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**SENTINEL LYMPH NODE BIOPSY
AS A DIAGNOSTIC TOOL IN
THE TREATMENT OF BREAST CANCER**

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ACADEMIC DISSERTATION

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List of Original Articles

- I** Leikola JP, Leppänen EA, von Smitten KAJ, Leidenius MHK. Adjusting the radioisotope tracer dose according to the body mass index does not enhance the visualization of axillary sentinel lymph nodes. *Acta Radiol* 47(7):646-9, 2006
- II** Leikola JP, Toivonen TS, Krogerus LA, von Smitten KAJ, Leidenius MHK. Rapid immunohistochemistry enhances the intraoperative diagnosis of sentinel lymph node metastases in invasive lobular breast carcinoma. *Cancer* 104(1):14-9, 2005
- III** Leikola J, Heikkilä P, von Smitten K, Leidenius M. The prevalence of axillary lymph node metastases in patients with pure tubular carcinoma of the breast and sentinel node biopsy. *Eur J Surg Oncol* 32(5):488-91, 2006
- IV** Leikola J, Heikkilä P, Pamilo M, Salmenkivi K, Von Smitten K, Leidenius M. Predicting invasion in patients with DCIS in the preoperative percutaneous biopsy. *Acta Oncol* 46(6):798-802, 2007
- V** Leikola J, Saarto T, Joensuu H, Sarvas K, Vironen J, Von Smitten K, Virkkunen P, Vanharanta B, Mäkelä P, Leidenius M. Ultrasonography of the axilla in the follow-up of breast cancer patients who have a negative sentinel node biopsy and who avoid axillary clearance. *Acta Oncol* 45(5):571-5, 2006

List of Abbreviations

AC	axillary clearance
ADH	atypical ductal hyperplasia
ALH	atypical lobular hyperplasia
BCT	breast conserving surgery
BMI	body mass index
CNB	core needle biopsy
DCIS	ductal carcinoma in situ
FNR	false negative rate
FS	frozen section
IDC	intraductal carcinoma
IHC	immunohistochemistry
ILC	invasive lobular carcinoma
ITC	isolated tumour cells
LCIS	lobular carcinoma in situ
LS	lymphoscintigraphy
MGR	mammography
NSN	non sentinel node
PTC	pure tubular carcinoma
ROLL	radio-guided occult lesion localization
SN	sentinel node
SNB	sentinel node biopsy
US	ultrasonography

Abstract

Aim

The purpose of this study was to evaluate the use of sentinel node biopsy (SNB) in the axillary nodal staging in breast cancer. A special interest was in sentinel node (SN) visualization, intraoperative detection of SN metastases, the feasibility of SNB in patients with pure tubular carcinoma (PTC) and in those with ductal carcinoma in situ (DCIS) in core needle biopsy (CNB) and additionally in the detection of axillary recurrences after tumour negative SNB .

Patients and methods

The study population was selected from 1580 clinically stage T1-T2 node-negative breast cancer patients, who underwent lymphoscintigraphy (LS), SNB and breast surgery between June 2000 and November 2004 at the Breast Surgery Unit, Department of Gastrointestinal and General Surgery of Helsinki University Central Hospital. LS was performed the day before surgery. In 178 patients, the isotope doses were adjusted according to body mass index (BMI) and in an adjoining branch of the study, 42 of the 80 patients without axillary hot spots in the LS received a second tracer injection. Intraoperatively a gamma probe and blue dye were used for identification of the SNs. Mastectomy or breast conserving surgery was accompanied by axillary clearance (AC) during the primary operation in patients with SN metastases or unsuccessful SN identification. In 438 patients, the intraoperative diagnosis of SLN metastasis was enhanced by using rapid immunohistochemistry (IHC) with a cytokeratin biomarker. Patients with false negative findings in the frozen section diagnosis underwent level I-II AC as a second operation.

The CNB and the surgical breast specimens were re-evaluated by an expert breast pathologist to confirm the correct histological diagnosis of PTC and DCIS. The CNB samples were

obtained from women, who participated in the biennial, population based mammography screening at the Mammography Screening Centre of Helsinki between June 2001 and November 2004.

In the follow-up, a cohort of 205 patients who avoided AC due to negative SNB findings were evaluated using ultrasonography one and three years after breast surgery.

Results

The visualization rate of axillary SNs was not enhanced by adjusting radioisotope doses according to BMI. The data published from the same study found, that in patients without axillary hot spots in LS, the intraoperative SN identification success rate was higher, 88%, with a second radioisotope injection than without it, 47%.

The sensitivity of the intraoperative diagnosis of SN metastases of invasive lobular carcinoma (ILC) was higher, 87%, with rapid, intraoperative immunohistochemistry (IHC) group compared to 66% without it. The sensitivity of the intraoperative diagnosis was similar in patients with other types of invasive cancer regardless of the use of rapid IHC, except for a marginal enhancement in the intraoperative diagnosis of isolated tumour cells.

The prevalence of tumour positive SN findings was 27% in the 33 patients with breast tumours diagnosed as PTC. Six of the nine patients with SN metastases had micrometastases, while three had macrometastases. The median histological tumour size was similar, 9 versus 10 mm, in patients with or without axillary metastases. After the histopathological review, six (22%) out of the limited number of 27 patients with true PTC had axillary metastases, with no significant change in the risk factors for axillary metastases.

Of the 67 patients with DCIS in the preoperative percutaneous biopsy specimen, 30% had invasion in the surgical specimen. The strongest predictive factor for invasion was the visibility of the lesion in ultrasound. However, due to the small number of patients in this study, the results are without statistical significance. Thirteen (50%) of the 26 patients with lesions visible in US had

invasion in their surgical specimens, while only 17% of the 41 patients without such a lesion had invasive or microinvasive cancer.

In the three year follow-up, axillary recurrence was found in only two (0.5%) of the total of 383 ultrasound examinations performed during the study, and only one (0.3%) of the 369 examinations performed at the scheduled study visits revealed cancer. None of the ultrasound examinations were false positive, and no study participant was subjected to unnecessary surgery due to ultrasound monitoring.

Conclusions

Adjusting the dose of the radioactive tracer according to patient BMI does not increase the visualization rate of SNs, but a second tracer injection can improve the rate of intraoperative SN identification, as shown in an adjoining publication. The intraoperative diagnosis of SN metastases is enhanced by rapid IHC particularly in patients with ILC. In this limited number of study patients, SNB seems to be a feasible method for axillary staging of pure tubular carcinoma in patients with a low prevalence of axillary metastases. SNB also appears to be a sensible method in patients undergoing mastectomy due to DCIS in CNB. It also seems useful in patients with lesions visible in breast US. During follow-up, routine monitoring of the ipsilateral axilla using US is not worthwhile among breast cancer patients who avoided AC due to negative SN findings.

Introduction

During the last decade, sentinel lymph node biopsy (SNB) has become a standard method for the axillary staging in breast cancer at many centres worldwide. It has led to the abolishment of axillary clearance (AC) as a routine treatment of choice in the clinically node negative axilla in numerous acknowledged institutes. This is much due to the fact, that SNB results in a significant reduction in physical and psychological morbidity when compared to AC. Such favourable consequences seem to apply equally in short and long term evaluations (Purushotham et al 2005, Mansel et al 2006, Leidenius et al 2005).

SNB as a reliable staging instrument for lymphatic involvement is universally accepted. However, at present there is no standard protocol for the localization or the histological evaluation of the sentinel nodes (SNs). Some 5 to 10 % of the axillas initially staged by SNB as tumour free can eventually turn out to be falsely judged (Lyman et al 2005).

The lack of consensus on the standard protocols of SNB leads to the inevitable conclusion that an optimal method for performing SNB is still to be found and proven worthy. Also, despite the attempts to collect reliable indications and contraindications (Cody 2007), controversies still remain on which breast cancer patients should be staged by SNB.

The purpose of the present study was to evaluate the feasibility of sentinel node biopsy in lymph node staging in breast cancer with special emphasis on the evaluation and improvement of the current methods used. A further attempt was made to distinguish indications for SNB, especially for breast cancer patients among whom axillary metastasis was not expected to occur.

Review of the Literature

1. Breast Carcinoma Pathogenesis

The proliferation of unevenly distributed epithelial cells with nuclei of varying shape and chromatin pattern, described as ductal hyperplasia, is often the first sign of breast pathology (Kenemans et al 2004). These cells are cytologically benign. There is however an increased risk of breast cancer as the transition from hyperplasia to atypical hyperplasia takes place. The next step in the progression to malignancy would be the development of carcinoma in situ, either ductal or lobular. This is defined as a proliferation of cells with cytological characteristics of malignancy, but without stromal invasion across the basement membrane. As cells detach from the basement membrane and invade the stroma, the tumour becomes invasive and through dissemination via blood and lymph vessels, invasive cells can give rise to metastases (Kenemans et al 2004). These are the steps in the classic model of multi-stage tumour development. Since cancer is a genetic disease, each step is considered to correlate with one or more distinct mutations in major regulatory genes.

In sporadic breast cancer, a serial stepwise accumulation of acquired and uncorrected mutations in somatic genes takes place, without any germline mutations playing a role. An early event in these sporadic tumours is the mutational activation of oncogenes, such as MYC, Int2, EMS1, CCND1 and ERBB2. Growth factors like EGF, TGF β and IGF-1 are also considered to play a role in the proliferation and growth of breast cancer, as well as invasion and cell adhesion genes, e.g. N-CAM or E-Cadherin and angiogenesis gene VEGF (Kenemans et al 2004). In hormone-dependent breast carcinogenesis, oestrogen receptor α gene is the most important growth factor receptor as estrogens can act as tumour initiator by causing direct DNA damage. Hereditary breast cancer is characterized by an inherited susceptibility to breast cancer on basis of an identified germline mutation in one allele of a high penetrance susceptibility gene, such as BRCA1, BRCA2, CHEK2, TP53 or PTEN (Wooster et al 2003). Inactivation of the second allele of these tumour suppressor genes would be an early event in this oncogenic pathway. Both in sporadic and

hereditary breast cancer, these early events can take place in a variety of specific genes (Kenemans et al 2004, Wooster et al 2003).

2. Premalignant and Malignant Breast Disease

Despite the multistep model of breast cancer progression presented above, the relation between premalignant lesions, malignant but preinvasive lesions and invasive cancer remains unclear. Most breast cancers arise from the same locality, the terminal duct lobular unit and their differences that characterize breast cancer morphology are manifestations of their differing molecular profiles (Wiechmann et al 2008).

Ductal carcinoma in situ (DCIS) is defined as a neoplastic proliferation of epithelial cells confined to the ductal-lobular system without tumour invasion through the basement membrane (Wiechmann et al 2008). It includes a heterogenous group of lesions with diverse morphologic and biologic features. The classification of DCIS is based on the histopathological assessment of features including nuclear grade, cell necrosis, cell polarization and architectural pattern (Wiechmann et al 2008). Also tumour size and presence or absence of calcifications should be noted. In the theory of linear breast cancer progression, low-grade DCIS is often considered to be preceded by atypical ductal hyperplasia (ADH), then progress to high-grade DCIS and eventually “dedifferentiate” to become invasive breast cancer. Since the majority of molecular changes observed in invasive cancer are already evident in DCIS, a theory of parallel disease has gained popularity. In this theory of cancer progression, low-grade DCIS progresses to low-grade invasive cancer and high-grade DCIS to high-grade invasive cancer. It must be noted, however, that these two theories of carcinogenesis do not seem to exclude one another (Wiechmann et al 2008).

The term “in situ lobular neoplasia” encompasses the non- invasive lobular proliferations, atypical lobular hyperplasia (ALH) and lobular carcinoma in situ (LCIS) (Alfonso et al 2008, Hanby et al 2008). In ALH the terminal duct lobular unit is partly or totally colonized by

small discohesive cells whose cytoplasm may contain variably conspicuous “private” acini. There is no expansion in the colonized units nor are their lumina obliterated by this proliferation. There is no widespread proliferation and no extension to ducts in a pagetoid undermining fashion. Conversely, in LCIS the colonized structures are expanded and the lumina lost even though the cytomorphological characteristics of the cells are similar. The distinction between LCIS and ALH is based on the percentage of acini in a lobular unit that are distended and filled by lobular cells (Alfonso et al 2008). The large spectrum of morphologically and cytogenetically diverse group of lobular neoplasms share the common molecular features that are the loss or downregulation of the adhesion molecule E-cadherin and a close relationship to low-grade ductal carcinoma (Hanby et al 2008, Alfonso et al 2008).

Invasive ductal carcinoma (IDC), as the most common type of invasive breast cancer, includes a wide range of histological appearances, from those with well-formed glands to those that have little or no evidence of specific differentiation. In the well-differentiated tumour cells, glandular formation is predominantly found, ranging from small regular oval forms with cuboidal to low columnar cells, to large irregular forms with cribriform configurations. However, the cells are arranged in irregular nests with little or no recognizable gland formation in less-differentiated tumours. The spectrum of histopathologic features of invasive lobular carcinoma (ILC) has considerably expanded over time. The classic form of ILC composes of small cells with uniform nuclei arranged in single files within the fibrous stroma and a targetoid arrangement around non-neoplastic ducts is often seen. The variations of this classic type may have alveolar or solid patterns and the cells may be pleomorphic, signet-ring and histiocytoid. Tubular carcinoma is a rare subset of invasive breast cancer, where small, well-formed ductal or tubular structures with open lumina lined by a single layer of cuboidal to low columnar cells with uniform nuclei. It is often regarded as a very well differentiated form of IDC, but it may also overlap ILC in the form of tubulolobular carcinoma. Other rare, specific histologic types of breast cancer include invasive cribriform,

mucinous, medullary, secretory carcinomas as well as adenoid cystic and metaplastic carcinomas (Roses 1999).

3. Lymphatic Staging in Breast Cancer

The status of axillary and internal mammary lymph nodes is the most significant prognostic factor for survival in breast cancer (Veronesi et al 1985). Some 75% of the lymphatic flow from the breast is directed to the ipsilateral axilla (Turner-Warwick et al 1959) and therefore the axillary nodal basin has been the main target in lymphatic staging in breast cancer. Axillary clearance (AC) has been the gold standard in axillary staging in breast cancer, providing valuable information about the planning of adjuvant therapy as well as excellent regional disease control as well (Morrow et al 1996). AC also provides a survival benefit of approximately 5%, independently of systemic adjuvant therapy (Orr et al 1999).

However, there exists significant arm morbidity after AC, also affecting the axillary-node-negative patients. Most of the patients with newly diagnosed breast cancer have an early stage disease. Consequently, axillary metastases are found in less than half of them (Blichert-Toft 2000). Therefore, less invasive, accurate methods for axillary staging in breast cancer are needed. Radiological methods such as axillary US, computed tomography scan and fluorodeoxyglucose positron emission tomography have been associated with too low sensitivity in detecting lymph node metastases in breast cancer (Veronesi et al 2006, Deurloo et al 2003). Nodal sampling has not gained widespread popularity, even though it has been advocated as a feasible and less invasive method than AC for axillary staging (Ahlgren et al 2002, Cserni 1999). Nevertheless, SNB has been introduced in breast surgery units world wide, since it has been suggested to be less invasive than AC and to provide accurate axillary staging (Giuliano et al 1995).

4. The Sentinel Node Concept

The sentinel node is the first draining node on the direct lymphatic drainage pathway from the tumour site (Morton et al 1992). The assumption is that there are no nodal metastases in the lymph node basin, for example in the axilla, if the sentinel node is tumour-negative. Furthermore, AC is regarded as unnecessary in breast cancer patients with tumour-negative axillary sentinel nodes. The number of sentinel nodes may be one or more (Valdes- Olmos et al 2001). In breast cancer, the sentinel nodes are located mainly in the ipsilateral axilla, but also in areas outside the axilla, most commonly in the internal mammary basin (Valdes- Olmos et al 2001).

5. Accuracy

A number of audit phase studies in which a back-up AC has been performed after harvesting of sentinel nodes have evaluated the accuracy of SNB in breast cancer (Bergkvist et al 2001, Miltenburg, et al 1999). These studies show, that the status of the sentinel nodes reliably reflects the nodal status of the entire axilla (Bergkvist et al 2001, Miltenburg, et al 1999). The false-negative rate (FNR) of SNB, that is the proportion of patients with negative sentinel nodes but with subsequently proven axillary metastases, has been approximately between 5% and 10% (Lyman et al 2005).

6. Sentinel Node Localization

The sentinel nodes can be localized using tracers that are transported from the injection site to the sentinel nodes through the lymphatic ducts. The tracers currently in use are Tc99m radioisotope labelled colloids and vital blue dyes, as separate or in combination. Lymphoscintigraphy (LS), the imaging of the sentinel nodes using a gamma camera, is usually performed 0.5–4 hours after the radioisotope injection.

There are several variations in sentinel node localizing techniques and an international consensus on the procedure is lacking. Different types of radioactive tracer itself with different dosages, concentrations and volumes are used. Some units perform SNB also without preoperative LS (McMasters et al 2000) or even using the blue dye only (Wong et al 2001). However, using blue dye as the sole sentinel node identification method may lead to higher FNRs (Wong et al 2001). Even though axillary sentinel nodes can be found without LS (McMasters et al 2000), it is most convenient in showing the number and location of the radiolabelled nodes, such that the surgeon is readily assured of all significant radiolabelled nodes being harvested. This is especially essential in teaching hospitals and outside highly specialized units. Although the blue dye is highly demonstrative, especially in experienced hands it may not provide substantial enhancement to the sentinel node identification rate when combined with the radioisotope localization (Derossis et al 2001). Nevertheless, the gold standard in SNB is considered to include preoperative LS, intraoperative sentinel node identification using a hand-held gamma probe and blue dye (Schwartz et al 2002).

7. Injection Site

There are several alternatives of the injection site of the tracer within the breast. The tracer may be injected superficially, that is sub- or intracutaneously, or sub- or periareolarly. The other possibility is the intraparenchymal injection, that is intra or- peritumoural injection.

The superficial techniques have been considered as superior resulting in practically 100% visualization of sentinel nodes in the axilla because lymphatic drainage from the skin is far richer than from the breast parenchyma (Martin et al 2001, McMasters et al 2001). However, sentinel nodes in the internal mammary chain are seldom visualized following a superficial injection of the tracer. This has led to the conclusion, that the drainage pattern from the skin is different to that from the underlying parenchyma (Roumen et al 1999, Valdes-Olmos et al 2000).

Thus, the advantage of the intraparenchymal injection is the option for nodal staging also outside the axilla, especially in the internal mammary basin. In addition, injecting well away from the tumour carries at least a theoretical risk that a watershed of lymphatics is crossed and the visualized node drains another area of the breast, not the tumour site (Valdes-Olmos et al 2000). Nevertheless, no difference has been observed in the false-negative rate when using superficial or intraparenchymal techniques (Martin et al 2001, McMasters et al 2001).

8. Tracers

It is difficult to find an ideal radiopharmaceutical for LS. The kinetics of particles in the lymphatic system are strongly dependent on their size. Very small particles migrate so readily that only a proportion remain in the first node, and secondary nodes are visualized as well, leading to visualization and unnecessary harvesting of numerous nodes . When using larger particles, the number of visualized second and third echelon nodes is diminished and only one or two sentinel nodes are identified (Paganelli et al 1998, De Cicco et al 1998, Wilhelm et al 1999, Noguchi 2002). The disadvantage of larger particles is their tendency to remain in the injection site and their failure to enter the lymphatic system, possibly resulting in the non-visualization of some sentinel nodes (Wilhelm et al 1999, Nieweg et al 1999). In theory, this could lead to increased false negative findings because if an SN does not take up enough radiocolloid to image with a gamma camera, it is unlikely to be detected with the probe intraoperatively (Goyal et al 2006).

Each tracer has some advantages, so an ideal radiocolloid is still to be found.

Therefore, different types of colloids are used in different parts of the world. In most parts of Europe, the Tc 99m labelled human albumin colloid and patent blue dye are widely used, while in the USA sulphur colloid and isosulfan blue dye are popular. In Asia, 99mTc-tin colloid is also used (Imoto et al 2004). A smaller particle size 99mTc-human albumin colloid is preferred in many

European centres (Rahusen et al 2000, Borgstein et al 1998, Valdes Olmos et al 2000, Valdes Olmos et al 2001, van der Ent et al 2001, Rink et al 2001, Heuser et al 2001, Leidenius et al 2004). Some studies favour ^{99m}Tc -human albumin with larger colloidal particle sizes because of less spillage of the tracer to the non-sentinel nodes (Paganelli et al 1998, De Cicco et al 1998).

9. Dose

Like optimizing the particle size, it is also difficult to optimize the tracer dosage for optimal visualization. Valdes-Olmos and co-workers found in their study that non-visualization of a single intratumoural tracer injection occurred almost always with doses less than 65 MBq, especially in elderly patients. (Valdes-Olmos et al 2000). Consequently, the required visualization rate has been reached using doses of 130 MBq on average (Tanis et al 2003).

Due to the richer lymphatic drainage from the skin, in general smaller doses are needed in the superficial injections (Mariani et al 2004.)

10. Preparation of the Tracer

Also the preparation of the radiocolloid influences the visualization of SN in LS. A comparative study has shown that a sentinel node visualization rate of 99% could be reached by optimizing the labelling protocol of ^{99m}Tc -nanocolloid (Valdes-Olmos et al 2001). By enhancing the particle concentration and adjusting the tracer dosage without increasing of the injection volume (0.2 ml), optimal sentinel lymph node visualization was observed in almost 90% of the patients receiving adjusted tracer injections (Valdes-Olmos et al 2001). Similarly, a nine times higher intraoperative count rate in the sentinel nodes was achieved with the highest concentration of ^{99m}Tc -colloidal albumin, with increased radiochemical labelling efficiency and stability (Gommans et al 2001).

11. Patient Related Factors

The risk of unsuccessful SN imaging has been shown to increase with patient age and body weight (Rousseau et al 2005, Mc Masters et al 2000, Derossis et al 2003). Also, tumour grade has been suggested to play a role in the non-visualization of the sentinel node in patients with breast cancer, visibility decreasing with increasing grade (Krausz et al 2001). Replacement of lymphatic tissue in SNs by tumour mass might lead to a reduction of radioisotope uptake (Goyal et al 2005).

12. Intraoperative Success Rates

The success rate in identifying sentinel nodes intraoperatively is largely reflected by the node localization methods used and the experience of the surgeon. Generally, the success rate has been above 96% (Kim et al 2006) , even though factors such as high patient body mass index, tumour location other than upper outer quadrant and non-visualization of SLN on pre-operative LS have been shown to significantly associate with failed localization (Goyal et al 2006).

Therefore the same factors leading to non-visualization are often also responsible for the intraoperative failure. High BMI certainly has a negative effect, since independent of the skill of the surgeon, an increase of one unit of BMI decreases the odds of successful mapping, and so detection of SNs pre- or intraoperatively, by approximately 5% (Cox et al 2002). Especially when combined with high age, obesity been shown to be hamper successful SN identification (Cox et al 2002, Derossis et al 2003, Sato et al 2003, Leppänen et al 2002). Accordingly, obese patients are more likely to undergo axillary dissection, because of failure in mapping (Derossis et al 2003). This is most undesirable, since patients with a high BMI have an especially high risk of developing arm lymphoedema after AC (Edwards et al 2000, Ozaslan et al 2004). In addition to patient age and BMI, the failure rate of pre- or intraoperative identification of SNs is significantly increased by the increasing number of metastatic axillary nodes (Wong et al 2002).

Nevertheless, the non-visualization of SNs in LS often leads to a failure in intraoperative SN localisation and clearance of a healthy axilla (Birdwell et al 2001, Haigh et al 2000, Goyal et al 2006). Due to the lower visualisation rate of SN in LS when using an intraparenchymal radioisotope injection, Krynycky and co-workers increased the visualisation of SNs by placing another tracer dose at the areolar-cutaneous junction, in addition to the intraparenchymal injection (Krynycky et al 2003). This problem has been also attempted to be solved by using a second tracer injection after a negative LS (Tanis et al 2002). This repeated intratumoural injection enhanced the visualization showing axillary SNs in 55% of these patients. Nevertheless, the impact of the second injection on the success rate of the intraoperative SN localisation has not been widely evaluated.

13. Histopathological assessment of sentinel node metastases

In addition to the SN localizing methods, there is a lack of an international standard of the histopathological examination of the sentinel nodes. Serial sectioning of the nodes has been recommended, but the number of sections as well as the section interval varies between the units (Cserni et al 2003). In addition to routine H&E staining, immunohistochemical staining is applied in many units, although not recommended in routine clinical practice (Cserni et al 2003). This is due to the fact, that there might be misinterpretations of the immunohistochemical staining. Both needle and excision biopsies of the breast tumour may lead to displacement and passive transport of tumour cells into regional lymph nodes. These passively transported tumour cells may be interpreted as metastases and mislead the nodal staging. Moreover, such false positive immunohistochemical results may originate from benign lesions. (Bleiweiss et al 2006). In addition, normal constituents of the lymph nodes, such as interstitial reticulum cells or plasma cells may stain with anti-cytokeratin antibodies mimicking cancer cells (Cserni et al 2006). On the other hand, there

is evidence that the use of immunohistochemistry may reduce the false-negative rate of SNB significantly (Lieberman 2000).

14. Enhanced Nodal Staging – Micrometastases and ITC

Meticulous histopathological examination of the sentinel lymph nodes is proposed to compensate for the false-negative results in SNB, because it reveals metastases that are undetected in AC (de Widt- Levert et al 2003, Giuliano et al 2001, Jakub et al 2003, Leidenius et al 2004). The metastases revealed by enhanced histopathological evaluation are most often micrometastases and isolated tumour cells (ITC) (de Widt- Levert 2003, Wong et al 2002). Micrometastases are defined as being larger than 0.2 mm but no larger than 2.0 mm in diameter, whereas ITC seen as single cells or cell deposits, are no larger than 0.2 mm. In addition, immunohistochemistry readily reveals metastases of invasive lobular carcinoma in sentinel nodes, which, in 24–41% of cases, remain undetected in routine histopathological evaluation of the AC specimens (Bussolati et al 1986, de Mascarel et al 2005, Leidenius et al 2004, Cserni et al 2006)

15. Prognostic Significance of Nodal Micrometastases

The prognostic impact of lymph node micrometastases has been evaluated by several retrospective studies with conflicting results (Cote et al 1999, Dowlatshahi et al 2001, Gray et al 2001, International (Ludwig) Breast Cancer Study Group 1990, Wilkinson et al 1982). In these retrospective studies, the nodes of tumour-negative AC specimens have been re-examined using serial sectioning or immunohistochemistry. However, many of these studies suffer from study samples that are too small or have inadequate histological methods (Cody et al 2004).

The meticulous histopathological assessment of sentinel nodes provides an excellent opportunity to estimate the prognostic impact of axillary micrometastases. So far, only few studies including patients with SNB have indicated that even minimal nodal involvement may diminish the

prognosis (Colleoni et al 2005, Kuijt et al 2005). These studies concluded that the overall 10 and 12 years survival worsened statistically significantly, 5 and 14% for patients with micrometastases compared to patients with node-negative breast cancer (Kuijt et al 2005, Bilchik et al 2007). In one of these studies, no significant difference was observed between the patients with micrometastases and those with a macrometastasis in one lymph node (Kuijt et al 2005). Over the years the histopathological analysis has greatly improved and in all of these studies, the frequency of micrometastases might have been underestimated in the lymph node-negative groups. When these study patients were treated, serial sectioning was not routinely used in each case (Kuijt et al 2005). Furthermore, in a portion of cases lymphatic staging using AC was performed. For these reasons, some cases with micrometastases have been assessed as node negative and the sizes of some axillary metastases have been underestimated . This is true for all studies not utilizing serial sectioning and immunohistochemical staining routinely in all study patients. Therefore, large prospective studies are still required to confirm the prognostic significance of nodal micrometastases.

16. Axillary Clearance in patients with sentinel node micrometastases

The value of AC has been questioned in patients with SN micrometastases and isolated tumour cells (ITC) because of low probability further metastases in AC in these patients. However, the estimated risk of non-sentinel node involvement in patients with sentinel node micrometastases and even with ITC has been approximately 10–15% (Cserni et al 2004). Table 1.

Table 1. The prevalence of non sentinel node (NSN) metastases among patients with SN micrometastases or isolated tumour cells (ITC)

Study	NSN micrometastases and ITC	NSN macrometastases	NSN metastases altogether
Cserni et al Br J Surg 2004	NA	NA	195/789 (25%)
Viale et al Ann Surg 2005	56/752	97/752	155/752 (21%)
Schrenk et al Br J Surg 2005	5/122	17/122	22/122 (18%)
Houvenaeghel et al J Clin Oncol 2006	NA	NA	94/700 (13%)
Leidenius et al EJSO 2005	14/84	8/84	22/84 (26%)
van Rijk et al Cancer 2006	6/160	18/160	24/160 (15%)

These further metastases are frequently large ones, even when the sentinel node finding is just a micrometastasis or ITC (Leidenius et al 2005, Viale et al 2001). The reason for this is not known exactly. The size of the SN metastasis may be underestimated, and the assumed micrometastases may represent only part of a larger one (Viale et al 2001). A more likely explanation is that the primary sentinel node with a large metastasis has not been identified, because of total or partial diversion of lymphatic drainage from the original sentinel node to a lesser involved one.

Omitting AC in patient with SN micrometastases or ITC carries an evident risk of missing prognostic information because NSN may hold a macrometastasis. Furthermore, omitting AC in these patients may lead to residual disease in the axilla in up to 25% of the patients (Leidenius et al 2005). On the other hand, there are few studies addressing a low axillary recurrence rate when omitting AC in these patients (Liang et al 2001, Fournier et al 2004). However, these few studies addressing it are too limited both in the size of the study groups and in the years of follow up to draw definite treatment protocols (Liang et al 2001, Fournier et al 2004). For these reasons, it is not advisable to omit AC even in patients with minimal sentinel node involvement outside prospective trials.

17. Intraoperative Diagnosis of Sentinel Node Metastases

Accurate intraoperative diagnosis of SN metastases is crucial for several reasons. In the majority of patients undergoing SLN biopsy, axillary staging and treatment are possible during the same operation. This has the positive effects of reducing the number of operations and hospital costs, since two different operations needed require more resources (Leidenius et al 2003, Rönka et al 2004). More over, reducing the axillary surgical interventions to just one might even play a role in diminishing morbidity following AC (Husen et al 2006). Two separate operations may also lengthen the time interval between surgery and adjuvant therapy.

In the intraoperative diagnosis, touch imprint cytology and frozen section (FS), separately or in combination, have been applied. Touch imprint cytology is very quick and less costly than FS (Motoruma et al 2000, Ratanawichitrasin et al 1999). Although the sensitivity may be too low for a method used alone (Leidenius et al 2003, Llatjos et al 2002), including the imprint cytology in the intraoperative diagnosis has been considered as feasible, because when positive, further assessment is avoided and the results can be announced to the operation theatre at once.

By using a laborious intraoperative FS method, a nearly 100% sensitivity in the intraoperative diagnosis has been reached (Veronesi et al 1999). This includes serial sectioning the whole SN for FS requiring resources from the pathology laboratories that are beyond the capability and financial constraints of most institutions. The sensitivity of the intraoperative diagnosis has been essentially lower when using other less laborious FS methods (Leidenius et al 2003, Weiser et al 2000, Mitchell 2004, Llatjos et al 2002). The sensitivity is high, over 90% in macrometastases but is essentially lower, some 60% in micrometastases and isolated tumour cells (ITC) (Leidenius et al 2003, Mitchell 2004, Llatjos et al 2002), but even as low as 17% (Weiser et al 2000). This seems to apply regardless whether FS, imprint or IHC is used. In addition to micrometastases and ITC, the sensitivity of the intraoperative diagnosis has been especially low in association with the metastases of invasive lobular carcinoma (ILC) (Leidenius et al 2003, Holck et al 2004, Viale et al 1999). A multi-institutional cohort study found that the nodal involvement in ILC was detected by IHC in some one third of the cases. The great majority of these cases were micrometastases or ITCs (Cserni et al 2006).

So far, attempts have been made to increase the diagnostic sensitivity with the implementation of intraoperative immunohistochemistry (D'Errico et al 2004, Karsten et al 2002, Nahrig et al 2003, Beach et al 2003). These studies are, however, based on a small study population and have been inconclusive in resolving the added value of IHC in histological subgroups of breast cancer.

18. Patient Selection in Sentinel Node Biopsy

18.1. Tumour Size and Multifocality

In the audit phase studies, conclusions concerning the sensitivity of SNB have been mainly made from patients with clinically axillary-node-negative, unifocal tumours not larger than 3 cm

(Schwartz et al 2002). Technically it is possible to perform a SNB also in patients with larger or multifocal and multicentric tumours. The validity of SNB has yet not been proven in this patient group (Schwartz et al 2002). Similar success and false-negative rates have reported in patients with large (Bedrosian et al 2000, Chung et al 2001, O'Hea et al 1998, Wong et al 2001, Goyal et al 2006) or multifocal tumours (Kumar et al 2003, Tousimis et al 2003) as in those with small and unifocal cancers. However, alarmingly high false negative rates of 25 to 33% in multifocal tumours have been reported (Ozmen et al 2002, Bergkvist et al 2005)

The prevalence of axillary metastases increases with increasing tumour size (Leidenius et al 2005, Viale et al 2005). The prevalence of axillary metastases has been reported to be especially high among patients with multifocal tumours (Andea et al 2004, Leidenius et al 2005). Due to the higher prevalence of axillary metastases in connection with large and multifocal tumours, there are evidently more numerous false-negative SNB cases associated with them, even though the false-negative rate is similar in small and large tumours. Actually, for large, T2 and T3 lesions, there is nearly 10% and 20% risk, respectively, of missing an axillary metastasis in a patients with a negative SN (Barone et al 2005). Rather few patients with a large tumour burden in the breast therefore avoid AC because of negative sentinel node findings. A large proportion of them may have residual disease in the axilla. The impact of this residual disease on axillary recurrences and survival in these patients is unknown.

For these reasons, the patients who benefit most from SNB are those with small primary tumours. The smaller is the primary tumour the lower is the prevalence of axillary metastases and the higher is the probability that the patient will avoid unnecessary AC.

18.2. Sentinel Node Biopsy in Patients with Favourable Subtypes of Breast Cancer

The incidence of axillary metastasis has been regarded to be very low among patients with “favourable” subtypes of breast cancer. Some 5% of the breast cancer population meet these favourable cancer’s criteria; nonpalpable, T1a and T1b, non-high nuclear grade tumours, without lymphovascular invasion (Jakub et al 2003). Therefore, nodal staging has been regarded as an unnecessary procedure among these patients. However, the staging of favourable breast cancer patients by SNB has not only reduced the morbidity compared with AC, but more importantly, has raised the accuracy in detecting axillary disease (4.4% vs. 0.6%) (Jakub et al 2003).

The low risk of nodal metastasis is particularly evident in some histological subtypes of breast cancer, like pure tubular carcinoma (PTC). PTC is a rare, well- differentiated histologic subtype of invasive breast cancer. The definition of this histologic subtype varies depending on the proportion of tubular formation. 90% or more of tubule formation is the mostly accepted definition (Fattaneh 2003, Ellis et al 1992), but even in the recent studies there is variation between 80% (Winchester et al 1996) and 95% (Cabral et al 2003).

The number of breast cancer patients meeting the criteria of PTC is very limited and therefore the factors concerning its’ metastasis have remained unresolved. Previous studies have reported the prevalence of lymph node metastases in PTC to be negligible in patients with a histological tumour size less than 1 cm (Fein et al 1997, Papadatos et al 2001, Maibenco et al 1999, McBoyle et al 1997). Generally speaking, there is possibly substantial morbidity to be expected from AC. Taking that as a fact and adding the findings of PTC’s low risk of axillary metastasis has led the investigators to conclude that the morbidity associated with AC would outweigh any potential benefit of axillary staging (Rutgers 2001, Kader et al 2001). However, due to its’

presumed low morbidity and accuracy in staging, the use of SNB has been suggested to be suitable for nodal staging among patients with favourable tumour subtypes, such as PTC (Wong et al 2002).

18.3 Sentinel Node Biopsy in Ductal Carcinoma In Situ

The number of patients diagnosed with ductal carcinoma in situ (DCIS) has increased due to the widespread use of screening mammography. The preoperative diagnosis of DCIS is usually made by core needle biopsy (CNB) or vacuum assisted biopsy after evident suspicion from the radiographs. However, limited sampling with such percutaneous biopsy techniques may lead to misdetection of cancer invasion. Because CNB represents only a small proportion of the tumour, some 10-20% of the patients with DCIS in CNB have invasive cancer in the breast resection or mastectomy specimen (Silverstein 2000).

Tumour palpability, mass lesion in mammography (MGR), lesion visibility in breast ultrasound (US), suspicion of microinvasion or high-grade histology as well as extensiveness of the disease are considered characteristics associated with elevated risk for invasion (Cody et al 2001).

The histopathological assessment of the surgical specimen may also fail detection of invasion. Accordingly, tumour positive SN findings have been observed in up to 14% of patients with “high risk “ pure DCIS and SN biopsy (Cox et al 2001, Klauber-Demore et al 2000, Mittendorf et al 2005, Pendas et al 2000, Yen et al 2005, Leidenius et al 2005). In some these DCIS patients with tumour-positive sentinel nodes, invasion has been detected in the histopathological review of the breast specimen (Cody et al 2001).

The majority of these tumour-positive SN findings have been micrometastases or ITC. In cases with AC, no additional metastases have been detected (Intra et al 2003, Kelly et al 2003, Klauber-DeMore 2001, Lara et al 2003). Furthermore, studies with a high prevalence of

tumour positive SN findings have included only small numbers of highly selected patients, not representing the entire disease spectrum of DCIS. The tumour positive SN findings have been clearly less common, just 3%, among unselected DCIS patients (Intra et al 2003). Nevertheless, the role of SN biopsy in patients with pure DCIS is still controversial.

19. Morbidity after Sentinel Node Biopsy

Randomized studies have shown that the rate of postoperative seroma formation or drain usage, as well as long-time morbidities like arm lymphoedemas, numbness and loss of sensitivity to light touch and pinprick are significantly less frequent among patients who undergo SNB as compared to the patients to whom an AC is performed (Purushotham et al 2005, Mansel et al 2006). Same studies proved equally beneficial effects of SNB concerning the scores reflecting immediate postoperative quality of life and psychological morbidity. Similar conclusions have been drawn in numerous non-randomized studies. Actually, SNB is associated with faster recovery and resumption of normal day-to-day activities (Burak et al 2002, Leidenius et al 2003, Swenson et al 2002, Mansel et al 2006, Leidenius et al 2003) and shorter hospital stay (Haid et al 2002, Leidenius et al 2003, Mansel et al 2006).

The prevalence of clinically significant lymphoedema seems negligible, just 1-2% , after sole SNB (Burak et al 2001, Haid et al 2002, Peintinger et al 2003, Rietman et al 2004, Rönkä et al 2005, Swenson et al 2002, Temple et al 2002). Approximately every fourth patient suffers at least some long-term arm symptoms, usually mild pain or sensory disorders (Burak et al 2001, Haid et al 2002, Peintinger et al 2003, Rietman et al 2004, Rönkä et al 2005, Swenson et al 2002, Temple et al 2002).

20. Axillary Metastases after Sentinel Node Biopsy

Eight percent false negative rate (Kim et al 2006, Lyman et al 2005), and therefore metastases may be sometimes left in the axilla when AC is omitted relying on tumour negative SN findings.

Concern has been raised that the proportion of cancer recurrences in the axilla could increase as a result from the use of the SNB. However, so far the axillary recurrence rate has been 0,9% (range 0 to 2,7%) following a negative SNB during a mean follow-up ranging from 22 to 65 months (Chung et al 2002, Schrenk et al 2001, Roumen et al 2001, Giuliano et al 2000, Veronesi et al 2005, Torrenga et al 2004, Naik et al 2004, de Kanter et al 2006) and there seems to remain a similar risk of recurrence and overall survival after AC and SBN (Veronesi et al 2003). However, this observation is mainly based on clinical follow-up performed solely with repeated physical examinations (Chung et al 2002, Schrenk et al 2001, Roumen et al 2001, Giuliano et al 2000, Veronesi et al 2005, Torrenga et al 2004, Naik et al 2004).

The sensitivity of preoperative physical examination in detection of axillary lymph node metastases is augmented by US, combined with examination of fine-needle biopsy aspirates (Torrenga et al 2004, Naik et al 2004). Therefore, axillary US may be beneficial also in the follow-up patients who have avoided AC due to negative SN findings.

In breast cancer, there is concern of increased false positive rates when US monitoring is used as compared to physical examination alone (Verbanck et al 1997). US used in combination with fine-needle aspiration cytology can improve the sensitivity and specificity of detecting lymph node metastases in the follow-up (Pamilo et al 1989, Tate et al 1989). The frequency of unnecessary surgical explorations of the axilla might also increase due to false positive findings in physical examinations or following false positive ultrasonography (US) of the axilla. Nevertheless, the role of US in the follow-up of SNB has not been widely evaluated so far.

Hypothesis of the study

- 1) The intraoperative success rate can be improved by adjusting tracer dose according to patients' BMI in addition to a repeated, second radioisotope tracer injection in patients with non-visualized SNs in preoperative lymphoscintigraphy.
- 2) The use of an immunohistochemical biomarker enhances the intraoperative detection of SN metastases.
- 3) SNB is a useful tool in the staging of breast cancer with minimal risk of nodal involvement, like tubular carcinoma.
- 4) Specific features predicting invasion can be identified in patients with DCIS in the preoperative biopsy.
- 5) Due to the minimal risk of axillary recurrence, routine US monitoring of the ipsilateral axilla is not worthwhile among breast cancer patients, whose axillary clearance has been omitted due to non-metastatic SNB findings.

Patients and Methods

The study was carried out at the Breast Surgery Unit, Department of Gastrointestinal and General Surgery of Helsinki University Central Hospital between June 2000 and November 2004, when in total 1580 patients with clinical stage T1-T2, clinically node negative breast cancer underwent LS and SN biopsy in our unit. In Study IV, the study patients consisted of women, who participated in the biennial, population based mammography screening at the Mammography Screening Center of Helsinki between June 2001 and November 2004 and had DCIS in the preoperative needle biopsy. The follow-up visits in Study V took place in the Breast Surgery Unit and the Department of Oncology. The project plan was approved by the Ethical Committee of the Department of Surgery, Helsinki University Central Hospital. The number of the patients and exclusion criteria in the studies are presented in Table 2.

Table 2. The study patients and exclusion criteria

No	Study	Patients	No in groups	No Excluded	Exclusion criteria
I	Optimizing SN visualization with BMI and 2nd injection (2001-2002)	<u>N=534</u> Grade I 188 II 221 III 99 NA 26 Stage T0 21 T1 380 T2 129 T3-4 4 Histology DCIS 21 Invasive ductal 281 Invasive lobular 138 Invasive others 94	Dose, 92Mbg, not adjusted according to BMI 356 (Group I) Dose, 80 or 100 or 140 Mbg, adjusted according to BMI 178 (Group II)	-	
II	Rapid IHC in the intraoperative diagnosis of SN metastases (2001-2004)	<u>N=995</u> Grade I 299 II 418 III 215 NA 14 Stage T0 48 T1a 30 T1b 199 T1c 461 T2-4 259 Histology DCIS 48 Invasive ductal 523 Invasive lobular 245 Invasive others 179	With rapid IHC 557 Without rapid IHC 438	270	The use of another rapid IHC technique
III	Axillary metastases in pure tubular cancer (2001-2004)	<u>N=27</u> Stage T1a-b 18 T1c 8 T2 1	-	-	
IV	SNB in patients with DCIS in CNB (2001-2004)	<u>N=67</u>	-	12	No DCIS in CNB according to the histopathological review
V	Axillary recurrences and axillary ultrasonography after SNB (2000-2001)	<u>N=205</u> Grade I 81 II 77 III 40 NA 7 Stage T1a 11 T1b 49 T1c 107 T2 38	-	-	

Lymphoscintigraphy

The day before surgery, lymphoscintigraphy was performed in all five studies a median of four hours after a single intratumoural injection of ^{99m}Tc labelled human albumin colloid Nanocoll® (Nycomed Amersham Sorin s.r.l. Saluggia, Italy), with particle size less than 80 nm in a volume of 0,2 ml.

In Study I, the “control” group (group I) received a median dose of 92 Mbq, whereas in group II the doses were adjusted according to BMI and equaled 80 or 100 or 140 Mbq. In studies I-V, the radioisotope was injected intratumourally by palpation control in patients with a clearly palpable tumour and was guided ultrasonographically or stereotactically when the tumour was not clearly palpable. In patients with a previous excision biopsy, the tracer was injected around the biopsy cavity. In all instances, the injection site was massaged for about one minute after the injection.

The activity of the injection syringe was measured before and after injection and the activity of the sterile gauze used to massage the injection area was measured as well. The actual amount of activity received by the patient was thus recorded (Study I).

In Study I, the dose received by patients in group II with BMI over 30 was 28 MBq larger than the patients in group I ($p < 0,001$). The patients with a BMI 30 or lower received similar doses in group I and group II (Table 3).

Table 3. The actual median dose of the radioactive tracer received by the study patients.

	Injection dose 92Mbq (N= 356)	Injection dose 80, 100 or 140Mbq (N= 178)	p
BMI			
<26	92 (50-123)	88 (70-104)	<i><0.001</i>
26-30	91 (60-110)	95 (66-116)	<i>0.008</i>
>30	89 (70-114)	117 (87-114)	<i><0.001</i>

Anterior and lateral views were obtained with a gamma camera (Toshiba GCA- 901A, Toshiba Corporation, Japan) using a 256 x 256 matrix and up to 5 min imaging time/frame. The localisation and number of all visible focal accumulations of radioactivity were recorded by an experienced nuclear medicine physician. When no axillary SN was visualized, especially in cases with an upper lateral tumour location, the breast was retracted medially and/or caudally to move the injection site further away from the axilla and a new anterior view was obtained.

In an adjoining study to Study I, 42 of the 80 patients without axillary hot spots in the LS received a second tracer injection with a median dose of 70 (36-110) MBq (Leikola et al 2006). The second injection was intratumoural in 30 (71%), subcutaneous in 7 (17%) and subareolar in 3 (7%) patients. In two patients (5%), the site of the second injection was not registered. No additional imaging was performed after an eventual second injection.

Surgery

Breast surgery (Studies I-V)

In all patients with breast conserving surgery (BCT), a wide local excision of the tumour was performed with a goal of 10 to 20mm free margins, including the underlying pectoral fascia and most often a slice of overlying skin. During primary surgery, the operation was converted to mastectomy if the tumour was not to be suitable for BCT because of multifocality or larger in size than evaluated preoperatively. Patient consent for conversion had been obtained preoperatively. When the margins were involved or close (< 3 mm), either mastectomy or re-resection was performed as a second operation in agreement with the patient.

In study IV, 43 (64%) patients underwent breast conserving surgery and 24 (36%) mastectomy during the primary operation. The type of surgery performed was decided in agreement with the patient. In seven patients a second operation was necessary due to insufficient margins. Two patients had a further resection while 5 underwent mastectomy. The total mastectomy rate was 43 % (29 patients). Altogether 16 of the 29 mastectomy patients underwent immediate breast reconstruction.

Sentinel node biopsy (Studies I-V)

At least 5 minutes before incision, 1ml of Patent Blue dye (Bleu Patenté V; Laboratoire Geuerbet, Aulnay-sous-Bois, France) was injected intratumourally. The SNs were harvested using a gamma probe and by searching for blue-stained lymphatic vessels and nodes. All focally radioactive and/or blue nodes in the axilla were harvested.

AC (Studies I-V)

Preoperative lymphoscintigraphy (LS), intraoperative gamma probe, and blue dye were used for identification of sentinel nodes (SNs). Axillary clearance (AC) was performed during the primary operation in patients with SN metastases in the frozen section as well as in patients with unsuccessful SN identification. Patients with false negative findings in the frozen section diagnosis underwent level I-II AC as a second operation.

In addition, AC was performed in one patient with tumour negative SN findings because of multifocality of primary tumour of PTC (Study III).

Axillary surgery in patients with DCIS in CNB (Study IV)

The indications for SNB in patients with DCIS in CNB included palpable tumor, mass lesion in mammography or breast ultrasound and an extensive lesion warranting mastectomy, but were individually decided by the operating surgeon in agreement with the patient. In study IV axillary surgery either during primary surgery or second operation was performed in 34 (51%) patients. Two patients underwent level I-II AC as a second operation due to invasive findings in the surgical specimen. Six patients underwent partial level I AC in connection with mastectomy and immediate breast reconstruction. Twenty-six (39%) underwent SN biopsy. Patients with tumor positive SN findings also underwent level I-II AC.

Histology

Core Biopsy Specimens (Study IV)

In Study IV, the CNB specimens were re-assessed by a pathologist specialized in breast pathology. The histological classification of DCIS was based primarily on the nuclear grade, and secondarily

on the presence of necrosis, as stated by the Van Nuys Classification (Silverstein et al 1995) The nuclear grade was classified from one to three as defined by the Consensus Conference on the Classification of Ductal Carcinoma in Situ or WHO classification (The Consensus Conference Committee 1997)

The cell size was evaluated as either small or large. Seven different architectural patterns were considered; comedo, cribriform, micropapillary, papillary, flat and solid (The Consensus Conference Committee 1997). Necrosis and microcalcifications were also notified. After the histopathological review, twelve patients were excluded. Eight of them had atypical ductal hyperplasia and one patient had lobular carcinoma in situ (LCIS) in their CNB, not DCIS. Three patients had invasive cancer already in CNB

The breast resection and mastectomy specimens (Studies I-V)

The breast resection and mastectomy specimens were oriented by the surgeon. In impalpable lesions, specimen radiograph was obtained in all resection specimens. In the resection specimens, the surfaces of the specimen were marked by different coloured inks. The samples were taken from the areas of microcalcifications including also the surrounding tissue and from any other abnormal area. From resection specimens, samples were also taken to include surgical margins to evaluate the resection margins microscopically. In the mastectomy specimens, further samples, in addition to suspect areas, were taken from all quadrants of the breast and from the nipple.

The breast specimens were assessed by experienced senior pathologists with special interest in breast pathology. The extension of cancer cells beyond the basement membrane into the adjacent tissues, with no single focus larger than 1mm in greatest dimension was considered microinvasion (American Joint Committee on Cancer 2002). The invasive tumours were graded by Elston and Ellis (Elston et al 1998).

In Study III, all breast specimens were reviewed by an expert breast pathologist to confirm the correct histological diagnosis with at least 90% tubular component. The tumour re-classification was performed according to the classification of International Agency for Research on Cancer (Tavassoli, Fattaneh 2003).

Sentinel nodes (Studies I-V)

The sentinel lymph nodes were labelled in the operation room indicating the site of origin and sent to the pathology laboratory as separate specimens. The pathologist removed all extracapsular fat and measured the nodes. The nodes were sliced into 1-1.5 mm thick sections perpendicular to their long axis, as described in our previous study (Krogerus et al 2004). The slices were arranged flat on pre-frozen Tissue-Tek ® OCTTM-compound. Touch preparations (imprints) from the surface were first made pressing the glass slide gently against the still soft surface of the slices, already frozen in place from the bottom. The imprints were then stained with toluidine blue. While the pathologist was examining the imprints, frozen sections were cut from 2 levels and stained with toluidine blue. During the intraoperative IHC era of the study, the adjacent antecedent section of the second cutting level was taken for rapid cytokeratin immunostaining according to the manufacturer's instructions.

As soon as malignant cells were seen, this was reported to the operation theatre. The remaining tissue from the frozen material was thawed, set in cassettes flat between sponges, fixed in buffered formalin and embedded in paraffin. H&E sections were made again from two levels. If no cancer cells were detected in the permanent H&E stain sections, an immunostain for cytokeratin was performed. The first specimens were stained with Cam 5.2 and later, with Cam 5.2 no longer available, with AE1/AE3- keratin stain as recommended by the antibody producers.

Postoperative examinations (Studies I-V)

After the intraoperative examinations, all the remaining sentinel node tissue was fixed directly into phosphate buffered 10% formalin. After fixation, the nodes were sliced and embedded wholly in paraffin. H&E sections were made from two levels of each lymph node. In addition to that, cytokeratin immunostaining was done from one level of the formalin fixed tissue in cases with a negative FS finding. Metastases of 2 mm or less, but larger than 0,2mm were considered micrometastases. Those not larger than 0,2 mm were called isolated tumor cells (ITC) (International Union Against Cancer 2002). If the micrometastasis was detectable in IHC only, the prerequisite for diagnosis was that immunostained cells were morphologically cancer cells.

AC specimens (Studies I-V)

Lymph nodes in the axillary clearance specimens were embedded wholly in paraffin. H&E sections were prepared from two levels, 200 um apart.

Adjuvant therapy (Study V)

Postoperative radiotherapy was given to the residual breast tissue after breast-conserving surgery using a linear accelerator to a cumulative dose of 50 Gy in 25 fractions, and a 10 Gy booster dose was delivered to the operative bed in 5 fractions for premenopausal women. Systemic adjuvant treatment consisted of six 3-weekly cycles of FEC (5-fluorouracil 600 mg/m², epirubicin 60 mg/m², and cyclophosphamide 600 mg/m², or of three 3-weekly cycles of docetaxel (80 to 100 mg/m²) or eight weekly cycles of vinorelbine (25 mg/m²) followed by three 3-weekly cycles of FEC given within a context of a prospective randomized trial. Women with oestrogen receptor or progesterone receptor positive cancer received tamoxifen 20 mg daily for 5 years.

Follow-up regimen (Study V)

Planned follow-up visits took place at one and three years after breast surgery. Physical examination, blood cell counts and blood chemistry, bilateral mammography, and US of the axilla were performed at these visits. The study participants had access for extra visits at the Department of Oncology whenever there was concern of breast cancer recurrence.

Ultrasonographic examination (Study I, IV, V)

The US examination of the breast and the ipsilateral axilla was carried out or supervised by a senior radiologist. Either an Echo Camera SSD-680 Aloka ultrasound system (Aloka Company Ltd., Tokyo, Japan) with a 7.5 megahertz linear array transducer or a Toshiba Power Vision 6000, SSA-370A (Toshiba Company Ltd., Tokyo, Japan) was used.

The ultrasonographic features considered as suspicious for malignancy in an axillary lymph node were two-dimensional enlargement giving a rounded appearance of the lymph node, an echo-poor central hilus, and eccentricity of the nodal cortex. If at least one of these features was present, a US-guided fine-needle aspiration biopsy was performed. Clinically suspicious nodes and those suspicious in a US examination, and nodes with atypical, suspicious, or malignant cytological findings were excised surgically for histological examination. All axillary recurrences were histologically confirmed.

Statistical methods

Proportional data was compared using chi-square or Fisher's exact tests. The medians and means were compared using the Mann-Whitney U-test. Two-tailed p-values <0.05 were considered statistically significant.

Results

The visualization of SNs in LS (Study I)

The visualization rate of axillary SNs in LS was 454/ 534 (85%) in the entire study population.

The visualization rate was 307/356 (86%) in patients receiving a similar tracer dose regardless BMI (group I) and 147/178 (83%) in patients receiving a dose adjusted according to BMI (group I) group II (p=0,303) (Table 4).). In both groups, the median number of axillary hot spots was 2 (range 1-7) among patients with visualised axillary nodes in LS. In patients with BMI over 30, the number of axillary SNs was higher in group II (p=0, 002) (Table 4)

Table 4. Non-visualization and median number of visualised axillary sentinel nodes according to the patient body mass index (BMI). The dose of the tracer was adjusted according to the patient body mass index in group II

	GROUP I (N=356)	GROUP II (N=178)	<i>p</i>
BMI			
<26			
Non-visualization	21/217 (10%)	13/113 (12%)	<i>0.703</i>
Median (visualised)	2 (1-7)	2 (1-6)	<i>0.702</i>
26-30			
Non-visualization	19/90 (21%)	13/41 (32%)	<i>0.197</i>
Median (visualised)	2 (1-5)	2 (1-5)	<i>0.380</i>
>30			
Non-visualization	9/49 (18%)	5/24 (21%)	<i>1.000</i>
Median (visualised)	1 (1-4)	3 (1-7)	<i>0.002</i>

The intraoperative success rate (Study I)

The axillary SN were identified in 496/534 (93%) patients. In 354/534 (66%) patients, both radioactive and blue SN were detected. In 136/534 (25%) patients only the radioactive SNs were found and in 6 (1%) of the 534 patients only the blue SNs were found intraoperatively. The median number of the harvested radioactive nodes was 2 (1-14) and the median number of the blue nodes was 2 (1-10).

The overall success rate in the intraoperative identification of SN in the axilla was substantially higher, 441/454 (97%) in patients with visualized axillary SN in the LS compared to the 55/80 (69%) patients without visualized SN, $p < 0.00005$. The radioisotope success rate differed significantly, 438/454 (96%) in patients with SN visualized in the axilla and 52/80 (65%) in those without axillary SN in the LS, $p < 0.00005$. Also the blue dye success rate was higher, 320/452 (71%), among patients with visualized SNs in the axilla, compared with the 40/80 (50%) in patients with non-visualization, $p = 0.0007$.

The overall intraoperative failure rate was 5/42 (12%) among the patients receiving a second tracer injection after non-visualization of axillary SN in the LS (Leikola et al 2006). Of these five patients, three had axillary metastases. 20/38 (53%) patients not receiving a second injection underwent AC because of non-identification of SN, resulting in a significant ($p = 0.0002$) difference between the patients with and without a second radioisotope injection. Seventeen (85%) of these 20 patients undergoing AC were axillary node negative. The radioisotope failure rate was 5/42 (12%) in patients receiving a second radioisotope injection and 15/38 (61%) in patients without a second injection, $p < 0.00005$. The blue dye failure rate was 19/42 (45%) after a second radioisotope injection and 21/38 (52%) without it, $p = 0.5021$ (Leikola et al 2006).

This study adjoining Study I also showed, that among patients without hot spots in LS , the use of a second injection increased the number of harvested radioactive SN among patients with successful intraoperative SN identification . The mean number of harvested radioactive SN was 2,3 (median 2, range 1-10) after a second injection, and 1,5 (median 1, range 0-9) in patients who did not receive a second injection., $p= 0.0114$ (Leikola et al 2006).

However, among all patients with successful intraoperative SN identification, the number of harvested radioactive SN was rather similar regardless of the use of a second injection. The mean number of radioactive nodes was 2,7 (median 2, range 0-14) in patients who did not receive the second injection and 2,3 (mean 2, range 1-10) in patients who received a second injection., $p= 0.0916$ (Leikola et al 2006).

The influence of rapid IHC in the intraoperative diagnosis of SN metastases (Study II)

Sentinel node metastases were found in 175/438 (40%) patients with rapid IHC (the IHC group) and in 219/557 (39%) patients without rapid ICH (the non-IHC group) The median size of SLN metastases was 3mm in both patient groups. The micrometastasis or ITC detected in the intraoperative examination was not found in the paraffin sections in 12/84 (14%) cases in the non-IHC group and 8/82 (10%) cases in the IHC group. SLNs were the only metastatic nodes in 150/219 (69%) in the non-IHC group and in 129/175 (74%) in the IHC group ($p =0,27$). Table 5.

Table 5. Sentinel lymph node metastases and the use of intraoperative immunohistochemistry (IHC)

	Non-IHC group (N=557)	IHC group (N=438)	p
Macrometastases	135/219 (62%)	93/175 (53%)	<i>0,101</i>
Micrometastases	84/219 (38%)	82/175 (47%)	<i>0,206</i>
Micrometastasis and ITC as only tumor positive finding	84/219 (38%)	82/175 (47%)	<i>0,206</i>
Micrometastases detected only in FS slides	12/84 (14%)	8/82 (10%)	<i>0,822</i>
Number of SLN metastases according to metastasis size			
< 0,2mm	5/219 (2%)	27/175 (15%)	
0,2-1,0mm	52/219 (24%)	42/175 (24%)	
1,1-2,0mm	27/219 (12%)	13/175 (8%)	

The sensitivity of intraoperative diagnosis in relation to the size of SN metastasis and the histological tumour characteristics (Study II)

The overall sensitivity of the intraoperative diagnosis was 79% in the IHC group and 78% in the non-IHC group, $p = 0.712$. Among patients with ILC, the sensitivity of the intraoperative diagnosis was higher, 87%, in the IHC group as compared to 66% in the non-IHC group ($p=0.02$). Patients with invasive ductal carcinoma (IDC) and other types of invasive cancer had similar sensitivity of intraoperative diagnosis in both patient groups. ITC, the smallest (<0,2mm) nodal metastases, were revealed intraoperatively only in the IHC group. The

sensitivity of the intraoperative diagnosis was similar in both patient groups regardless of histological tumour stage or grade. Table 6.

Table 6. The characteristics of the primary tumour and the sensitivity of intraoperative diagnosis in relation to the size of SN metastasis (Study II)

	NON-IHC GROUP	IHC GROUP	p
<u>Tumour histology</u>			
DCIS	0/0 (0%)	3/3 (100%)	-
IDC	106/127 (83%)	67/85 (79%)	0,470
ILC	39/59 (66%)	45/52 (87%)	0,015
other invasive	25/33 (76%)	24/35 (69%)	0,594
<u>Tumour stage</u>			
T0	0/0 (0%)	3/3 (100%)	-
T1a	1/1 (100%)	4/5 (80%)	1,000
T1b	19/29 (66%)	14/20 (70%)	1,000
T1c	78/109 (71%)	45/62 (73%)	0,861
T2-4	73/82 (89%)	71/83 (86%)	0,478
<u>Tumour grade</u>			
I	49/67 (73%)	20/31 (65%)	0,536
II	82/106 (77%)	69/86 (80%)	0,694
III	38/45 (84%)	43/50 (86%)	0,832
N.A.	4	6	
<u>SLN metastases size (mm)</u>			
<0,2	0/5 (0%)	13/27 (48%)	0,064
0,2-1,0	27/52 (52%)	25/42 (60%)	0,534
1,1-2,0	16/27 (59%)	10/13 (77%)	0,316
>2,0	127/135 (94%)	91/93 (98%)	0,206
<u>Overall intraoperative sensitivity</u>	170/219 (78%)	139/175 (79%)	0,712

Axillary staging with SNB in PTC (Study III)

The SN were successfully harvested in all 33 patients with a median of 3 (range 1-10) SN. The median total number of retrieved axillary nodes was 16 (15-24) in patients with AC.

Nine of the 33 patients had axillary metastases. The median number of metastatic nodes was 1 (1-3). The median size of the SN metastases was 0,5 mm (mean 1,7 mm, range 0,4-5mm).

Micrometastases as the only tumour positive SN finding were observed in six patients, while three patients had larger metastases. None of the patients had isolated tumour cells in their SN. Metastases in the non-sentinel nodes were found in two patients. Both of them had 3mm SN metastases. Intraoperative frozen section revealed SN metastases in seven patients. Both patients with false negative frozen section findings had SN micrometastases.

The median histological tumour size was similar, 9- 10 mm, in patients with or without axillary metastases and smaller than 10mm in 17 patients. Five of these had axillary metastases. Among these five patients, the median size of the SN metastasis was 0,5mm (mean 1,3 mm, range 0,4-3mm). Axillary metastases were equally frequent in patients with palpable and non-palpable tumours. The median age of patients with axillary metastases seemed somewhat younger, 54 (44-71) years than those with tumour negative SN findings with the median age of 57 (39-80) years, but the difference was not statistically significant (Table 7).

The tumour specimens' histopathological review revealed that 5 of the 33 tumours lacked sufficient tubularity to be classified as PTC. The prevalence of or the risk factors for axillary metastases were not remarkably influenced by the histopathological review.

Table 7. The influence of patient and tumour characteristics on the prevalence axillary metastases in 33 patients with pure tubular carcinoma (PTC) of the breast

	Patients with SN metastases (N=9)	Patients without SN metastases (N=24)	p
Median age in years (range)	54 (44-71)	57 (39-80)	<i>0.808</i>
The histological size of the primary tumour (mm)*	9 (6-12)	10 (3-26)	<i>0.700</i>
Histological tumour stage			
T1a-b	5	15	
T1c	4	8	
T2	0	1	<i>0.795</i>
Palpable tumours	4	11	<i>0.944</i>
Tumour location			
Upper lateral	3	15	
Upper medial	3	7	
Lower lateral	2	1	
Lower medial	1	1	
Central	0	0	<i>0.739</i>

*median (range)

Invasive cancer in patients with DCIS in CNB (Study IV)

Forty-seven (70%) of the 67 patients with DCIS in the preoperative needle biopsy had pure DCIS. Eight (12%) patients had microinvasive DCIS and 12 (18%) had invasive carcinoma. According to the histopathological assessment of the surgical specimens, the median size of the invasive component was 9mm (4-15mm) among the 12 patients with invasive cancer.

Two patients with pure DCIS had tumour positive SN findings, one patient had a micrometastasis and the other had ITC. One patient with microinvasive DCIS had ITC in a single SN. Two patients with invasive cancer had SN metastases, one had a micrometastasis and the other had a larger metastasis. All these patients underwent level I-II AC. Only the patient with invasive cancer and SN macrometastasis had further metastases in her AC specimen.

The strongest predictor for invasive carcinoma or microinvasive DCIS was the visibility of the lesion in breast US. Thirteen (50%) patients with lesions visible in US had invasion in their surgical specimens, while only 7 (17%) patients without such a lesion had invasive or microinvasive cancer, $p=0.006$. Invasion in the surgical specimen was also detected more often, in 13 (35%) patients with high nuclear grade DCIS in the preoperative CNB than in patients with medium or low nuclear grade, 7 (17%) patients, $p=0.0418$. Invasion seemed also more common, in 12 (43%) patients with comedo type DCIS in CNB than in patients with non-comedo histology, 8 (21%) patients, $p=0.062$. Table 8

Table 8. The risk of invasive cancer or microinvasive DCIS in the 67 patients with DCIS in the preoperative core needle biopsy (CNB) specimen

Risk factor	Invasive or microinvasive cancer	p
<u>Tumour size in MGR</u>		
over 30mm	9 / 25 (36%)	<i>0.421</i>
under 30mm	11/42 (26%)	
<u>Tumour palpability</u>		
Palpable	6 / 12 (50%)	<i>0.160</i>
Non-palpable	14 /55 (25%)	
<u>Lesion in MGR</u>		
Mass lesion	7 / 19 (37%)	<i>0.555</i>
No mass lesion	13/48 (27%)	
<u>Growth pattern of DCIS in CNB</u>		
Comedo	12 / 28 (43%)	<i>0.062</i>
Non-comedo	8/39 (21%)	
<u>Large cell DCIS in CNB</u>		
Yes	18/51 (35%)	<i>0.1450</i>
No	2/16 (13%)	
<u>Nuclear Grade</u>		<i>0.0418</i>
1-2	7/32 (17%)	
3	13/35 (37%)	
<u>Lesion visibility in breast US</u>		
Visible	13 / 26(50%)	<i>0.006</i>
Non- visible	7/41(17%)	

Twenty-five of the patients had a lesion not visible in US and non-comedo type DCIS in their CNB. Only 2 (8%) of these patients had invasive cancer or microinvasive DCIS in their surgical specimens. Combining other low risk features to non-visibility in US or with each other was not helpful in identifying minimal risk of invasion.

The role of US in the follow-up (Study V)

Nine of the 205 study patients died during the follow up period three of whom died from breast cancer without signs of axillary recurrence. Four patients developed distant breast cancer recurrence without findings of axillary recurrence. Eleven other patients did not complete the 3-year follow-up program, but all were alive at the end of the study according to the files of the Finnish Cancer Registry.

At the one year follow-up visit, the US examination of the axilla was performed in 186 patients. No breast cancer recurrences were detected in these nor in mammography nor in the clinical examination. The presence of enlarged, but ultrasonographically nonsuspicious axillary lymph nodes led to a fine-needle aspiration biopsy to be taken from two patients. These fine-needle aspirates contained only benign cells, and both patients had two subsequent follow-up US examinations with normal findings.

At the 3-year follow-up visit, axillary US examination was performed in 183 patients. The US revealed suspicious axillary lymph nodes in one patient. The axillary recurrence was confirmed both in a fine-needle aspiraton cytology and histologically in the surgical specimen. No other needle or surgical biopsies were taken from any of the patients. One patient had, however, a repeat US examination following detection of enlarged, ultrasonographically nonsuspicious lymph nodes.

Between the scheduled study visits, nine patients had axillary US performed, six due to axillary pain, two due to presence of palpable nodes, and one patient visited the outpatient clinic a year too early by mistake. A fine -needle aspiration cytology was taken from two of these patients, and revealed axillary recurrence in the patient who visited the outpatient clinic one year too early. Thus, breast cancer was detected in only two (0.5%) of the total of 383 US examinations performed during the study, and in only one (0.3%) of the 369 examinations performed at the scheduled visit times.

Discussion

The Visualization of the Sentinel Nodes in Lymphoscintigraphy and the Intraoperative Success Rate (Study I)

Numerous previous studies have revealed that both the visualization rate of the axillary SN in LS as well as the intraoperative success rate in SN harvesting are lower among obese patients (Cox 2002, Derossis et al 2003). Therefore, the aim was to enhance the visualization rate of the axillary SN by adjusting the tracer dose according to the patients' BMI. Disappointingly, this study could not improve the visualization rate of axillary SNs in these patients. When the most obese patients received a higher dose of radioactive tracer, only the number of visualized axillary SNs increased. Higher tracer doses tend to cause an excessive number of hot spots, some of which no longer can be regarded as SNs. This could lead to the weakening of the fundamental principle of SN biopsy: an accurate axillary staging with minimized morbidity. On the other hand, high sensitivity of SN biopsy requires that all true SNs are visualized as hot spots (Wong et al 2001).

It is noteworthy, that there was a considerable difference between the intended and actual tracer doses received by the patients in whom the dose was adjusted according to the BMI. The most probable reason for this is that the volume of tracer used was small, 0.2ml in all patients. Some of the liquid tracer always remains in the syringe and the needle. Furthermore, some of the injected volume is absorbed into the sterile gauzes used to massage the injection area. Since the concentration of the radioactive Tc99m within the 0.2ml was higher in the more obese group, more radioactive material was lost due to the reasons explained. Hence a loss of nearly 20% of the

intended tracer radioactivity should be taken into consideration, when arbitrating the different lymphatic mapping techniques.

The results concerning in particular the most obese patients might have been different had considerably higher doses been used, e.g. >200 MBq. Also the optimal tracer volume is unresolved so far (Tanis et al 2001, Berman et al 1999, Lloyd et al 2001). Until these problems are solved, the optimal tracer dose, especially for obese patients, remains open.

In a case of non- visualization in LS, delayed imaging and re-injection have been shown to increase the visualization rate (Tanis et al 2002). Accordingly, the further results of this study showed that an additional injection enhanced the harvesting of SNs in patients with non-visualized SN (Leikola et al 2005). It especially reduced the need of AC in node negative patients. The second injection was most often performed intratumorally such as for the initial one. In the study presented, further LS imaging could not be obtained after the second injection due to tight perioperative scheduling.

The blue dye identification rate was remarkably lower in patients with non-visualized axillary SN, without differences between patients with or without a second radioisotope injection. The improvement in the success rate after a second radioisotope injection was therefore solely due to enhanced radioisotope identification. Generally, the blue dye success rate was rather low, most probably because only 1ml of dye was injected just 5 minutes before the incision, according to known routine SN biopsy protocol. Furthermore, blue dye is regarded as a secondary method to radioisotope localization in our unit. It may not add much to the radioisotope identification, especially in experienced hands (Derossis et al 2001). As a teaching hospital, our unit still uses blue dye, because it it can be highly demonstrative.

Besides weak lymphatic circulation, lymphatic obstruction due to axillary metastases, may also result in non-visualization in LS. In these cases, a second radioisotope injection after a negative LS is a non-physiological way to force the radioisotope to the axillary nodes and may

result in false negative findings. Therefore, careful palpation of the open axilla in order to harvest suspicious lymph nodes is warranted, particularly in these cases.

The superficial injections have been increasingly adopted because these provide better visualization rates of axillary SN in LS (McMasters et al 2001, Chagpar et al 2004, Martin et al 2001). In the Breast Surgery Unit, the intratumoural injection is used for two reasons. Firstly, the internal mammary chain can not be visualized with the superficial injection techniques (Roumen et al 1999). Secondly, favourable results have been obtained by radio-guided occult lesion localization for non-palpable breast cancer (Rönka et al 2004), so patients undergoing ROLL will receive ultrasonographically guided intratumoural tracer injection anyway.

The Intraoperative Diagnosis of Sentinel Node Metastases (Study II)

The macrometastases of IDC do not usually pose problems in the intraoperative diagnosis (Leidenius et al 2003). It is therefore not surprising, that no improvement in the intraoperative diagnosis was noted in this most common histopathologic type of breast cancer by intraoperative immunohistochemistry.

The second most common type of breast cancer, ILC, has rising incidence rates, especially among women over 50 years of age (Li et al 2003, Li et al 2000). This has recently been attributed to hormone replacement therapy (Li et al 2000). ILC as a histological subtype of breast cancer remains challenging for pathologists (Holck et al 2004, Bussolati et al 1986). ILC metastasizes to lymph nodes in a scattered fashion, lacking severe cytological atypia. Metastatic cells tend to fill the sinuses, mimicking sinus histiocytosis (Bussolati et al 1986). Adding IHC to the routine paraffin H&E diagnostic methods has been shown to provide substantial advantage in revealing ILC metastasis from such morphological camouflage. In SNB, the use of IHC reduces the FNR by 10-36% and can therefore be recommended to be routinely used especially in patients with ILC (Bussolati et al 1986, Turner et al 1998; 68 Cote et al 1999; Cserni et al 2006).

Not surprisingly, this study's results indicate that the use of rapid IHC cytokeratin biomarker analysis significantly reduced the FNR in the intraoperative diagnosis of the ILC metastases. With the incorporation of the cytokeratin biomarker the sensitivity of the intraoperative diagnosis of ILC metastases equaled that obtained in IDC. On the other hand, no remarkable differences were observed in the intraoperative diagnosis of micrometastases with or without rapid IHC. However in the case of detection of the smallest micrometastasis, there appeared to be a small benefit of rapid IHC. The cases with micrometastasis noted only in the frozen section slides were few. Therefore, the incorporation of IHC as an additional biomarker seems to have a negligible upstaging effect.

The overall sensitivity of the intraoperative diagnosis was not enhanced by the use of rapid IHC in IDC, because only 25% of the patients had ILC. This is in agreement with previous reports (Viale et al 1999, Zurrida et al 2001). However, in these previous reports, the results were not subclassified according tumour histology. Neither was sensitivity of the intraoperative diagnosis reported separately for metastases of ILC. As regards to micrometastases, adding intraoperative IHC to such tedious cryosectioning technique as used in Milan seems unlikely to improve the already high sensitivity (Viale et al 1999). In the case of micrometastases, the number of tissue sections examined is of primary importance when compared to the addition of biomarkers in IHC for finding the small metastatic sites (Viale et al 1999). However, the value of rapid IHC maybe contributory in centers that incorporate fewer sections in their intraoperative diagnostic procedures and who routinely experience a lower sensitivity for the intraoperative detection of the disease.

The intraoperative examination of sentinel lymph node specimens is challenging and tedious work. Confirming the negative result may be especially time consuming; the pathologist is forced to evaluate multiple cytologic and morphologic criteria in benign cells mimicking cancer cells. Part of this difficulty is attributed to the fact that blue dye, as well as the colloid medium for the radioactive tracer are ingested by the histiocytes, making the endothelial cells swell and

resulting in a pathologic phenotype which appears suspicious for malignancy. Furthermore, follicular centres in the lymph nodes may be cut in a plane that simulates a focus of poorly differentiated carcinoma. Differential brown staining of IHC greatly simplifies the pathologic decision because it is easier and quicker when cancer cells are stained brown by cytokeratin marker to enhance the differential diagnosis. Even though unspecific staining in dendritic cells (Xu et al 2000) is rather common, a differential diagnosis between benign and malignant immunostained cells is not usually a confounding histopathologic dilemma. In addition, the small scattered metastatic lobular cancer cells are rather difficult to tell apart from lymphatic cells without IHC.

Sentinel Node Biopsy in Pure Tubular Carcinoma (Study III)

In the present study, the prevalence of axillary metastases in PTC was high, when compared with the 7% observed in a meta-analysis (Papadatos et al 2001), most probably because the meticulous histological examination of SN reveals metastases that are not detected in standard lymph node processing (de Widt-Levert et al 2003, McMasters et al 1998, Leidenius et al 2004). Accordingly, the prevalence of metastases in the present study was only 6%, when excluding the micrometastases from the data. Furthermore, metastases have been detected in 17% of PTC patients when using SN biopsy in axillary staging (Wong et al 2002).

The assumption, that the risk of axillary nodal involvement is negligible in patients with a PTC tumour less than 10 mm (Rutgers 2001, Fein et al 1997, Maibenco et al 1999, McBoyle et al 1997, Papadatos et al 2001) was not supported by findings of this study. In fact, neither the tumour size nor the patient age had any influence at all on the risk for metastases. The aggressiveness of the tumour (Peters et al 1981) as well as the risk for metastases (Green et al 1997, Papadatos et al 2001) increase with the decreasing proportion of tubular morphology. However, as regards to this study, results for axillary metastases were not altered after the histopathological reclassification of the tumour specimens.

As stated earlier, the nodal involvement is the most powerful prognostic factor in breast cancer (Fisher et al 1985, Rutgers 2001). As regards to PTC, the effect of nodal disease on disease-free or overall survival has been controversial (Livi et al 2005, Winchester et al 1996, Elson et al 1993, Maibenco et al 1999, Diab et al 1999, Kitchen et al 2001, McBoyle et al 1997, Cabral et al 2003). The low incidence of nodal involvement and the excellent disease-free survival rates of pure tubular carcinoma reported earlier have led some investigators to support the use of SNB only in selected cases (Mendez et al 2005). On the other hand, even a large study population of more than

4000 patients was unable to provide any constellation of predictive features that would identify patients at a low, i.e. significantly under 10% risk of SLN metastases to be safely spared SNB and therefore such staging procedure is highly recommended to all eligible patients with invasive breast cancer (Viale et al 2005). Accordingly, the results of the present and an earlier study (Wong et al 2002), show that axillary metastases in PTC may be more common than previously assumed (Papadatos et al 2001, McBoyle et al 1997, Winchester et al 1996). SN biopsy appears therefore as a feasible axillary staging method in PTC, providing also valuable data for further evaluation of prognosis and natural history of this rather uncommon histological subtype of breast cancer.

SN as the only tumour positive nodes was found among vast majority of our PTC patients with axillary metastasis. Usually, the risk for non-sentinel node metastases is substantial even in patients with SN micrometastases (Leidenius et al 2005). Considering that the data concerning the size and the number of nodal metastases as well as the definite features of the primary tumour are available only in the postoperative phase, AC is recommended in cases where the SN metastases are detected in the intraoperative diagnosis. Since none of the PTC patients with SN micrometastases had non-sentinel node metastases, AC as a second operation after false negative frozen section findings might be omitted in this patient group. However, one must keep in mind, that the very limited number of patients in the present and in the vast majority of the previous studies (Wong et al 2002, Papadatos et al 2001, Cabral et al 2003, Winchester et al 1996, McBoyle et al 1997) addressing PTC, renders the conclusions rather uncertain. It is therefore essential to collect the reported figures of PTC patients for a meta-analysis and thus hopefully obtain a more reliable estimate of the frequency of SN metastases and the need for AC.

Ductal Carcinoma In Situ (Study IV)

In the present study, the prevalence of invasive cancer undetected in CNB was rather high, 30%, when also cases with microinvasive cancer are included. Since the proportion of cases initially diagnosed as DCIS in CNB and upstaged to invasive cancer after surgery can be 30 % or even more (Goyal et al 2006), it seems reasonable to make attempts to identify predictors or risk factors of invasion among these patients. Such predictors could then be used to omit multiple operations and unnecessarily extensive axillary surgery.

In this study, the strongest predictor for invasive cancer was the visibility of the lesion in breast US. This is an issue less addressed in literature in association high risk DCIS. In general, no other imaging modality than MGR has an established role in the diagnostic work-up of DCIS. Nevertheless, the present results as well as the findings in previous studies (Yang et al 2004, Moon et al 2002) indicate that US is beneficial in the evaluation of patients with DCIS, especially as an adjunctive tool to MGR.

Comedo type histology and a high nuclear grade have also been also considered to carry a high risk of invasion (Yen et al 2005, Renshaw 2002). Also the presence of lobular cancerization on CNB has been suggested to be a significant, independent predictor of invasion (Huo et al.2006). In the present study, the first two of these risk factors seemed to provide predictive value alike. However, factors such as extensive or mass lesion in MRG tumour were not such strong predictors for invasion in the present study, unlike in some previous reports (Pandelidis et al 2003, Jackman et al 2001, Yen et al 2005, Darling et al 2000, Huo et al 2006, Goyal et al 2006). Nevertheless, in many studies no factors predicting invasion have been identified (Cox et al 2002, Mittendorf et al 2005, Lee et al 2000). These differing findings explain why, at present, there exists no consensus on these prognostic factors and the issue remains controversial.

Because the end-point of the present study was invasion in the surgical specimen, two patients with pure DCIS in the surgical specimen had tumour positive SN findings but were not included among cases with invasion. However, the surgical specimens of these two patients have been reassessed, without signs of invasion, in connection with our previous study (Leidenius et al 2006). Nevertheless, the small study population in the present study is the most important factor rendering the conclusions rather uncertain.

The demonstrated 13% prevalence of tumour positive SN findings among patients with microinvasive DCIS was similar as in previous studies (Cox et al 2002, Klauber-Demore et al 2000, Intra et al 2003). Actually, the prevalence of tumour positive SN findings was comparable among the patients with microinvasive DCIS and in those with invasive cancer. For these reasons, the results are in agreement with Adamovich and co-workers (Adamovich et al 2003), that SN biopsy appears as feasible in the staging and treatment of patients with microinvasive DCIS. SN biopsy has been considered useful in patients with extensive DCIS warranting mastectomy. If invasion is detected in the mastectomy specimen, further SN biopsy is technically impossible. Also the risk of missing invasion due to sampling error in the histopathological work-up is higher in extensive lesions (Leidenius et al 2006). In the Breast Surgery Unit of HUCH, all patients with pure DCIS and tumour positive SN findings are those undergoing mastectomy (Leidenius et al 2006). Furthermore, SN biopsy may not add much morbidity to that observed after mastectomy, with or even without immediate breast reconstruction.

In patients with DCIS undergoing breast conserving surgery, the prevalence of SN metastases has been as low as 2%, (Veronesi et al 2005). In such cases with localised DCIS, routine SN biopsy is not advisable, and in a case of invasive local recurrence, a previous SN biopsy may hamper a further one. Therefore, even more important than recognizing high risk lesions is to identify patients with lesions including a minimal risk for invasion in order to avoid unnecessary SN biopsies. In the light of this limited patient population it might be worthwhile to observe the

absence of factors such as visibility in US and comedo or high nuclear grade histology in the preoperative CNB.

Previous breast surgery disrupts the lymphatic ducts draining to the axilla from the tumour site and influences lymphatic drainage patterns (Estourgie et al 2007). This may lead either to unsuccessful SN identification or to false negative results (Feldman et al 1999). However, the false negative rate, (Wong et al 2002, Tafra 2001) as well as the regional recurrence rate (Luini et al 2005) have been similar after excision biopsy and needle biopsy. Therefore, if invasion is detected in the breast resection specimen, SN biopsy can usually be performed as a second operation. The exception to this is DCIS located in the upper lateral part of the breast. Here the resection may disrupt the lymphatic drainage to the axilla, similarly as in mastectomy. In these cases, SN biopsy can usually be performed through the skin incision used for the breast resection.

Overall, the risk of SN metastases appears to be low although even 40% of the breast specimens originally diagnosed as DCIS in CNB may be invasive or microinvasive cancer. The use of intraoperative frozen section diagnosis might therefore be considered as not worthwhile. However, in our unit, the sensitivity in the intraoperative diagnosis is high, almost 80% (Study II) enabling us to minimize the need for AC as a second operation. This is of special importance when mastectomy is combined with an immediate breast reconstruction using a latissimus dorsi-flap or an abdominal flap with a microvascular anastomosis in the axilla (Meretoja et al 2007). In these cases, a subsequent axillary operation is technically difficult to perform.

The histopathological review revealed that 15% of the initial DCIS findings in CNB were either invasive carcinoma, atypical ductal hyperplasia or lobular carcinoma in situ. This finding emphasises the importance of accurate histopathological work-up of CNB specimen in order to determine the appropriate extent of surgery both in the breast and in the axilla.

Axillary Recurrences and Axillary Ultrasonography after Sentinel Node Biopsy (Study V)

The risk of axillary recurrence when omitting AC due to tumour negative SN findings seems very low, some 0,3% in this study, at least during a short or medium time follow-up. In agreement with the other studies (Chung et al 2002, Schrenk et al 2001, Roumen et al 2001, Giuliano et al 2000, Veronesi et al 2005, Torrenga et al 2004, Naik et al 2004, de Kanter et al 2006), the present findings suggest that the axillary recurrence rate is low when axillary dissection is omitted following a negative SNB.

In general, the axillary recurrences after SNB have been clearly less common than could be assumed on the basis of average 8% FNR. Previous large series (Fisher et al 1985) suggested that about half of the nonoperated metastases will manifest clinically later. The difference in the axillary recurrence rates in the eighties and in the SNB era is most probably due to a different study population. Although the 40% prevalence of axillary metastases in Fisher's study is similar as in many SNB studies, a substantial proportion, some 30-40% of the tumour positive SN findings are micrometastases and ITC. In addition, many SNB patients do have occult, screen detected, low grade tumours. Furthermore, the vast majority of the SNB patients avoiding AC are operated with breast conserving techniques and therefore receive postoperative radiotherapy. The breast radiotherapy field frequently overlaps the lower part of the axilla. Also the modern systemic adjuvant treatment has a favourable impact on improving both local and regional disease control. The low axillary recurrence rates are mainly based on the clinical examination on the axilla (Chung et al 2002, Schrenk et al 2001, Roumen et al 2001, Giuliano et al 2000, Veronesi et al 2005, Torrenga et al 2004, Naik et al 2004). In several studies the sensitivity of the US has been found to be greater than that of clinical examination in the detection of lymph node metastases (Naik et al 2004, Rossi et al 1997, Leppänen et al 2002). Therefore also the sensitivity of the clinical

examination in detecting axillary recurrences is most probably low. Our policy has been to add the axillary US in the follow-up of breast cancer patients avoiding axillary clearance following a negative SNB. In the present study, clinically occult recurrence in the axillary lymph nodes was discovered by US in two patients, but less than 1% of the US examinations resulted in detection of recurrent breast cancer.

A few studies suggest that the use of axillary US results in no overall diagnostic improvement due to an increased rate of false positive findings (Verbanck et al 1997, Bruneton et al 2006). The present study found enlarged, although morphologically not suspicious nodes only in three of the scheduled US studies. Even though fine-needle aspiration cytology was normal in two of these cases, the specificity of the US examination was still relatively high in the present study (Pamilo et al 1989, Tate et al 1989). No false positive findings in fine-needle aspiration cytology were noticed, which is in line with studies that suggest a generally high specificity for fine-needle aspiration in the diagnosis of breast cancer (Kuenen-Boumeester et al 2003)

Two-dimensional enlargement giving a rounded appearance to the lymph node, an echo poor central hilus, and eccentricity of the nodal cortex have traditionally been considered as features suspicious for metastasis in US (Naik et al 2004, Sapino et al 2003, Lernevall 2000]. The size of the lymph nodes may be of less importance than anticipated (Obwegeser et al 2000). In a recent study on preoperative US examination of the axilla the maximum lymph node cortex thickness was the most important feature that predicted metastatic involvement (Deurloo et al 2003).

It can be concluded that serial monitoring of the ipsilateral axilla using US resulted only in a few needle biopsies, and rarely in repeat US examinations. Furthermore, no false positive ultrasonographic or fine-needle biopsy findings were detected, and none of the patients was subjected to unnecessary surgery of the axilla due to US monitoring. However, the probability of finding axillary recurrence with routine axillary US is low in patients whose axillary clearance has been omitted following a negative SNB. The results of this study are in agreement with other

studies using US monitoring after SNB, with follow-up times between 25 and 65 months (Paajanen et al 2006, Kanter et al 2006, Snider et al 2006). Because the detection rate of axillary cancer recurrence by US has been very low, less than 1%, the routine monitoring of the ipsilateral axilla using US seems not to be worthwhile among these patients.

Conclusions

The visualization rate of SNs in LS was not enhanced by adjusting the dose of the radioactive tracer according to patient BMI. However, a study performed on the same study population concludes, that the failure rate in the intraoperative SN identification can be minimized using a second tracer injection in patients without axillary hot spots in LS.

The intraoperative diagnosis of SN metastases is enhanced by rapid IHC in patients with ILC. Rapid IHC may also improve the intraoperative diagnosis of the smallest micrometastases.

Among the limited number of PTC patients presented in this study, SNB is a feasible method for axillary staging in patients with a low prevalence of axillary metastases, such as PTC.

It is also a sensible method in patients undergoing mastectomy due to DCIS in CNB as well as among selected patients undergoing breast conserving surgery. It seems especially useful in patients with lesions visible in breast US, although larger series are needed to draw definite conclusions.

Due to the very low detection rate of axillary recurrence, routine monitoring of the ipsilateral axilla using US is not worthwhile after negative SNB.

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