB who did not receive ALND, treated at the European Institute of Oncology (IEO), Milan, Italy. The overall 5 years' survival was 97.5%. Except for radiotherapy, adjuvant medical therapy was not reported. Despite these promising results the authors suggest that a subset of patients might be at high risk of developing overt axillary disease. In the present series 17% of the patients with micrometastases had more than one involved node and three of these patients had further metastases in the ALND specimen. Follow-up on the patients in the present study has not been performed yet why overall survival cannot be compared.

A study from the Danish Breast Cancer Cooperative Group[14] identified 1,577 patients with micrometastases treated in 2002-2008 and found that the proportion of positive sentinel nodes, lympho-vascular invasion, the hormone receptor status and the location of the tumour in the upper lateral quadrant of the breast were risk factors for non-sentinel node metastases. Based on these factors a model identified 5% of the patients whose risk of non-sentinel node metastases was nearly 40%. Their model, however, was unable to identify a subset with very low risk of non-sentinel node metastases, and screening status was not considered.

Several authors have advocated that older women with minimal metastases to the SNB, especially with smaller oestrogen receptor-positive tumours, might not benefit from completion ALND[15]. The present study shows that the method of detection seems to be a better predictor of additional metastases in completion ALND specimens in patients with micrometastases than age alone because there was no difference in age between micrometastatic patients that were screen-detected compared with symptomatic breast cancer.

The patients with screen-detected breast cancer and micrometastases in the SNB and further metastases in the axillary specimen in the present study are possibly outliers. One was diagnosed at prevalence screening at the age of 40 years and had a hereditary history of breast cancer. Another had two invasive foci and all three had large tumour size. Reflecting these facts the results of the present study show that completion ALND can safely be omitted in screen-detected patients with a tumour size of around 15 mm or smaller.

The axillary recurrence rate in SNB-negative patients without ALND is as low as 1% in 5 years[16] despite the fact that it is well known and accepted that about 5-8%[5,17]of patients have regional metastases at the time of the SNB even though the SNB is negative. In patients with micrometastases the situation is different. Pepels et al.[18] reported 1,028 patients with micrometastases without ALND; the 5 year rate of axillary recurrence occurring at any time was

5.6% with a hazard ratio of 4.39 (95% CI, 1.46–13.24) adjusted for age, tumour size, histological grade, hormone receptor status, adjuvant systemic therapy and irradiation of the breast. Galimberti et al.[13] found a cumulative incidence of axillary recurrence of only 1.6% (95% CI 0.7–3.3) at 5 years in 377 selected patients, with a single SNB with micrometastases, treated at the European Institute of Oncology in Milan.

By construction of nomograms[19-21] several authors have tried to predict the presence of metastases in completion ALND specimens after a SNB with micrometastases The present study did not include all these data, such as multifocality, and we were therefore unable to validate any of the published nomograms. All other nomograms are constructed to predict further metastases in completion ALND specimens in patients with macrometastases[19]. These nomograms can only be used after validation in local populations because they perform very diversely in different populations[22]. Overall predictors of additional metastases in the completion ALND specimens after positive SNBs are largely the same as the predictors of positive SNBs[19].

This study has some drawbacks. Because micrometastases are relatively rare it is difficult to obtain a large study population, and the present study comprises only 143 patients with micrometastases. Despite the small number of patients the difference in positive completion ALND specimens between screen-detected and symptomatic patients is statistically significant. Moreover the SNB was examined by four different histopathological methods but this did not seem to influence the results.

This study uses data from a register for the public mammography screening programme that was constructed for statistical purposes and not for research purposes. Therefore the definition of "screen-detected" might not be the same throughout the 10 years of the study as the screening unit was re-organised during the last three years. The attendance rate during the study period increased which is why more women were detected clinically in the first part of the study. Furthermore women aged 40-45 years were also invited during the last part of the study period. These facts might explain the difference in frequency of screen-detected patients during the period.

Omitting completion ALND in metastatic breast cancer patients eliminates the prognostic information obtained earlier on from the number of positive axillary nodes. Presuming that the size of the largest metastases can be used instead of the number of positive nodes as a more detailed prognostic marker the present data show that this information will be available in 85-90% of patients treated by SNB. The SNB method itself has a false-negative rate of around 5-8% and therefore more detailed prognostic information will also be available in 80-85% of patients when completion ALND is omitted.

With the purpose of avoiding the side effects of axillary dissection there is a trend towards substituting axillary surgery with adjuvant therapy. It must be remembered that all types of treatments[23] including SNB, adjuvant endocrine therapy[24,25], chemotherapy[26] and radiation therapy[27,28] have side effects. Women with breast cancer might have different needs to avoid particular side effects, and therefore the patient's own opinion is important when treatment alternatives are offered. Furthermore overtreatment with adjuvant medical therapy must also be avoided and efforts should be made to accurately define women in whom minimal surgical intervention is sufficient therapy.

In conclusion, despite the small number of patients with micrometastases in the sentinel nodes in this large cohort of breast cancer patients, these results support the contention that completion ALND can safely be omitted in screen-detected breast cancer patients with micrometastases in SNBs, at least in those with tumours smaller than 15 mm.

Conflict of interest statements. None

Acknowledgements. Thanks to the Unilabs Mammography Units for providing data about referral status (screening or clinical) and to Pär-Ola Bendahl for statistical assistance. Thanks to the Swedish Breast Cancer Organisation (BRO), the Swedish Cancer Society, Swedish Research Council, the Gunnar Nilsson Cancer Foundation, the Mrs. Berta Kamprad Foundation, Skåne

County Council's Research and Development Foundation and Governmental Funding of Clinical Research within the National Health Service (ALF) for supporting the study by funds.

References

1. Benson JR, Wishart GC. Role of axillary clearance for patients with sentinel node-positive early breast cancer. Br J Surg 2011; 98:1499-1500.

2. Avril A, Le BG, Lorimier G et al. Phase III randomized equivalence trial of early breast cancer treatments with or without axillary clearance in post-menopausal patients results after 5 years of follow-up. Eur J Surg Oncol 2011; 37:563-570.

3. Bilimoria KY, Bentrem DJ, Hansen NM et al. Comparison of sentinel lymph node biopsy alone and completion axillary lymph node dissection for node-positive breast cancer. J Clin Oncol 2009; 27:2946-2953.

4. Yi M, Giordano SH, Meric-Bernstam F et al. Trends in and outcomes from sentinel lymph node biopsy (SLNB) alone vs. SLNB with axillary lymph node dissection for node-positive breast cancer patients: experience from the SEER database. Ann Surg Oncol 2010; 17 Suppl 3:343-351.

5. Bergkvist L, Frisell J. Multicentre validation study of sentinel node biopsy for staging in breast cancer. Br J Surg 2005; 92:1221-1224.

6. Grabau D, Ryden L, Ferno M et al. Analysis of sentinel node biopsy - a single-institution experience supporting the use of serial sectioning and immunohistochemistry for detection of micrometastases by comparing four different histopathological laboratory protocols. Histopathology 2011; 59:129-138.

7. UICC International Union Against Cancer. TNM Classification of Malignant Tumours. L H Sobin, M K Gospodarowicz, and C Wittekind. 7th. 2009. Wiley-Blackwell.

8. AJCC Cancer Staging Manual. Edge, S. B., Byrd, D. R., Compton, C. C., Fritz, A. G., Greene, F. L., and Trotti, A. 2010. Springer Verlag.

9. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology 1991; 19:403-410.

10. Bloom HJ, Richarson WW. Histological grading and prognosis in breast cancer; a study of 1409 cases of which 359 have been followed for 15 years
1. Br J Cancer 1957; 11:359-377.

11. Barry M, Kell MR. Re-evaluating the role of axillary lymph node dissection in screen-detected breast cancer patients. Breast 2012; 21:58-60.

12. Farshid G, Pradhan M, Kollias J et al. A decision aid for predicting non-sentinel node involvement in women with breast cancer and at least one positive sentinel node. Breast 2004; 13:494-501.

13. Galimberti V, Botteri E, Chifu C et al. Can we avoid axillary dissection in the micrometastatic sentinel node in breast cancer? Breast Cancer Res Treat 2012; 131:819-825.

14. Tvedskov TF, Jensen MB, Lisse IM et al. High risk of non-sentinel node metastases in a group of breast cancer patients with micrometastases in the sentinel node. Int J Cancer 2012. [Epub ahead of print]. Accessed on 07/31/2012. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22344558

15. Feigelson BJ, Acosta JA, Feigelson HS et al. T1 breast carcinoma in women 70 years of age and older may not require axillary lymph node dissection. Am J Surg 1996; 172:487-489.

16. Andersson Y, de BJ, Jonsson PE et al. Axillary recurrence rate 5 years after negative sentinel node biopsy for breast cancer. Br J Surg 2012; 99:226-231.

17. Gill G. Sentinel-lymph-node-based management or routine axillary clearance? One-year outcomes of sentinel node biopsy versus axillary clearance (SNAC): a randomized controlled surgical trial. Ann Surg Oncol 2009; 16:266-275.

18. Pepels MJ, de BM, Bult P et al. Regional recurrence in breast cancer patients with sentinel node micrometastases and isolated tumor cells. Ann Surg 2012; 255:116-121.

19. Meretoja TJ, Strien L, Heikkila PS et al. A simple nomogram to evaluate the risk of nonsentinel node metastases in breast cancer patients with minimal sentinel node involvement. Ann Surg Oncol 2012; 19:567-576.

20. Houvenaeghel G, Bannier M, Nos C et al. Non sentinel node involvement prediction for sentinel node micrometastases in breast cancer: nomogram validation and comparison with other models. Breast 2012; 21:204-209.

21. Mittendorf EA, Hunt KK, Boughey JC et al. Incorporation of sentinel lymph node metastasis size into a nomogram predicting nonsentinel lymph node involvement in breast cancer patients with a positive sentinel lymph node. Ann Surg 2012; 255:109-115.

22. Cserni G, Boross G, Maraz R et al. Multicentre validation of different predictive tools of non-sentinel lymph node involvement in breast cancer. Surg Oncol 2012; 21:59-65.

23. Andersen KG, Kehlet H. Persistent pain after breast cancer treatment: a critical review of risk factors and strategies for prevention. J Pain 2011; 12:725-746.

24. Amir E, Seruga B, Niraula S et al. Toxicity of adjuvant endocrine therapy in postmenopausal breast cancer patients: a systematic review and meta-analysis. J Natl Cancer Inst 2011; 103:1299-1309.

25. Lorizio W, Wu AH, Beattie MS et al. Clinical and biomarker predictors of side effects from tamoxifen. Breast Cancer Res Treat 2012; 132:1107-1118.

26. Azim HA, Jr., de AE, Colozza M et al. Long-term toxic effects of adjuvant chemotherapy in breast cancer. Ann Oncol 2011; 22:1939-1947.

27. Darby S, McGale P, Correa C et al. Effect of radiotherapy after breast-conserving surgery on 10-year recurrence and 15-year breast cancer death: meta-analysis of individual patient data for 10,801 women in 17 randomised trials. Lancet 2011; 378:1707-1716.

28. Wernicke AG, Shamis M, Sidhu KK et al. Complication Rates in Patients With Negative Axillary Nodes 10 Years After Local Breast Radiotherapy After Either Sentinel Lymph Node Dissection or Axillary Clearance. Am J Clin Oncol 2011. [Epub ahead of print]. Accessed on 07/31/2012. Available at http://www.ncbi.nlm.nih.gov/pubmed/22134519

Table 1. Patients and tumour characteristics according to method of presentation in patients treated by sentinel node biopsy.

		Screen-detected n	%	Symptomatic presentation n	%	p value
Total		757		701		
Age	Median (range)	61 (40-74)		59 (23-90)		0.128
Tumour size, mm	Median (range)	14 (1-90)		18 (0-70)		0.000
Tumour type	Ductal	629	83	556	79	0.068
	Lobular	98	13	100	14	
	Other	30	4	45	6	
Histological grade	Grade 1	225	30	151	22	0.000
	Grade 2	385	51	344	50	
	Grade 3	139	19	199	29	
	Missing	8		7		
Oestrogen receptor	Positiv >10%	670	89	594	85	0.032
	Negative ≤10%	82	11	102	15	
	Missing	5		5		
Progesterone receptor	Positiv >10%	551	73	480	69	0.071
	Negative ≤10%	201	27	216	31	
	Missing	5		5		
Type of surgery	Breast-concerving	514	68	356	51	0.000
	Mastectomy	243	32	345	49	
Number of sentinel nodes	Median (range)	2 (1-9)		2 (1-8)		0.597
Number of sentinel nodes with metastases	Median (range)	1 (1-4)		1 (1-5)		0.847
Size of sentinel node metastases, mm	Median (range)	5 (0.21-30)		5 (0.21-30)		0.981
Number of axillary nodes		13 (5-34)		13 (4-34)		0.527
Sentinel node status						
Negative		495	65	386	55	0.001
ITC <0.2 mm / <200 cells		36	5	39	6	
Micrometastases >0.2 mm / >200 cells and ≤2.0 mm		62	8	81	12	
Macrometastases > 2.0 mm		164	22	195	28	

Table 2. Patients and tumour characteristics according to method of presentation in patients where sentinel node biopsy showed micrometastases.

		Screen-detected		Symptomatic presentation		p value
		n	%	n	%	
Total		62		81		
Age	Median (range)	59 (40-73)		59 (30-88)		0.754
Tumour size, mm	Median (range)	14 (1-39)		20 (5-51)		0.001
Tumour type	Ductal	56	90	66	81	0.303
	Lobular	5	8	11	14	
	Other	1	2	4	5	
Histological grade	Grade 1	23	38	16	20	0.012
	Grade 2	30	49	40	49	
	Grade 3	8	13	25	31	
	Missing	1		0		
Oestrogen receptor	Positive >10%	55	89	74	93	0.437
	Negative ≤10%	7	11	6	8	
	Missing	0		1		
Progesterone receptor	Positive >10%	47	76	64	80	0.549
	Negative ≤10%	15	24	16	20	
	Missing	0		1		
Type of surgery	Breast-conserving	40	65	40	49	0.071
	Mastectomy	22	35	41	51	
Number of sentinel nodes	Median (range)	3 (1-6)		2 (1-5)		0.539
Number of sentinel nodes with micrometastases	Median (range)	1 (1-3)		1 (1-3)		0.604
Size of micrometastases, mm	Median (range)	1 (0.21-2.0)		1 (0.21-2.0)		0.799
Number of axillary nodes	Median (range)	13 (5-25)		13 (4-29)		0.180

Table 3. Number of patients with positive nodes in completion axillary dissection (ALND) specimen according to size of metastases in sentinel nodes.

	Screen-detected				Symptomatic presentation				
	n	n with ALND specimen	n with positive ALND specimen	%	n	n with ALND specimen	n with positive ALND specimen	%	p value
SN with metastases >0.2/> 200 cells and ≤2.0 mm	62	61	3	5	81	79	18	23	0.013
Number of positive sentinel nodes									
1	50	49	2	4	68	67	16	24	0.014
2	10	10	0	0	11	10	2	20	
≥3	2	2	1	50	2	2	0	0	
SN with metastases >2.0 mm	164	164	73	45	195	193	110	57	0.027
Number of positive sentinel nodes									
1	97	97	31	32	115	114	57	50	0.020
2	47	47	29	62	53	52	31	60	
≥3	16	16	11	69	21	21	17	81	

Table 4. Number of patients with micrometastases in relation to method of detection and to number of patients with positive nodes in completion axillary dissection (ALND) specimen according to tumour size and method of sentinel node examination.

	Screen-detected				Symptomatic presentation			
	n	n with ALND specimen	n with positive ALND specimen	%	n	n with ALND specimen	n with positive ALND specimen	%
Tumour size								
1-10 mm	16	16	0	0	6	7	1	14
11-20 mm	34	33	1	3	40	39	10	26
21-30 mm	10	10	0	0	26	25	7	28
>30 mm	2	2	2	100	9	9	0	0
Method of sentinel node examination								
Method 1	11	11	0	0	15	15	7	47
Method 2	7	7	1	14	6	6	2	33
Method 3	13	13	0	0	23	22	2	9
Method 4	31	30	2	7	37	36	7	19

Sentinel node examination: Method 1; 1 HE and 1 CK, method 2; 1 HE and 1 CK and 2 HE at random levels, Method 3; 3 HE at levels of 200 μm, method 4; 3 HE and 3 CK levels of 200 μm. HE = haematoxylin, CK = cytokeratin.

Table 5. Logistic regression analysis of patients with micrometastases in sentinel nodes treated by completion axillary dissection (n=139). The dependent variable is the presence of metastases in the axillary specimen.

		95% CI**.for OR	
	OR*	Lower	Upper
Symptomatic presentation *vs.* screen-detected	5.1	1.4	19
Tumour size per mm	1.0	0.98	1.1
Histological grade 2 vs 1	1.5	0.42	5.4
Histological grade 3 vs 1	0.98	0.22	4.3
Mastectomy vs breast conserving therapy	0.80	0.29	2.2

* OR = Odds Ratio. ** CI = confidence interval.

Of 143 patients with micrometastases 3 patients had no completion axillary dissection, and the histological grade was missing for 1 patient because of too small tumour size.

Figure 1. Breast cancer study cohort

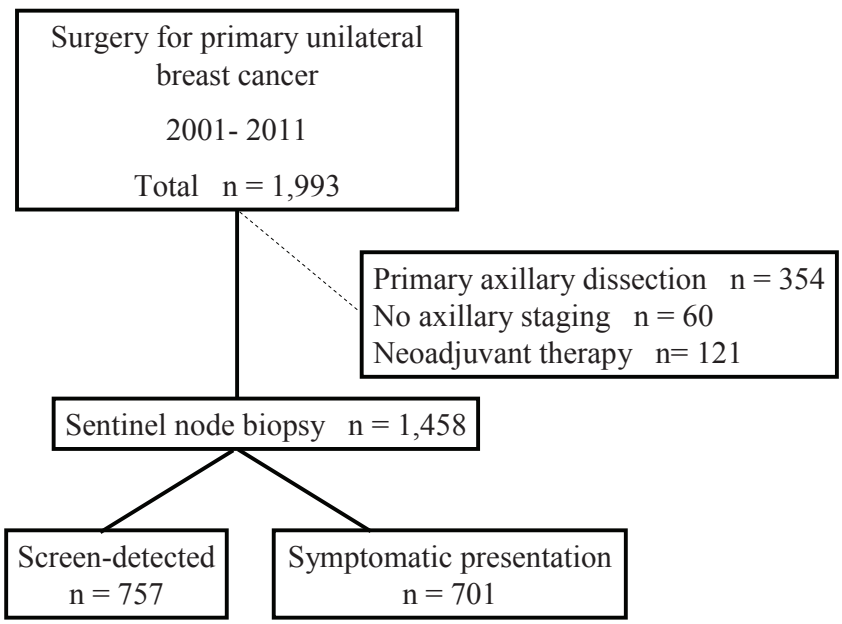


Figure 2. Percentage of patients with sentinel node biopsy detected by screening or symptomatic presentation according to year of surgery

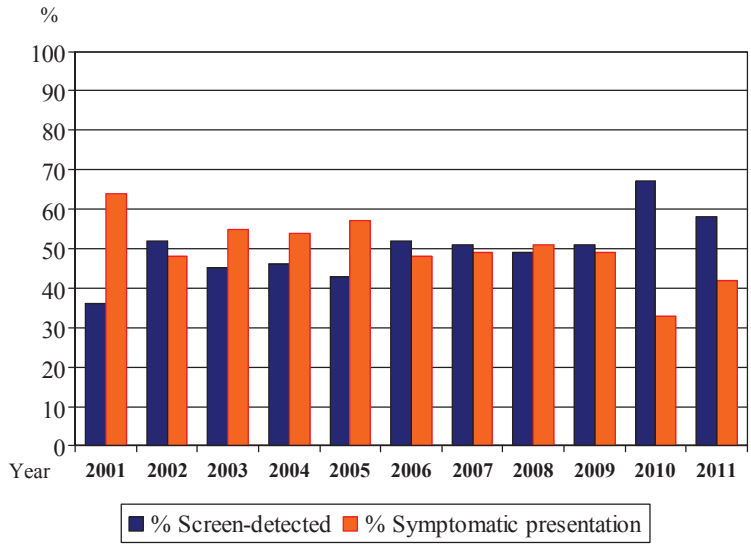
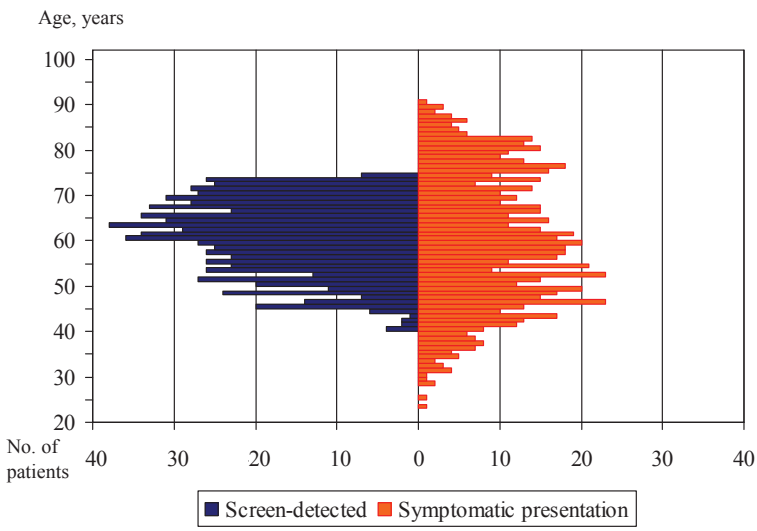


Figure 3. Ages of patients with sentinel node biopsy detected by screening and by symptomatic presentation



Paper IV

The prevalence of immunohistochemically determined oestrogen receptor positivity in primary breast cancer is dependent on the choice of antibody and method of heat-induced epitope retrieval – prognostic implications?

Grabau D[1], Bendahl P-O[2], Rydén L[3], Stål O[4], Fernö M[2]. For the South and South-East Swedish Breast Cancer Groups

1. Department of Pathology, Skåne University Hospital, Lund, Sweden. Clinical Science, Lund University, Lund, Sweden.
2. Division of Oncology, Department of Clinical Sciences, Lund University, Lund, Sweden.
3. Department of Surgery, Skåne University Hospital, Lund, Sweden. Clinical Science, Lund University, Lund, Sweden.
4. Department of Clinical and Experimental Medicine, Division of Oncology, Faculty of Health Sciences, Linköping University, Linköping, Sweden

Corresponding author: Dorthe Grabau, Dept. of Pathology, Skåne University Hospital, SE-221 85 Lund, Sweden.
Fax: +46 46 143397
Phone number: +46 46 173511
E-mail address: Dorthe.Grabau@skane.se

Abstract

Background. Oestrogen receptor (ER) status is important for the choice of systemic treatment of breast cancer patients. However, most data from randomised trails on the effect of adjuvant endocrine therapy according to ER status are based on the cytosol methods. Comparisons with immunohistochemical methods have given similar results. The aim of the present study was to examine whether different ER antibodies and heat-induced epitope retrieval (HIER) methods influence the prevalence of ER-positivity in primary breast cancer.

Material and methods. This study is based on patients included in a clinical trial designed to compare the effect of two years of adjuvant tamoxifen *vs.* no adjuvant systemic treatment in premenopausal women. From 1986 to 1991, 564 patients from two study centres in Sweden were enrolled and randomised. Patients were randomised independently of ER status. In the present study, ER status was assessed on tissue microarrays with the three different ER antibody/HIER combinations: 1D5 in citrate pH 6 (n=390), SP1 in Tris pH 9 (n=390) and PharmDx in citrate pH 6 (n=361).

Results. At cut-offs of 1% and 10% respectively, the prevalence of ER-positivity was higher with SP1 (75% and 72%) compared with 1D5 (68% and 66%) and PharmDx (66% and 62%). At these cut-offs, patients in the discordant groups (SP1-positive and 1D5-negative) seem to have a prognosis intermediate between those of the double-positive and double-negative groups. Comparison with the ER status determined by the cytosol-based methods in the discordant group also showed an intermediate pattern. The repeatability was good for all antibodies and cut-offs, with overall agreement ≥93%.

Conclusion. The present study shows that the choice of antibody and HIER method influences the prevalence of ER-positivity. We suggest that this be taken into consideration when choosing a cut-off for clinical decision making.
Key words: Breast cancer, oestrogen receptor antibodies, prevalence, prognosis.

Introduction

In breast cancer, the determination of oestrogen receptor (ER) status in tumour tissue is important in the choice of adjuvant therapy. For patients with ER-positive breast cancer, adjuvant endocrine therapy is indicative, whereas for patients with ER-negative disease this treatment is not beneficial [1,2]. Not only the presence of ER, but also the level of ER-positivity, seem to be associated with the sensitivity to endocrine therapy [3].

Over the years, the methods for ER analysis have changed. They started with ligand-binding assays (LBA) and enzyme immunoassay (EIA) in cytosol samples from fresh frozen tissue. These techniques were replaced by immunohistochemical (IHC) methods in formalin-fixed paraffin-embedded tissue, using monoclonal antibodies. Most of our knowledge about the importance of ER as a predictive marker for the response to endocrine therapy, especially about long-term clinical follow-up, is based on cytosol techniques. However, in a number of studies, ER has also been measured with IHC, alone or in addition to LBA/EIA, and related to clinical outcome after endocrine therapy. The overall conclusion is that LBA/EIA and IHC provide similar predictive value for the response to endocrine treatment [4-7].

Though ER has been used for decades now as a treatment-predictive test, no standardisation of the IHC test has emerged and thresholds predicting response to endocrine treatment have varied. To distinguish ER-positive and ER-negative breast cancer in clinical routine, the previous commonly applied cut-off of 10% positive tumour cells has recently been replaced by 1%, according to international recommendations [8,9]. Another classification, the Allred score, considers not only the percentage of positive cells but also the staining intensity of positive cells [10]. The continuous development of the IHC method has reached a limit with very intense dark staining using new epitopes and powerful staining systems. Pathology departments, adhering to quality assurance programmes such as United Kingdom External Quality Assurance System (UK NEQUAS) or NordiQC, often obtain excellent marks for the staining when using the most modern methods for ER assessment. However, these quality assurance programmes only consider the quality of the IHC staining itself in a limited number of cases. They do not consider a possible influence on the prevalence of ER-positivity and its predictive value. To improve the accuracy of testing, the American Society of Clinical Oncology/College of American Pathologists recommended that only methods with pre-analytical and analytical components conformed exactly to clinically validated assays or compared with the clinically validated assays showing 90% concordance for the ER-positive category and 95% concordance for the ER-negative category, with positivity defined as ≥1% stained nuclei, be used to predict response to endocrine therapy [9]. If methodological changes [11] (e.g. new tissue processing, new antibodies, changes in heat-induced epitope retrieval (HIER) and/or detection methods) are implemented without such validation [12], the long-term consequences will influence the prevalence of ER-positivity and the predictive value of ER. The importance of the choice of antibody for the prevalence of ER-positivity has been demonstrated in a study by Cheang and co-workers [13] where the proportion of ER-positive breast cancers increased from 63% to 71% when SP1 antibody was used instead of 1D5. In that study [13], SP1 was found to be a better prognostic marker for breast cancer-specific survival than 1D5.

The aim of the present study was to compare three different antibodies (1D5, SP1, and the ER part of ER/PR PharmDx™, in this study denoted PharmDx) with different HIER in relation to the prevalence of ER-positivity and recurrence-free survival (RFS) in a well-defined cohort of breast cancer patients with primary stage II breast cancer.

Material and Methods

Study design

The patients included in the present study were enrolled in a clinical trial designed to compare the effect of two years of adjuvant tamoxifen (TAM) *vs.* no adjuvant systemic treatment in premenopausal women with stage II breast cancer. From 1986 to 1991, 564 patients were randomised from two study centres in Sweden. Patients were randomised independently of ER and progesterone receptor status. The characteristics of this trial have previously been described in detail [14]. Adjuvant poly-chemotherapy was administered to fewer than 2% of the patients.

Tissue microarray

From representative areas of the formalin-fixed and paraffin-embedded invasive breast cancer tissue samples, two core biopsies (0.6 mm in diameter) were punched out and mounted into the recipient block using a tissue array machine in accordance with the manufacturer's instructions (Beecher Instruments, MD, USA). The tissue microarray (TMA) were sectioned and stained for the ER clones 1D5, SP1 and PharmDx.
Paraffin-embedded material for TMA was initially available for 500 of 564 patients in the study [15] (Figure 1). Due to intensive use of the TMA blocks for other studies, some of the core biopsies were missing and of the 500 cases in the TMA, 1D5 and SP1 were evaluable in 390 (69%) cases and PharmDx, which was stained later, in 361 (64%) cases. All three antibodies were available for scoring in 321 cases (Figure 1). Comparing patients with and without TMA scores for 1D5 and SP1, no significant differences appeared in histological type or grade, tumour size or lymph node status. However, patients with evaluable TMAs were slightly older than patients without TMA data, at 45 years (26-57 years) and 43 years (25-55 years), respectively.

Immunohistochemical analyses

After deparaffinising, sections stained with 1D5 were pre-treated in a microwave oven in citrate buffer pH 6. The primary antibody 1D5 (DAKO, Glostrup, Denmark) diluted 1:35 was incubated for 25 minutes. The detection system was labelled streptavidin biotin-horseradish peroxidase (LSAB-HRP) used in a DAKO TechMate500+, and the reaction product was visualised with diaminobenzidine (DAB). For SP1, HIER was performed in microwave oven in Tris buffer pH 9. The primary antibody (RM-9101-S, clone SP1, Neomarkers, AH Diagnostics, Stockholm, Sweden) was diluted 1:100 and incubated for 25 minutes. The Envision system (K5001, DAKO, Glostrup, Denmark) was used in a DAKO TechMate500+, and the reaction product was visualised with DAB. PharmDx (ER/PR PharmDx kit code K4071, DAKO, Glostrup, Denmark) was applied with HIER in epitopal retrieval solution (citrate pH6) in an autoclave for 5 minutes at 125 °C. Staining was performed in a DAKO Techmate 500/1000 automated staining instrument (BioTek Solutions, Winooski, VT, USA), and visualisation was based on dextran technology.

Cytosol-based method

ER was analysed in cytosol samples with LBA or EIA. ER-positive and negative samples were classified as previously described [14].

Evaluation

ER status was assessed in the invasive component, at least 10 cancer cells being required for scoring. The stainings for the antibodies 1D5, SP1 and PharmDx were categorised at seven levels with cut-offs of 0%, 1%, 10%, 25%, 50%, 75% and 90% positive cells. In the following only two cut-offs, 1% and 10%, were considered for ER status (negative *vs.* positive). For repeatability, 50% was also considered because this cut-off has been suggested as defining a patient group with a good response to endocrine therapy [8]. PharmDx was also scored as 0: negative, 1: >0 and ≤1%, 2: >1%

and ≤10%, 3: >10% and ≤33%, 4: >33% and ≤66% or 5: >66% and the intensity was scored as 0: negative, 1: weak, 2: moderate or 3: strong to produce the Allred score by adding the intensity score to the percentage score. One pathologist (DG) evaluated the staining results on two different occasions (repeatability). To compare different antibodies, the highest score from the two TMAs was used in each case. The pathologist was blinded to the clinical data, the follow-up and the results of the other scorings. For evaluation of the prognostic value of ER, cut-offs of 1% and 10% were used for 1D5, SP1 and PharmDx and an Allred score 3 where cases with a score ≥3 were considered positive.

Statistical analysis

The Mann-Whitney test was used for the two-group comparison of continuous variables, the chi-square test for the comparison of categorical variables, and the logrank test for the comparison of survival curves. All tests were two-sided and p-values <0.05 were considered significant. McNemar's test was used to test equality of paired proportions. The overall agreement, i.e. the proportion of samples with the same receptor status, was calculated, as were exact 95% confidence intervals (CIs) based on the binomial distribution. Furthermore, positive and negative agreements were used as summary measures. When one of the two assays compared can be regarded as a reference, the positive agreement was defined as the proportion of positive specimens that were also positive with the test assay. Negative agreement was defined similarly [12]. When the same assay was evaluated twice, to assess repeatability, symmetric definitions of positive and negative agreement with approximate 95% CIs were used, following Graham and Bull [16]. The upper limits of the approximate CIs exceeded 1.00 in a few cases but were then set to 1.00. In the analysis of RFS, the event was defined as local, regional, or distant recurrence or breast cancer death. Long-term follow-up is available, but only the first five years after diagnosis were used because the effect of ER is known to diminish with time. It should be emphasised that our study was not designed to have the power to study the prognosis of the discordant group in relation to the double-positive or double-negative groups or the effect of adjuvant TAM *vs.* no TAM. The statistics packages Stata version 12 and SPSS version 20 were used.

Results

Repeatability of staining evaluation

A comparison of evaluations 1 and 2 for each antibody at different cut-offs is shown in Table 1. The repeatability was excellent with overall agreement between 93% and 100% for 1D5, SP1 and PharmDx respectively at cut-offs 1%, 10%, and 50%. The positive agreement (≥93%) and negative agreement (≥92%) were also acceptable.

Agreement between antibodies

The proportion of ER-positive cases for SP1 was significantly higher than that for 1D5 at all cut-offs. At cut-offs of 1%, 10% and 50%, a total of 29, 24 and 47 patients were positive with SP1 but negative with 1D5, whereas only 3, 0, and 0 patients showed the opposite pattern (Table 2). As a consequence, the positive agreement was high above 99%, whereas the negative agreement was lower: 77%, 82%, and 74% at cut-offs of 1%, 10% and 50%, respectively. In the subgroup where ER was also analysed with PharmDx (n=321), the overall agreement was similar at cut-offs of 1% and 10% for PharmDx compared with 1D5 and SP1 (Table 3), but at a cut-off of 50% the overall agreement was higher between PharmDx and 1D5 than between PharmDx and SP1. The intensity of positive cases, stained with PharmDx, was distributed as follows: 107 (42%) patients with weak, 108 (43%) with moderate, and 39 (15%) with strong intensity. The concordance in ER status between the Allred score and the 10% cut-off for PharmDx was 95% (92-97%). The addition of intensity to the percentage of positive cells resulted in 19 cases changing from negative with PharmDx to positive with the Allred score, whereas no cases were positive with PharmDx and negative with the Allred score. At a cut-off of 1%, no patients had discordant ER status.

Prevalence of ER-positivity
At a cut-off of 1%, the prevalence of ER-positivity was significantly ($p<0.001$) higher with SP1 (75%; 292/390) compared with 1D5 (68%; 266/390) and PharmDx (66%; 211/321). The corresponding figures for a cut-off of 10% were 72%, 66% and 62%, and for a cut-off of 50% were 67%, 55% and 41% (Table 2 and Table 3).

Recurrence-free survival
Breast cancer patients with tumours positive (cut-offs 1% and 10%) with both 1D5 and SP1 had a better five-year RFS compared with those negative with both antibodies. This was true for the entire patient cohort as well as for the TAM-treated sub-group (Figure 2a-b and 2d-e). For patients without adjuvant TAM the difference in RFS was not significant (Figure 2c and 2f). Discordant patients that were 1D5-negative, but SP1-positive, showed an intermediate RFS (Figure 2a-f). When 1D5 and SP1 were compared with PharmDx, similar results were obtained (data not shown). The staining intensity was also related to RFS (Figure 3a). When only the positive cases were included, staining intensity correlated weakly with RFS ($p=0.12$). When the intensity was added to the percentage score, to obtain the Allred score, 19 cases became positive. These 19 patients had a prognosis intermediate between that of patients positive with Allred and PharmDx and that of patients negative with Allred and PharmDx (Figure 3b).

Patient and tumour characteristics
The characteristics of the discordant group (1D5-negative/SP1-positive) were compared with those of the group positive (cut-offs 1% and 10%) with both 1D5 and SP1 and the group negative with both antibodies (Table 4 and Table 5). The discordant patients were more often of histological grade 1 or 2 compared with the double-negative patients, and this difference was statistically significant ($p<0.01$). At a cut-off of 1% the discordant group differed also significantly from the double-positive group ($p<0.01$). The discordant patients were also significantly more often node-positive than were the double-negative patients ($p=0.01$) at a cut-off of 10%. The double-positive patients were more often ER-positive with the cytosol-based methods compared with the double-negative patients (cut-off 1%: 84% *vs.* 8% and cut-off 10%: 85% *vs.* 11%). The discordant patients showed an intermediate level of ER-positivity based on the cytosol methods at both cut-offs (29% and 37%), significantly different from both the double-positive patients ($p<0.01$) and the double-negative patients ($p=0.01$). No differences appeared in age, tumour size, or histological type.

Discussion
Three antibody/HIER combinations, 1D5 with HIER in citrate pH 6, SP1 in Tris pH 9, and PharmDx in citrate pH 6, were compared for the prevalence of ER-positivity in patients with clinically stage II breast cancer participating in a clinical trial comparing two years of adjuvant TAM *vs.* no adjuvant systemic treatment. The overall pairwise agreement varied between 93% and 100%. Staining with SP1 yielded a higher percentage of ER-positivity than did 1D5 and PharmDx at cut-offs of both 1% and 10%. It should be noted that the overwhelming majority of discordant patients went from negative with 1D5 to positive with SP1 (n=29) at a cut-off of 1% whereas only three patients went in the opposite direction. At a cut-off of 10%, all discordant patients (n=24) went from negative with 1D5 to positive with SP1. Patients 1D5-negative SP1-positive showed an intermediate clinical outcome (RFS). The same pattern appeared when comparing PharmDx at a 10% cut-off with the Allred score, where the addition of intensity resulted in a discordant group with an intermediate RFS.
The ER/PR guidelines [9,12] underline the details of concordance studies when a new method of determination of ER status is being tested. To be considered acceptable, positive agreement must be ≥90% and negative agreement ≥95%; positive results are defined as ≥1% immunoreactive cells

compared with a clinically validated assay. In the present study the differences between the antibodies were greater with negative agreements of only 77%, 82% and 74% comparing SP1 with 1D5 at a cut-off of 1%, 10% and 50%. The positive agreements between 1D5, SP1 and PharmDx were all >90%. This appears to be antibody- and HIER method-related because the repeatabilities within the antibodies were acceptable.

Similarly to the results of the present study, which shows an increase of 7 percentage point with SP1 compared with 1D5 at a cut-off of 1%, the ER-positivity increased by 8 percentage points when SP1 was used instead of 1D5 with a cut-off of 1% in the study by Cheang et al. [13] who reported a large population-based study from British Columbia, Canada. That study included 1,450 patients who had ER assessment by the dextran-coated charcoal method (DCC) method. In fact, the material used for TMA to examine ER by IHC was from frozen stores of residual tissue in excess of the requirements of the DCC assay [17], i.e. those patients with enough tumour tissue to make frozen samples. Consequently, the patients had a more advanced disease stage than population-based patient material. In the analysis of recurrence-free survival and breast cancer-specific survival, the cases positive with SP1 and negative with 1D5 followed the positive cases. In the present study, the discordant group had an outcome intermediate between the double-positive and double-negative groups, but no significant differences were found.

The cut-off for IHC-detected ER-status has not been determined in randomised studies but has evolved by comparison with the cytosol based methods method [7]; the correlation is about 0.85 [18]. Today, 1% is the generally accepted cut-off used for clinical decision making [19]. In the randomised Stockholm adjuvant tamoxifen trial, including postmenopausal patients (STO-3), only 7 of 777 patients had ER expression between 1 and 10%. In the present study 9 of 390 patients were classified between 1 and 10% with 1D5, 11 of 390 with SP1 and 19 of 361 with PharmDx, respectively. In the STO-3 trial using a cut-off of 10%, the predictive value of ER status determined by IHC was comparable with that of the DCC method [6]. In a population-based registry in Sweden including more than 6,800 patients, the percentage of ER-positivity at a cut-off of 10% was 86% in 2011 (personal communication). The more sensitive antibodies and more aggressive HIER methods may contribute to a higher prevalence of ER-positivity [20].

In the present study, the addition of intensity does not add prognostic information. It should also be mentioned that no method to determine the exact amount of protein by weight in IHC sections exists and the influence of fixation and HIER on protein loss is largely unknown [21], which makes the estimation of intensity unreliable.

Another study compared ER status assessed by 1D5 and SP1 on whole sections from 508 breast carcinomas including in situ cancer and metastases, finding only two cases positive for SP1 and negative for 1D5 [22]. In that study, both antibodies had HIER in citrate pH 6; the dilution was 1:100 for 1D5 and 1:200 for SP1. The authors suggest that because SP1 has an 8-fold higher affinity for its epitope than does 1D5, it may simply detect more positive cells than does 1D5 when protein levels are extremely low. The low discordance rate of 2/508 might also be attributed to the fact that SP1 was examined in citrate pH 6 instead of Tris pH 9.

Rhodes et al. [20] examined the influence of HIER buffer and heating time, and showed for the ER-antibodies 6F11 and SP1 that HIER in Tris EDTA, pH 9 and a longer heating time resulted in higher Allred scores than for HIER in citrate pH 6 and shorter heating times. In the present study, Tris pH 9 was used with SP1, and this more sensitive IHC method and the higher affinity for its epitope resulted in an increase of SP1-positive cases of 7 percentage points at a cut-off of 1%, in line with the 8 percentage points increase reported by Cheang et al. [13]. It seems the antibody and HIER method is more important in the increasing prevalence of ER-positive patients than intra-rater variability (repeatability). This is reflected in the present study, where one pathologist showed good repeatability when scoring the antibodies twice, and in a recent study of patients referred for a second opinion where the ER status was changed in only 2 of 405 patients [23].

Using 1D5 in EDTA buffer at pH 8, Lau et al. [24] reported 18% of 55 lung carcinomas and 72% of 50 breast carcinoma to be ER-positive using a cut-off of >0. In another study, Gomez-Fernandez et

al. [25] also examined lung carcinomas. HIER was citrate pH 6 and the results showed 27% of 92 cases positive for SP1, 14% positive for 6F11 and 8% positive for 1D5. The higher affinity for the epitope of SP1 also causes more lung adenocarcinomas to be ER-positive, information very important to the diagnostic pathologist because ER status is involved in the distinction between primary lung adenocarcinomas and breast cancer metastases.
In conclusion, the present study shows that the choice of antibody and HIER method influences the prevalence of ER-positivity. We suggest that this be taken into consideration when choosing a cut-off for clinical decision-making.

Acknowledgements
We are indebted to participating departments of the South and South-East Swedish Breast Cancer Groups for providing samples and clinical follow-up. Thanks to Kristina Anderson for performing staining with 1D5 and SP1 and to Kristina Lövgren for performing the staining with PharmDx. Thanks to Dako for providing the antibody ER PharmDx. The study was supported by funding from the Swedish Cancer Society, the Swedish Research Council, the Gunnar B Nilsson Cancer Foundation, the Mrs. Berta Kamprad Foundation, the Anna and Edwin Bergers foundation, Skåne University Hospital Research Foundation, Skåne County Council's Research and Development Foundation, and Governmental Funding of Clinical Research within the National Health Service.

Conflicts of interests. None

References

[1] Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 2005; 365(9472): 1687-717.

[2] Goldhirsch A, Wood WC, Coates AS, Gelber RD, Thurlimann B, Senn HJ. Strategies for subtypes--dealing with the diversity of breast cancer: highlights of the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011. Ann Oncol 2011; 22(8): 1736-47.

[3] Harris L, Fritsche H, Mennel R, Norton L, Ravdin P, Taube S et al. American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer. J Clin Oncol 2007; 25(33): 5287-312.

[4] Ferno M, Andersson C, Fallenius G, Idvall I. Oestrogen receptor analysis of paraffin sections and cytosol samples of primary breast cancer in relation to outcome after adjuvant tamoxifen treatment. The South Sweden Breast Cancer Group. Acta Oncol 1996; 35(1): 17-22.

[5] Chebil G, Bendahl PO, Idvall I, Ferno M. Comparison of immunohistochemical and biochemical assay of steroid receptors in primary breast cancer--clinical associations and reasons for discrepancies. Acta Oncol 2003; 42(7): 719-25.

[6] Khoshnoud MR, Lofdahl B, Fohlin H, Fornander T, Stal O, Skoog L et al. Immunohistochemistry compared to cytosol assays for determination of estrogen receptor and prediction of the long-term effect of adjuvant tamoxifen. Breast Cancer Res Treat 2011; 126(2): 421-30.

[7] Harvey JM, Clark GM, Osborne CK, Allred DC. Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. J Clin Oncol 1999; 17(5): 1474-81.

[8] Goldhirsch A, Ingle JN, Gelber RD, Coates AS, Thurlimann B, Senn HJ. Thresholds for therapies: highlights of the St Gallen International Expert Consensus on the primary therapy of early breast cancer 2009. Ann Oncol 2009; 20(8): 1319-29.

[9] Hammond ME, Hayes DF, Dowsett M, Allred DC, Hagerty KL, Badve S et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer (unabridged version). Arch Pathol Lab Med 2010; 134(7): e48-e72.

[10] Phillips T, Murray G, Wakamiya K, Askaa J, Huang D, Welcher R et al. Development of standard estrogen and progesterone receptor immunohistochemical assays for selection of patients for antihormonal therapy. Appl Immunohistochem Mol Morphol 2007; 15(3): 325-31.

[11] Yaziji H, Taylor CR, Goldstein NS, Dabbs DJ, Hammond EH, Hewlett B et al. Consensus recommendations on estrogen receptor testing in breast cancer by immunohistochemistry. Appl Immunohistochem Mol Morphol 2008; 16(6): 513-20.

[12] Fitzgibbons PL, Murphy DA, Hammond ME, Allred DC, Valenstein PN. Recommendations for validating estrogen and progesterone receptor immunohistochemistry assays. Arch Pathol Lab Med 2010; 134(6): 930-5.

[13] Cheang MC, Treaba DO, Speers CH, Olivotto IA, Bajdik CD, Chia SK et al. Immunohistochemical detection using the new rabbit monoclonal antibody SP1 of estrogen receptor in breast cancer is superior to mouse monoclonal antibody 1D5 in predicting survival. J Clin Oncol 2006; 24(36): 5637-44.

[14] Ryden L, Jonsson PE, Chebil G, Dufmats M, Ferno M, Jirstrom K et al. Two years of adjuvant tamoxifen in premenopausal patients with breast cancer: a randomised, controlled trial with long-term follow-up. Eur J Cancer 2005; 41(2): 256-64.

[15] Jirstrom K, Ryden L, Anagnostaki L, Nordenskjold B, Stal O, Thorstenson S et al. Pathology parameters and adjuvant tamoxifen response in a randomised premenopausal breast cancer trial. J Clin Pathol 2005; 58(11): 1135-42.

[16] Graham P, Bull B. Approximate standard errors and confidence intervals for indices of positive and negative agreement. J Clin Epidemiol 1998; 51(9): 763-71.

[17] Dowsett M. Estrogen receptor: methodology matters. J Clin Oncol 2006; 24(36): 5626-8.

[18] Wolff AC, Dowsett M. Estrogen receptor: a never ending story? J Clin Oncol 2011; 29(22): 2955-8.

[19] Nofech-Mozes S, Vella ET, Dhesy-Thind S, Hagerty KL, Mangu PB, Temin S, Hanna WM. Systematic review on hormone receptor testing in breast cancer. Appl Immunohistochem Mol Morphol 2012; 20(3): 214-63.

[20] Rhodes A, Sarson J, Assam EE, Dean SJ, Cribb EC, Parker A. The reliability of rabbit monoclonal antibodies in the immunohistochemical assessment of estrogen receptors, progesterone receptors, and HER2 in human breast carcinomas. Am J Clin Pathol 2010; 134(4): 621-32.

[21] Shi SR, Shi Y, Taylor CR. Antigen retrieval immunohistochemistry: review and future prospects in research and diagnosis over two decades. J Histochem Cytochem 2011; 59(1): 13-32.

[22] Brock JE, Hornick JL, Richardson AL, Dillon DA, Lester SC. A comparison of estrogen receptor SP1 and 1D5 monoclonal antibodies in routine clinical use reveals similar staining results. Am J Clin Pathol 2009; 132(3): 396-401.

[23] Kennecke HF, Speers CH, Ennis CA, Gelmon K, Olivotto IA, Hayes M. Impact of routine pathology review on treatment for node-negative breast cancer. J Clin Oncol 2012; 30(18): 2227-31.

[24] Lau SK, Chu PG, Weiss LM. Immunohistochemical expression of estrogen receptor in pulmonary adenocarcinoma. Appl Immunohistochem Mol Morphol 2006; 14(1): 83-7.

[25] Gomez-Fernandez C, Mejias A, Walker G, Nadji M. Immunohistochemical expression of estrogen receptor in adenocarcinomas of the lung: the antibody factor. Appl Immunohistochem Mol Morphol 2010; 18(2): 137-41.

Table 1. Repeatability for 1D5 (n=390), SP1 (n=390) and PharmDx (n=361). The figures below each of the nine two-by-two tables for overall agreement are, from left to right, the ratios (numerator/denominator), the percentages and the exact binomial 95% confidence intervals. If the cell counts in these two-by-two tables are labelled a, b, c and d, from left to right beginning with the first row, negative agreement is defined as 2a/(2a+b+c) and positive agreement as 2d/(2d+b+c), measures that are symmetric in evaluation order. For the latter measures, approximate 95% confidence intervals based on the delta method are presented.

	1D5		SP1		PharmDx	
			Evaluation 1			
Cut-off 1%	-	+	-	+	-	+
Evaluation 2 -	124	3	98	1	122	7
+	1	262	2	289	7	225
Overall agreement	(124+262)/390	99 (97-99)	(98+289)/390	99 (98-100)	(122+225)/361	96 (94-98)
Negative agreement		98 (97-100)		98 (97-100)		95 (92-97)
Positive agreement		99 (99-100)		99 (99-100)		97 (95-99)
Cut-off 10%	-	+	-	+	-	+
Evaluation 2 -	133	2	109	1	141	7
+	0	255	0	280	1	212
Overall agreement	(133+255)/390	99 (98-100)	(109+280)/390	100 (99-100)	(141+212)/361	98 (96-99)
Negative agreement		99 (98-100)		100 (99-100)		97 (95-99)
Positive agreement		100 (99-100)		100 (99-100)		98 (97-99)
Cut-off 50%	-	+	-	+	-	+
Evaluation 2 -	166	3	131	2	216	15
+	25	196	4	253	1	129
Overall agreement	(166+196)/390	93 (90-95)	(131+253)/390	98 (97-99)	(216+129)/361	96 (93-98)
Negative agreement		92 (89-95)		98 (96-100)		96 (95-98)
Positive agreement		93 (91-96)		99 (98-100)		94 (91-97)

Table 2. Agreement of evaluations of the highest scores for 1D5 and SP1 (n=390). The figures below each of the nine two-by-two tables are, from left to right, the ratios (numerator/denominator), the percentages are the exact binomial 95% confidence intervals. 1D5 is used as a reference when calculating negative and positive agreements.

			1D5	
Cut-off 1%			-	+
	SP1	-	95	3
		+	29	263
Overall agreement			(95+263)/390	92 (89-94)
Negative agreement			95/124	77 (68-84)
Positive agreement			263/266	99 (97-99)
Cut-off 10%			-	+
	SP1	-	109	0
		+	24	257
Overall agreement			(109+257)/390	94 (91-96)
Negative agreement			109/133	82 (74-88)
Positive agreement			257/257	100 (99-100)
Cut-off 50%			-	+
	SP1	-	131	0
		+	47	212
Overall agreement			(131+212)/390	88 (84-91)
Negative agreement			131/178	74 (66-80)
Positive agreement			212/212	100 (98-100)

Table 3. Agreement of evaluations of the highest scores for PharmDx, 1D5 and SP1 (n=321). The figures below each of the nine two-by-two tables are, from left to right, the ratios (numerator/denominator), the percentages are the exact binomial 95% confidence intervals. 1D5 and SP1, respectively, are used as reference categories when calculating negative and positive agreements.

		1D5			SP1	
Cut-off 1%		-	+		-	+
PharmDx	-	93	17	PharmDx	76	34
	+	10	201		4	207
Overall agreement		(93+201)/321	92 (88-94)		(76+207)/321	88 (84-91)
Negative agreement		93/103	90 (83-95)		76/80	95 (88-98)
Positive agreement		201/128	92 (88-95)		207/241	86 (81-90)
Cut-off 10%		-	+		-	+
PharmDx	-	100	21	PharmDx	86	35
	+	10	190		5	195
Overall agreement		(100+190)/321	90 (87-93)		(86+195)/321	88 (83-91)
Negative agreement		100/110	91 (84-96)		86/91	95 (88-98)
Positive agreement		190/211	90 (85-94)		195/230	85 (79-89)
Cut-off 50%		-	+		-	+
PharmDx	-	140	49	PharmDx	103	86
	+	5	127		3	129
Overall agreement		(140+127)/321	83 (79-87)		(103+129)/321	72 (67-77)
Negative agreement		140/145	97 (92-99)		103/106	97 (92-99)
Positive agreement		127/176	72 (65-79)		129/215	60 (53-67)

Table 4. Patient and tumour characteristics by group according to 1D5/SP1 results at a cut-off of 1%.

	1D5+/SP1+ n=279	%	1D5-/SP1+ n=42	%	1D5-/SP1- n=64	%	p -/+ vs +/+	p -/+ vs -/-
Age, years								
≤40	47	18	4	14	28	29	0.46	0.39
41-45	82	31	13	45	34	36		
46-50	101	38	8	28	24	25		
>50	33	13	4	14	9	9		
Tumour size, mm								
≤10	18	7	0	0	4	4	0.07	0.65
11-20	97	37	8	28	20	21		
21-30	110	42	12	41	41	43		
>30	38	14	9	31	30	32		
Tumour type								
Ductal	225	86	25	89	80	84	0.05	0.67
Lobular	27	10	0	0	2	2		
Other	9	3	3	11	13	14		
Missing	2		1		0			
Histological grade								
1	41	16	1	3	2	2	<0.01	<0.01
2	143	55	10	34	8	9		
3	77	30	18	62	77	89		
Missing	2		0		8			
Lymph node status								
Negative	63	24	6	21	37	39	0.29	0.17
1-3 positive nodes	141	54	12	43	37	39		
≥4 positive nodes	59	22	10	36	21	22		
Missing	0		1		0			
ER (cytosol method)								
Positive	179	84	7	29	6	8	<0.01	0.01
Negative	33	16	17	71	74	93		
Missing	51		5		15			

Excluded are 3 patients with 1D5+/SP1-.

Table 5. Patient and tumour characteristics by group according to 1D5/SP1 results at a cut-off of 10%.

	1D5+/SP1+ n=257	%	1D5-/SP1+ n=24	%	1D5-/SP1- n=109	%	p -/+ vs +/+	p -/+ vs -/-
Age, years								
≤40	45	18	4	17	32	29	0.62	0.21
41-45	80	31	8	33	42	39		
46-50	100	39	7	29	26	24		
>50	32	12	5	21	9	8		
Tumour size, mm								
≤10	17	7	1	4	4	4	0.82	0.35
11-20	94	37	9	38	23	21		
21-30	109	42	9	38	46	42		
>30	37	14	5	21	36	33		
Tumour type								
Ductal	220	86	21	91	89	82	0.66	0.41
Lobular	26	10	1	4	4	4		
Other	9	4	1	4	16	15		
Missing	2		1		0			
Histological grade								
1	41	16	1	4	2	2	0.12	<0.01
2	140	55	12	50	10	10		
3	74	29	11	46	89	88		
Missing	2		0		8			
Lymph node status								
Negative	61	24	2	9	45	41	0.10	0.01
1-3 positive nodes	138	54	12	52	41	38		
≥4 positive nodes	58	23	9	39	23	21		
Missing	0		1		0			
ER (cytosol method)								
Positive	175	85	7	37	10	11	<0.01	0.01
Negative	32	15	12	63	82	89		
Missing	50		5		17			

Figure 1. Flowchart of patients.

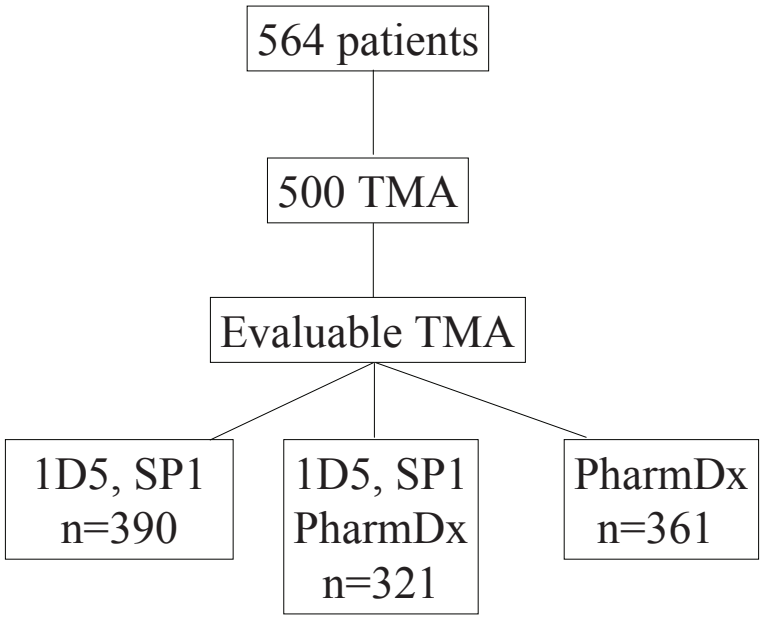


Figure 2. Recurrence-free survival, according to ER status assessed by 1D5 and SP1 at cut-offs of 1% (left panel) and 10% (right panel), for all patients (a and d), for TAM-treated patients (b and e) and for patients not treated with TAM (c and f).

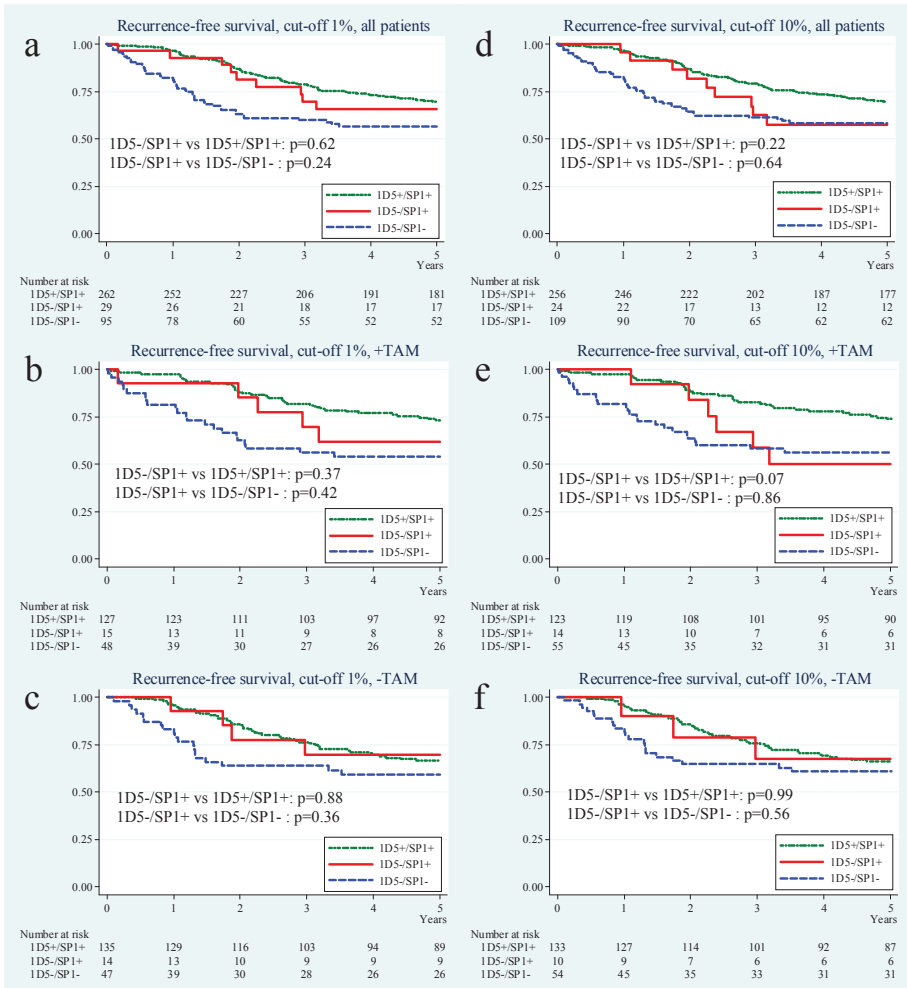
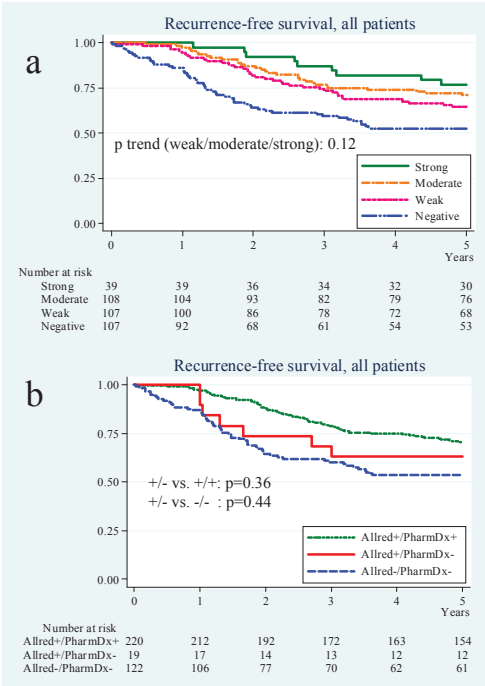


Figure 3. Recurrence-free survival according to intensity of ER (a) and ER status assessed by PharmDx at a cut-off of 10% and Allred score (b), (n=361).



LUND UNIVERSITY
Faculty of Medicine

LUND UNIVERSITY, FACULTY OF MEDICINE
DOCTORAL DISSERTATION SERIES 2012:69
ISSN 978-91-87189-32-6 ISBN 1652-8220