Sentinel Node Biopsy in Breast Cancer

Clinical and Immunological Aspects

JANA DE BONIFACE
Dissertation presented at Uppsala University to be publicly examined in Aulan, Ingång 21, Centrallasaretet, 72189 Västerås, Friday, June 1, 2007 at 13:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English.

Abstract


The most important prognostic factor in breast cancer is the axillary lymph node status. The sentinel node biopsy (SNB) is reported to stage the axilla with an accuracy > 95 % in early breast cancer. Tumour-related perturbation of T-cell function has been observed in patients with malignancies, including breast cancer. The down-regulation of the important T-cell activation molecules CD3-ζ and CD28 may cause T-cell dysfunction, anergy, tolerance and deletion.

The expression of CD3-ζ and CD28 was evaluated in 25 sentinel node biopsies. The most pronounced down-regulation was seen in the paracortical area, where the best agreement between both parameters was observed. CD28 expression was significantly more suppressed in CD4+ than in CD8+ T-cells.

From the Swedish sentinel node database, 109 patients with breast cancer > 3 cm planned for both SNB and a subsequent axillary dissection were identified. The false negative rate (FNR) was 12.5%. Thirteen cases of tumour multifocality were detected on postoperative pathology. The FNR in this subgroup was higher (30.8%) than in patients with unifocal disease (7.8%; P = 0.012).

From the Swedish SNB multicentre cohort trial, 2246 sentinel node-negative patients who had not undergone further axillary surgery were selected for analysis. After a median follow-up time of 37 months (range 0-75), 13 isolated axillary recurrences (13/2246; 0.6%) were found. In another 14 cases, local or distant failure preceded or coincided with axillary relapse (27/2246; 1.2%).

In conclusion, the immunological analysis of the sentinel node might provide valuable prognostic information and aid selection of patients for immunotherapy. SNB is encouraged in breast cancer larger than 3 cm, if no multifocal growth pattern is present. The axillary recurrence rate after a negative SNB in Sweden is in accordance with international figures. However, a longer follow-up is mandatory before the true failure rate of the SNB can be determined.

Keywords: breast cancer, tumour immunology, sentinel node biopsy, CD3-zeta, CD28

Jana de Boniface, Centre for Clinical Research, Centrallasaretet, Uppsala University, SE-72189 Västerås, Sweden

© Jana de Boniface 2007

ISSN 1651-6206
urn:nbn:se:uu:diva-7890 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-7890)
Here in this book
Some answers you’ll find
But alas! So many more questions...
List of publications

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


Reprints were made with the permission of the publishers.
Contents

Introduction...................................................................................................11
Historical notes on breast cancer and axillary surgery.......................11
The sentinel lymph node biopsy...............................................................13
Host immune response to malignant tumours .....................................14
CD3-ζ: the zeta chain of the T-cell receptor ........................................16
CD28: co-stimulatory receptor on T-cells .............................................17
The sentinel node biopsy in immunological research ..........................18

Aims of this thesis.........................................................................................20

Patients..........................................................................................................21
Paper I and II ............................................................................................21
Paper III ....................................................................................................21
Paper IV ....................................................................................................22

Methods ........................................................................................................23
Identification of sentinel node ...............................................................23
Pathological and immunological assessment .......................................23
Follow-up ..................................................................................................24
Definitions ...............................................................................................24
Statistical methods ..................................................................................25
Ethics ..........................................................................................................26

Results...........................................................................................................27
Paper I ......................................................................................................27
Paper II .....................................................................................................28
Paper III ....................................................................................................29
Paper IV ....................................................................................................29

Discussion.....................................................................................................31
Clinical aspects of the sentinel node biopsy .........................................31
Immunological aspects of the sentinel node biopsy ...............................34

Conclusions...................................................................................................37
Future perspectives .....................................................................................38

Sammanfattning på svenska..........................................................................40
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
</tr>
<tr>
<td>AR</td>
<td>Axillary recurrence</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CTLA</td>
<td>Cytotoxic T-lymphocyte-associated protein</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>FNR</td>
<td>False negative rate</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NSLN</td>
<td>Non-sentinel lymph node</td>
</tr>
<tr>
<td>PABAK</td>
<td>Prevalence Adjusted Bias Adjusted Kappa</td>
</tr>
<tr>
<td>SLN</td>
<td>Sentinel lymph node</td>
</tr>
<tr>
<td>SNB</td>
<td>Sentinel node biopsy</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TIL</td>
<td>Tumour-infiltrating lymphocytes</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>ZAP</td>
<td>Zeta-chain-associated protein kinase</td>
</tr>
</tbody>
</table>
Introduction

Historical notes on breast cancer and axillary surgery

The oldest known description of breast cancer was discovered on the *Edwin Smith Papyrus* in Egypt, dating back to approximately 1600 BC (Figure 1). The treatment applied to eight breast tumours or ulcers (the term *cancer* is not used) was performed with a tool called “the fire drill”, a type of cauterisation instrument. The writing, however, states “there is no treatment”. By the second century AD, the Roman doctor Galen (129-ca.200) advised surgical treatment for early, “superficial” breast cancer, which would comprise removal of all cancerous growth until only healthy tissue remained. Naturally, surgery in those days involved a substantial hazard to the patient’s life.

The understanding of the natural history of breast cancer rapidly grew after the discovery of the lymphatic system by Jean Pecquet (1622-1674), Thomas Bartholin (1616-1680) and Olof Rudbeck the Elder (1630-1702) in the early 1650s. The evolving concept that the cancer would spread via the regional lymph nodes seemed to suggest that locally extensive surgery might be a potential cure for breast cancer. Consequently, breast cancer operations that included excision of regional lymph nodes started appearing in the 17th and 18th centuries. The *radical mastectomy*, involving removal of the breast, the underlying chest muscles and the lymph nodes of the axilla, was finally described by William Stewart Halsted (1852-1922), professor of surgery at Johns Hopkins University, during the last decade of the 19th century (Figure 2).

*Figure 1. Edwin Smith Papyrus*  
*Figure 2. William S. Halsted*
Halsted’s radical mastectomy was to dominate breast cancer surgery far into the 1970s. It was claimed by some that an even more radical approach, additionally removing internal mammary and infraclavicular nodes, would further improve recurrence rates. However, the severe disfigurement created by these procedures also raised doubts as to whether such extensive surgery was in fact necessary. In 1948, Patey and Dyson described the modified radical mastectomy, omitting the removal of the chest muscles, yet still clearing the entire axilla. Unfortunately, none of the methods adopted were subjected to scientific evaluation at that time, and most of the argumentation was based on mere anecdotalism.

It is important to remember that the role of axillary surgery at the time of its development was purely therapeutic, as adjuvant treatments were lacking. Its significant morbidity, comprising chronic pain, arm lymphedema, sensory disturbances and limitation of arm mobility, was by many regarded acceptable as long as extensive surgery was believed to be the only treatment offering a potential for cure. But with the arrival of increasingly potent adjuvant drugs, the diagnostic significance of axillary surgery as a staging instrument gained in importance. In the late 1960s, Bernard Fisher formulated the hypothesis that breast cancer might be a systemic disease, the outcome of which would not merely depend on the extent of locoregional treatment. As a result, the B-04 clinical trial of the National Surgical Adjuvant Breast and Bowel Project (NSABP) was initiated in August 1971. So far, its 6-, 10- and 25-year results have been published: no survival benefit was reported for patients treated with radical mastectomy compared to those receiving total mastectomy (not including the removal of regional lymph nodes or chest muscles) with or without postoperative irradiation. Thus, it was suggested that a primary axillary dissection would not alter survival even in patients with positive axillary lymph nodes.

A parallel development in the 1970s was the introduction of population-based screening programs, resulting in a reduction of tumour size at the time of diagnosis. Due to the linear relation between tumour size and axillary involvement, the percentage of patients presenting with nodal metastases at primary operation started decreasing, too. Thus, a growing number of breast cancer patients were at risk of suffering the debilitating effects of axillary dissection without gaining any therapeutic benefit. Nevertheless, the prevalence and number of axillary lymph node metastases was, and is still today, one of the most important prognostic factors for disease recurrence and survival. Thus, accurate axillary staging was deemed a necessity, but the extent of surgery required to obtain it remained to be determined. At a time when quality of life issues were being given increasing attention, this was a dilemma in need of an innovative solution.

Attempts to limit axillary surgery evolved that aimed at accurate staging of the axilla without carrying the morbidity of a full axillary node dissection. In 1975, the pectoral node biopsy was described by Cant. Lymph nodes
found in the axillary tail during mastectomy without axillary clearance were hoped to represent the status of the remaining axilla, which nevertheless was shown to be inaccurate. Subsequently, other methods were developed, including several forms of *axillary sampling*, evaluating three\(^\text{15}\), four\(^\text{16}\) or five\(^\text{17}\) nodes from the axilla with varying reliability.

### The sentinel lymph node biopsy

The sought-for innovation came with the concept of the *sentinel node* (Figures 3 and 4). It is defined as the first lymph node receiving lymphatic drainage, and thus also metastasising cancer cells, on a direct pathway from the tumour site. The term *sentinel node* was already mentioned in a publication on tumours of the parotid gland in 1960\(^\text{18}\). However, the lymph node described then was simply defined by its regular appearance at the confluence of two facial veins. But already at that time, the concept was to use it as a "marker" lymph node evaluating the presence or absence of nodal metastasis. It was Cabanas in 1977 who first described the sentinel node biopsy (SNB) in patients with penile carcinoma\(^\text{19}\). Preoperative lymphangiograms were performed to locate the sentinel lymph node (SLN), and further nodal dissection was discouraged if the biopsy did not show any evidence of metastasis. At about the same time, Donald Morton and Alistair Cochran and their team at the John Wayne Cancer Institute in California began searching for the optimal staining procedure for the lymphatic system, which led to the presentation of a vital blue dye injection technique to the Society for Surgical Oncology in 1990. The news was received unenthusiastically to say the least\(^\text{20}\). The distinct scepticism was reflected in the rejection of the original manuscript describing the technique by several renowned journals, until it could finally be published in 1992\(^\text{21}\).

![Figure 3. The SNB technique](image1)

![Figure 4. Intraoperative identification](image2)
During the 1990s, numerous international studies aimed to validate the accuracy of the sentinel node technique, complementing it with a confirmatory axillary dissection. A false negative result was defined as a negative SNB followed by an axillary dissection revealing metastases in non-sentinel lymph nodes (NSLN), and the false negative rate (FNR) as the percentage of false negative cases among all node-positive cases. Results varied considerably: FNRs ranged from 0% to 40%, with a median FNR of 7% 22,23. In 2001, the Philadelphia Consensus Conference recognised the SNB procedure to be “a suitable replacement for axillary dissection as a staging and diagnostic procedure in T1 and T2 (usually ≤3 cm) breast cancers”. However, it was recommended that surgeons should perform a concomitant axillary dissection until they could document a detection rate of more than 95% and a FNR of less than 5% 24.

The impact on survival and regional control that the omission of further axillary surgery in sentinel node-negative patients might bring about has, as yet, not been evaluated in randomised clinical trials. A large multicentre trial (NSABP B-32) comparing the SNB as a single staging procedure with conventional axillary surgery is currently underway 25. One earlier randomised study 26 did not provide enough statistical power to compare both treatments adequately 27. As a surrogate parameter, the incidence of axillary recurrences (AR) in sentinel node-negative patients has been reported in numerous trials. After a relatively short median follow-up time (range 14-57 months), axillary recurrences seem to be rare events. AR rates range from 0% to 3.6% internationally 26,28-51. However, considering the slowly growing nature of breast cancer, long-term results will have to be awaited before determining the true failure rate of the staging procedure.

The applicability of SNB for tumours larger than 3 cm is a matter of debate. The initial FNR in breast cancer patients with T2 and T3 tumours reported from the Memorial Sloane-Kettering Cancer Center was as high as 18% 52. However, in a subsequent publication from the same institution this figure had dropped to 3% in T2 tumours 53, and it was suggested that the observed improvement reflected the growing experience with the method. Other series confirmed the accuracy of the SNB in large tumours 54-56. It was nevertheless pointed out that the higher incidence of axillary metastases in patients with larger tumours would inevitably lead to a higher absolute number of false negative cases, despite an invariably low false negative rate 57.

Host immune response to malignant tumours

The observation of immunological reactivity against malignant tumours dates back to the beginning of the 20th century. It was not until the 1950s and 1960s that more advanced techniques emerged, allowing more refined experimental testing of the processes observed. The term immunosurveillance
was postulated by Burnet in 1970\textsuperscript{58}, suggesting that it was one of the major roles of the host immune system to continuously fight the development of cancer. Even though this theory was soon rejected due to a lack of available evidence in its favour, it has undergone a renaissance in recent years\textsuperscript{59}. Lately, it has been proposed that both the adaptive and the innate immune system not only participate in preventing tumour evolvement but also play a role in shaping the immunogenicity of those tumours that eventually develop. This hypothesis has been denominated \textit{cancer immunoediting}\textsuperscript{60}. Three phases of the process have been suggested, namely “elimination” of evolving neoplastic cells, “editing” of those cells that survive the first line of defence, and finally “escape”, in which the tumour by several mechanisms eludes recognition and destruction by the host immune system\textsuperscript{61}. As the tumour escape pathways possibly constitute the major cause for the relatively disappointing clinical results that novel immunotherapies frequently yield, they represent one of the most challenging aspects of cancer immunology today\textsuperscript{59,62-64}.

The common conception of adaptive immunity against tumours suggests that signals from transformed cells cause the attraction and activation of antigen-presenting dendritic cells (DCs)\textsuperscript{65}. After antigen recognition, immature DCs will undergo maturational changes while migrating to the draining lymph nodes in order to elicit an appropriate T-cell response. Activated antigen-specific CD8\textsuperscript{+} T-cells will differentiate into cytotoxic T-cells with the potential to destroy tumour cells (Figure 5). Possible tumour escape mechanisms can involve any step of this process, from decreased tumour antigen presentation to abnormal expression of co-stimulatory or adhesion molecules by DCs, alteration in signal transduction molecules, and, ultimately, the induction of suppressive, tolerogenic or anergic T-cells\textsuperscript{59}.

Suppressed and dysfunctional immunoreactivity has been reported in several other types of cancer\textsuperscript{66}, frequently correlating with the stage of the disease\textsuperscript{67-69}. However, breast cancer was long regarded as being relatively non-immunogenic. Historically, the finding of enhanced sinus histiocytosis in the axillary lymph nodes was suggested to be a positive prognostic factor\textsuperscript{70,71}, but it could not establish itself against more powerful predictors such as axillary node status and primary tumour characteristics. While the occurrence of tumour-infiltrating lymphocytes (TIL) was shown to predict a better prognosis in malignant melanoma\textsuperscript{72}, ovarian\textsuperscript{73}, esophageal\textsuperscript{74} and colorectal cancer\textsuperscript{75}, conflicting data exist concerning breast cancer. One group reported the occurrence of TIL to be associated with a poor prognosis\textsuperscript{76}, while another found it a positive prognostic variable, at least in women aged under 40 years\textsuperscript{77}. However, no classification of different lymphocyte subsets was attempted, nor the activation status of the cells determined, which renders any interpretation difficult. With the refinement of immunological cellular essays, more evidence for immunosuppressive properties of breast cancer was gathered. The functional competence of peripheral T-lymphocytes was found
to be impaired in comparison to healthy donors\textsuperscript{78}. Natural killer cells (NK cells) were observed to be increasingly dysfunctional in more advanced disease stages\textsuperscript{79}. On peripheral blood T-lymphocytes, down-regulation of the immunological signalling molecules CD3-ζ (CD3-zeta), ZAP-70 and p56\textsuperscript{ck}, and a dysfunctional reaction to stimulation with interleukin-2 (IL-2) and anti-CD3 was described\textsuperscript{80}. Peripheral blood monocytes underexpress ICAM-1 (CD54), B7.1 (CD80) and B7.2 (CD86), and the production of tumour necrosis factor alpha (TNF-α) is decreased\textsuperscript{81}.

**Figure 5.** Host immune response to malignant tumours (schematic drawing)

**CD3-ζ: the zeta chain of the T-cell receptor**

The zeta-chain (CD3-ζ) is a transmembrane signalling molecule of the T-cell receptor (TCR; Figure 6) with a short extracellular component and a longer cytoplasmic tail containing three ITAMs (immunoreceptor tyrosine-based activation motifs). Because CD3-ζ mostly appears as a homodimer, two ζ chains will be found within one TCR complex, with six phosphorylation sites or ITAMs. CD3-ζ is essential for the transduction of T-cell activation signals provided by antigen-presenting cells (APC) such as dendritic cells. On ligation of the TCR, rapid tyrosine-phosphorylation of the ITAMs oc-
curs, serving as a binding site for the kinase ZAP-70, ultimately leading to the activation of transcription factors.

CD3-ζ is down-regulated in dysfunctional tumour-infiltrating and peripheral T-cells in several neoplastic diseases. This phenomenon is paralleled by suppressed expression of ZAP-70 and p56lck, decreased production of TNF, abnormal calcium flow through the cell membrane and increased T-cell apoptosis. Treatment with IL-2 can upregulate the expression of CD3-ζ in T-cells. The most emphasised down-regulation of CD3-ζ was observed in areas of tumour regression after immunotherapy of metastatic malignant melanoma. Several studies point towards a correlation between the decreased expression of CD3-ζ, tumour progress and shorter survival. Additionally, it may prove a relevant prognostic marker for the response to immunotherapy.

Figure 6. T-cell receptor (TCR) before (left) and after phosphorylation (right)

CD28: co-stimulatory receptor on T-cells

CD28, a member of the immunoglobulin family, is found on the surface of T-lymphocytes, while its natural ligands, B7.1 (CD50) and B7.2 (CD54), amongst others, are expressed on antigen-presenting cells (Figure 7). In healthy individuals, CD28 is detected on 95% of CD4+ T-cells but only on 50% of CD8+ T-cells. The expression of CD28 increases with activation. Its ligation constitutes an essential part of T-cell activation by providing a
co-stimulatory signal during antigen/MHC complex presentation. CD28’s counterpart, the receptor molecule CTLA-4, delivers an inhibitory signal, thereby limiting its effects98. CD28 ligation lowers the threshold for T-cell activation, and enables a sustained T-cell proliferation by means of an increased IL-2 production99. If no co-stimulatory signal is provided, the interaction between T-cell and APC will lead to only a transient proliferative response, clonal anergy, or deletion100,101.

Figure 7: The interaction between APC and T-cell

In breast cancer, a significantly smaller percentage of peripheral T-lymphocytes are CD28+ compared to healthy controls102. In colorectal cancer, tumour-infiltrating T-lymphocytes lack CD28 expression in contrast to T-cells in healthy colon interstitium103. In metastatic malignant melanoma, T-cells exhibiting the profoundest down-regulation of CD28 are located in areas of tumour regression after biochemotherapy104. In chronic lymphocytic and hairy cell leukaemia, CD28 expression on dysfunctional peripheral T-cells is diminished in comparison to healthy individuals105,106.

The sentinel node biopsy in immunological research

The host immune response to tumours is initiated in regional lymph nodes, more precisely in the sentinel lymph node (Figure 8). Here, antigen-presenting cells arriving from the tumour site initiate clonal T-cell proliferation, which predominantly takes place in the T-cell areas of the lymph node. Most studies on the immunological status of the sentinel node concern malignant melanoma patients, describing reduced expression of dendritic cell
maturational markers and their corresponding T-cell receptors, possibly due to the increased production of several immunosuppressive cytokines (reviewed in 107). In breast cancer sentinel lymph nodes (SLN), cell populations expressing B7.1, B7.2, CD40 and HLA-DR are decreased compared to non-sentinel lymph nodes (NSLN)108. While the latter study used flow cytometry analysis, it might be even more valuable to perform immunological analyses on tissue sections to enable differentiation between functionally distinct areas within the nodal architecture. This type of research has long been embraced by Alistair Cochran and colleagues109-112. In breast cancer, the paracortical area and the density of paracortical dendritic cells in SLNs is decreased in comparison to NSLNs, with a predominance of immature dendritic cells113. Thus, immunological functions seem to be more disturbed in SLNs than in NSLNs, a distance-related impact that might suggest the existence of tumour-derived immunosuppressive signals reaching the SLN via the lymphatic system.

Figure 8. Schematic drawing of the lymph node structure
Aims of this thesis

- To study and describe the expression of the zeta chain (CD3-ζ) of the T-cell receptor in different areas of the sentinel lymph node from breast cancer patients.

- To study and describe the expression of the co-stimulatory molecule CD28 on CD8+ and CD4+ T-cells in the sentinel lymph node and correlate its distribution with that of the zeta chain (CD3-ζ).

- To determine the false negative rate and detection rate of the sentinel node procedure for breast cancer larger than 3 cm.

- To report the current axillary recurrence rate and disease-free survival in sentinel node-negative patients in whom no axillary clearance was performed.
Patients

Paper I and II
Twenty-five female patients with palpable, unifocal, invasive breast cancer less than 3 cm in diameter underwent sentinel node biopsy at the Central Hospital in Västerås, Sweden, as part of their surgical treatment. Neoadjuvant therapy, ongoing pregnancy, palpable axillary lymph nodes suggestive of nodal metastases and known allergy to blue dye or isotope were exclusion criteria. In some sentinel node sections from two patients, tumour growth was too abundant to allow evaluation of the remaining lymphatic tissue. In paper I, one of these patients had to be excluded from analysis, and in paper II, the specimens of both patients were affected. In another two patients, the technical quality of sections stained for CD28 expression was too poor to permit immunological assessment. Thus, nodal sections from 24 and 21 patients (paper I and paper II, respectively) could be immunologically analysed.

Paper III
Patients with invasive breast cancer larger than 3 cm were identified from the national sentinel node database in Sweden. Only patients who were planned for a sentinel node biopsy followed by an axillary dissection of levels I and II, regardless of the outcome of the sentinel node biopsy, were eligible. Apart from the difference in tumour size, exclusion criteria for papers I and II applied. Overall, we could identify 109 patients from the database. Twenty-three cases derived from the initial SNB learning phase and 68 from the SNB validation phase. After introduction of the SNB as a single staging procedure, patients with tumours larger than 3 cm were not routinely operated with SNB in Sweden. Therefore, an additional 18 patients with such tumours were recruited between 2004 and 2006. Operations were performed by 28 surgeons of all levels of experience at 20 Swedish hospitals.
Paper IV

Between September 2000 and January 2004, 3534 patients with invasive, clinically node-negative breast cancer ≤ 3 cm were prospectively enrolled in a multicentre study for sentinel node biopsy as a single staging procedure. Twenty-six Swedish hospitals and 131 surgeons contributed to patient accrual. Patients with ongoing pregnancy, known allergy to the blue dye or isotope used during the SNB procedure, previous breast surgery or preoperatively diagnosed multifocal disease, and those requiring neoadjuvant treatment, were not eligible. As the aim of the analysis was to evaluate the outcome of the SNB as the only axillary staging procedure, only sentinel node-negative patients who did not undergo a completion axillary dissection were included. Furthermore, patients in whom the postoperative pathology examination found a tumour size larger than 3 cm, benign or in situ disease only, or who were followed up outside Sweden, were excluded (Figure 9). Thus, the final analysis comprised 2246 patients.

![Flow-chart for selection of patients for paper IV.](Figure 9)

Initially considered for inclusion
N = 3534

- Benign (3) or in situ (68) disease only, tumours > 30 mm (127)
  N = 198
- Sentinel node not identified
  N = 101
- Sentinel node biopsy positive
  N = 906
- Miscellaneous reasons for exclusion, e.g. patient followed up outside Sweden
  N = 20
- Axillary dissection performed due to multifocality, palpable nodes, isotope activity, patient preference, non-sentinel metastatic lymph nodes; N = 63

Included in the present analysis
N = 2246
Methods

Identification of sentinel node

Radioactive isotope (40-60 MBq Technetium-99 nanocolloid, Solco Nanocoll®; Nycomed, Amersham, UK) was injected peritumourally, sub- or intracutaneously, followed by gentle massage of the area. Injection techniques could vary between participating institutions. For non-palpable tumours, mammographic guidance was used. Preoperative lymphoscintigraphic images were most frequently obtained 5 and 45-60 minutes after injection, and repeated after 2-3 hours if no sentinel node had been identified before. In a similar fashion, 1 ml blue dye (Patent Blue V®; Guerbet, Paris, France) was injected 5 to 15 minutes before incision, and the area gently massaged.

During surgery, sentinel lymph nodes were identified by a handheld gamma probe and/or by their uptake of blue dye. Hot and/or blue nodes were defined as sentinel nodes and sent fresh to pathology. In the event that no sentinel node could be detected, a routine axillary dissection of anatomical levels I and II was performed. Part of the sentinel node procedure was also to palpate the axilla thoroughly in order not to miss lymph nodes suspicious of metastasis that nevertheless had accumulated neither isotope nor colour. Only in paper IV was axillary dissection omitted in case the sentinel node biopsy was found to be negative. In the remaining studies, axillary clearance was performed regardless of the result of the SNB.

Pathological and immunological assessment

Frozen sections were obtained from the sentinel nodes and examined during operation. Nodes larger than 4 mm in diameter were bisected and at least one or two sections obtained from each part. For definitive pathology, at least three sections were taken from each sentinel node or each part of the bisected node. Staining was performed with haematoxylin and eosin (HE), and, if no metastatic cells could be identified, with additional immunohistochemical staining (IHC) using cytokeratin antibodies. Non-sentinel lymph nodes were only evaluated on routine HE sections.

For papers I and II, additional snap-frozen sections were obtained and sent to Linköping University for immunological double-staining. After fixation and blocking against unspecific binding, the first staining was per-
formed with primary antibodies against CD3 (*papers I and II*), CD4 and CD8 (only *paper II*). DAB (3,3’-diaminobenzidine, D-5637, Sigma, Stockholm, Sweden) was used as a substrate to obtain a brown colour. Negative control slides were manufactured using mouse IgG (Sigma, Stockholm, Sweden). To detect the expression of CD3-ζ and CD28, tissue sections were then incubated with mouse monoclonal antibodies to these substances. The technique used resulted in a bright red staining for CD3-ζ and CD28. Thus, the higher the expression of these molecules was, the more would the red colour dominate, while areas of low expression would appear brown.

The expression of CD3-ζ and CD28 was differentially evaluated in three areas of the sentinel node: the primary follicles and germinal centres (B-cell activation areas) and the paracortex (T-cell activation area). For *paper II*, the expression of CD28 in CD4+ and CD8+ T-cell subsets was compared. Whole sections were always analysed. Slides were assessed in a semiquantitative manner by two investigators (A.H., B.G). The percentage of positive cells in each area was estimated, and the degree of expression of CD28 and CD3-ζ determined *high* (more than 75% positive), *moderate* (50-75% positive) or *low* (less than 50% positive).

**Follow-up**

In *paper IV*, follow-up after primary operation was scheduled annually. Even though both clinical examination and mammography had been postulated in the study protocol, the method of follow-up could vary between participating centres. A few institutions reported telephone interviews combined with self-examination or mammography without any clinical examination. All follow-up data were reported to the study database at the Centre for Clinical Research of Uppsala University at the Central Hospital in Västerås. Before the present data analysis was conducted, a complete list with all included patients per institution was sent out to each centre for an updated report of unfavourable events and latest follow-up dates. Additionally, one of the authors (J.d.B.) was granted access to hospital files at on-site visits for data validation.

**Definitions**

The *false negative rate* is the percentage of sentinel node-negative patients among all axillary lymph node-positive patients. The *detection rate* is defined as the percentage of procedures in which a sentinel node could be successfully retrieved among all attempted sentinel node procedures.

In *paper IV*, any recurrences diagnosed within the same 2-month interval were regarded as concurrent to each other. Axillary recurrences were defined
as isolated if the axilla was the sole initial site of relapse. The definition of a locoregional axillary relapse comprises the above, and additionally, those patients in whom a local relapse in the ipsilateral breast was found prior or concurrently to the axillary relapse. The overall axillary relapse group additionally included those patients in whom systemic and/or extra-axillary lymphatic metastases were detected prior to, or concurrently to, the axillary recurrence. A local recurrence was defined as a new cancer in the breast on the previously operated side.

Statistical methods

In papers I and II, the Mann-Whitney U test was used for analysis of correlations between tumour and patient characteristics and the expression of CD28 and CD3-ζ. For comparison of the expression of the same marker in different areas of the same sentinel lymph node, the Kruskal-Wallis test was applied. The same is true for comparison of the CD28 expression in the CD4+ and CD8+ T-cell subgroups.

The expression of CD28 and CD3-ζ within the same area of the lymph node was compared using the Prevalence-adjusted Bias-adjusted Kappa (PA-BAK). It is interpreted like Cohen’s Kappa\(^\text{\textsuperscript{114}}\). Values indicate excellent (> 0.80), good (0.61-0.80), moderate (0.41-0.60), fair (0.21-0.40) and poor agreement (0.00-0.20). A value below zero suggests an agreement that is less than expected by chance.

In papers III and IV, the impact of individual tumour and patient characteristics on detection and false negative rates (paper III) and on recurrence and survival rates (paper IV) was analysed performing univariate logistic regression. To study each significant factor’s relative impact on those parameters, multivariate logistic regression was used. For survival analysis, the Kaplan-Meier and Cox proportional multiple regression models with 95% confidence intervals (CI) were applied. For overall survival, all causes of death were included, whereas in the calculation of breast cancer specific survival, death from breast cancer was the endpoint, and dates of death from other causes or last follow-up were censoring dates. For disease-free survival, the time to any first recurrence was calculated.

For paper IV, the null hypothesis was that the 5-year axillary recurrence rate would be ≥ 3% (H\(_0\): p ≥ 0.03). This figure was based on the published 5-year axillary recurrence rate of 1% after negative axillary dissection in Sweden\(^\text{\textsuperscript{115}}\). Taking into account the benefits of decreased morbidity that the sentinel node method offers in comparison to conventional axillary clearance, a 5-year axillary recurrence rate of less than 3% in sentinel node-negative patients was regarded as acceptable. It was calculated that the inclusion of at least 2000 patients was necessary to reject H\(_0\) with a power of 87%, if a true
axillary recurrence rate of 2% at 5 years was assumed. With true axillary recurrence rates falling below 2%, statistical power would quickly increase.

For all analyses, a $P$ value less than 5% was regarded significant. The SPSS® (SPSS Inc., Chicago, Illinois) program was used throughout this thesis.

Ethics
The study designs were approved by the ethics committees at the University of Uppsala and the University Hospital of Linköping (papers I and II), at the Karolinska Institutet, Stockholm (paper IV), and the local ethics committees of each participating region in papers III and IV.
Results

Paper I

Sections of 24 sentinel node biopsies were available for analysis. Expression varied considerably comparing different patients’ tissue samples but also comparing different functional areas within the same sentinel node (Figure 10).

In the B-cell follicles, where B-cell activation takes place and germinal centres appear once antigen confrontation leads to clonal proliferation, the degree of down-regulation was generally low. Only in 18 patients, sufficient numbers of germinal centres were found to allow immunological assessment. In the majority of these cases, CD3-ζ expression was notably suppressed. In the paracortex, the main T-cell activation area of the lymph node, the strongest down-regulation was found (Table 1). No correlations to tumour histopathological parameters or clinical data were identified. Statistical analysis of subgroups was not deemed meaningful due to small sample size.

Figure 10. CD3-ζ expression in germinal centers (upper row) and paracortex (lower row) of the sentinel lymph node. Low (left side), moderate (upper right) and high (lower right) degree of CD3-ζ down-regulation.
Table 1. Down-regulation of CD3-\(\zeta\) in different areas of the sentinel lymph node from 24 breast cancer patients. Numbers signify cases (percent).

<table>
<thead>
<tr>
<th>Degree of down-regulation</th>
<th>B-cell follicles (primary follicles)</th>
<th>Germinal centers (secondary follicles)</th>
<th>T-cell activation area (paracortex)</th>
</tr>
</thead>
<tbody>
<tr>
<td>low</td>
<td>14 (58)</td>
<td>3 (17)</td>
<td>1 (4)</td>
</tr>
<tr>
<td>moderate</td>
<td>4 (17)</td>
<td>2 (11)</td>
<td>4 (17)</td>
</tr>
<tr>
<td>high</td>
<td>6 (25)</td>
<td>13 (72)</td>
<td>19 (79)</td>
</tr>
</tbody>
</table>

**Paper II**

Sections of 21 sentinel node biopsies could be analysed for the occurrence and distribution of CD28+ T-cells within the nodal architecture. The subset of CD8+ T-cells generally showed a markedly higher CD28 expression than CD4+ T-cells. This difference was especially emphasised in the paracortex and the germinal centres, where CD28 expression on CD4+, but not on CD8+ T-cells, was strongly down-regulated \((P < 0.001)\). This difference was not notable in the B-cell areas, the primary follicles, where both T-cell subsets showed the least degree of CD28 down-regulation. In comparison with CD3-\(\zeta\) expression in the same areas of the same biopsies, the agreement between both markers was highest in the paracortex, reaching a PABAK value of 0.62 (Table 2). However, this was only true for CD4+ T-cells. It was confirmed that those CD3+ T-cells expressing low levels of CD3-\(\zeta\) in paper I represented mostly CD4+ T-cells.

Table 2. The paracortex of the sentinel lymph node: degree of CD28 expression on CD4+ T-cells in comparison with CD3-\(\zeta\) expression (depicted are 20 samples in which both staining modalities were analysable)

<table>
<thead>
<tr>
<th>Expression of CD3-(\zeta)</th>
<th>Expression of CD28 on CD4+</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>High</td>
<td>0</td>
</tr>
<tr>
<td>Moderate</td>
<td>0</td>
</tr>
<tr>
<td>Low</td>
<td>0</td>
</tr>
</tbody>
</table>
As in paper I, no correlations to tumour histopathological parameters or clinical data were found. Statistical analysis of subgroups was not performed considering the small sample size.

Paper III

The tumour size of 109 breast cancer patients identified from the sentinel node database ranged from 3.1 to 8.0 cm (median 4 cm), including 94 T2 (86%) and 15 T3 tumours (14%). The sentinel node could be identified in all but 6 cases, resulting in a detection rate of 94.5%. Axillary lymph node metastases were found in 67 patients, of whom the sentinel node had not been identified in 3 cases. Eight of the remaining 64 patients had a negative sentinel node biopsy, resulting in a false negative rate of 12.5% (8/64). Most of these cases occurred during the earlier years of the inclusion period, but possibly due to the small numbers in the subgroups, this observation was not statistically significant. The false negative rate did not differ between individual surgeons or contributing institutions.

On postoperative pathological examination, multifocal tumour growth was detected in 16 cases. These had not been diagnosed by preoperative investigations. It was noted that the false negative rate among patients with multifocal tumours (30.8% (4/13)) was significantly higher than in the remaining patients with unifocal disease (7.8% (4/51); \( P = 0.012 \)). At the same time, the prevalence of nodal metastases appeared to be increased (81% (13/16) versus 58% (54/93); not significant). On multiple regression analysis, multifocality was the only significant prognostic factor for a false negative biopsy (\( P = 0.026 \)).

Paper IV

After a median follow-up time of 37 months (range 0-75), the axilla was the sole initial site of relapse in 13 cases (13/2246; 0.6%). In 7 cases, axillary recurrence was combined with local recurrence in the ipsilateral breast, and in a further 7 cases, it occurred together with a generalisation of the disease. Thus, 27 cases of clinically detectable axillary recurrence were identified (27/2246; 1.2%). The median time from operation to axillary relapse was 21 months (range 4-51). The estimated cumulative incidence of isolated axillary recurrence at 5 years was 1.1% (95% CI 0.2-1.9; see Figure 11). On multiple regression analysis, the only significant risk factor for axillary relapse was Elston histological grade (\( P = 0.006 \)). The results of the contributing institutions and individual surgeons did not differ significantly.

Overall, 117 recurrences in 91 patients were reported. Seven extra-axillary nodal recurrences and 55 cases of distant relapse were found. After
breast-conserving treatment, 27 local recurrences were diagnosed, and one patient developed a chest wall recurrence after mastectomy.

Cancer-related death occurred in 38 cases. Even here, the only significant prognostic factor on Cox proportional regression analysis was Elston histological grading ($P = 0.003$). Overall 5-year survival was 91.6%, disease-free 5-year survival 92.1% and breast cancer-specific 5-year survival 94.7%.

Figure 11. Cumulative isolated axillary recurrence-free survival after negative sentinel node biopsy with 95% confidence interval.
Discussion

Clinical aspects of the sentinel node biopsy

It has been a long journey from the first anecdotal, hardly auspicious attempts at breast cancer treatment via Halsted’s era of extensive and debilitating surgery to our time, embracing the principle of best possible cure while respecting the patient’s quality of life, functionality and cosmesis. The importance of the patient’s perspective on treatment modalities has steadily grown since the end of World War II, a trend that has not only led to the development of breast-conserving treatment but also to a general trend towards less invasive surgery. Another seminal step forward during the last century was that from “non-science to science”\textsuperscript{2}: the insight that scientific hypotheses could be tested by means of randomised clinical trials, resulting in the principles of evidence-based medicine. Potent adjuvant drugs were found to increase survival in both node-negative and node-positive breast cancer patients\textsuperscript{116}, further eroding the surgical monopoly. The development of the sentinel node biopsy concept is one of the most enthralling aspects of these historical paradigm shifts in surgical science.

The application of the sentinel node method was adopted at such a speed that it has all but replaced conventional axillary clearance in early breast cancer today. After a period of early validation studies, determining the rate of false negative results by performing a subsequent confirmatory axillary clearance, the sentinel node method has been recommended as a single axillary staging method in biopsy-negative patients, provided that the false negative rate would not exceed 5\%\textsuperscript{24}. Yet once axillary clearance is omitted, only the occurrence of clinically detectable axillary relapse will give us a clue about potentially false negative procedures, and will thus be a surrogate quality indicator for the axillary surgery performed. It is important nevertheless to stress that the axillary recurrence rate is not equivalent to the false negative rate, as has been suggested in some publications, because this would underestimate the true figure significantly\textsuperscript{117}. Additionally, not every nodal metastasis left behind in the axilla will necessarily develop into clinically overt disease\textsuperscript{5,118-120}. The axillary recurrence rate after a negative sentinel node biopsy should therefore be compared to the same rate after conventional axillary clearance in node-negative patients. The first sufficiently large randomised trial of that kind is underway and will hopefully contribute to further clarification on the subject\textsuperscript{25}.
In the meantime, axillary recurrence rates (range 0-3.6%) following a negative sentinel node biopsy seem promisingly low. However, most current publications derive from expert multidisciplinary teams in specialised high-volume centres, and only one multicentre study including five surgical departments and 479 patients could be identified\(^1\). At the same time, an own literature research of early validation studies revealed a considerable difference between false negative rates from single institutions (6.9%, range 1-13.7%; 14 studies) and multicentre trials (10.9%, range 7.2-22%; 11 studies). It is therefore important to study the axillary recurrence rate in a setting where even small community hospitals with only a few cases per year are represented, so that results may truly reflect the heterogeneous situation that prevails in most countries. In paper IV, six of 25 hospitals accrued less than 15 cases to the overall study, and 72 of 131 surgeons performed less than 10 procedures each. Nevertheless, the axillary recurrence rate observed lies well within the range of the above stated international publications.

There is reason to believe that not every axillary relapse after a negative sentinel node biopsy signifies failure of the axillary staging method. When a local recurrence in the operated breast is previously or concurrently found, the axillary recurrence is likely to be a consequence of the new breast cancer rather than of inadequate axillary surgery. It can also be disputed whether an axillary relapse coinciding with distant disease spread should be regarded as a surgical treatment failure or as a result of particularly aggressive primary tumour properties. Thus, isolated axillary recurrences appear to be the only reliable quality indicator of the sentinel node biopsy. In paper IV, axillary relapse was therefore analysed in the categories isolated, locoregional, and overall. The cumulative 5-year incidence of locoregional axillary relapse of 1% after negative axillary clearance in an unselected Swedish population\(^{115}\) is included in the 95% confidence interval (0.7-2.6) of the corresponding figure of 1.6% after negative sentinel node biopsy.

Even before the sentinel node era, axillary recurrences were a relatively rare event, occurring in approximately 1-3% of breast cancer patients 5 years after adequate axillary node dissection\(^{115,121,122}\). Accordingly, there are few studies on the treatment and outcome of axillary relapse. The subsequent distant failure rate of patients with axillary recurrence was 50% in a series reported by Newman et al.\(^{123}\). In a Dutch study, 44 of 59 patients who had been diagnosed with an axillary recurrence subsequently died, having developed distant metastases in 36 cases\(^{124}\). Survival rates after diagnosis of regional lymph node recurrence were 72% and 43% at 5 and 10 years, respectively, compared to 91% and 81% for patients without recurrence\(^{125}\). In British Columbia, 220 breast cancer patients with isolated axillary recurrences had an overall 5-year survival of 49.3% and a median survival time of 4.9 years calculated from the date of relapse\(^{126}\). On the opposite scale lies the significant reduction of postoperative morbidity following the sentinel node procedure compared to conventional axillary clearance\(^{127,129}\).
It is important to identify those patients who might be put at risk of incorrect staging, and thereby inadequate adjuvant treatment, by the omission of a conventional axillary clearance. To this end, it is necessary to determine risk factors for a false negative sentinel node biopsy, but also predictors for the prevalence of axillary metastases: a higher risk of axillary metastases will yield a higher absolute number of false negative cases in spite of an unchanged false negative rate. Factors suggested to increase the incidence of false negative biopsies include histological grade of the tumour, tumour size, tumour multifocality, inexperience with the method, upper outer quadrant tumour location, and a low number of retrieved sentinel nodes. Several of the mentioned tumour characteristics constitute predictors for axillary node metastases as well. Thus, the potential benefit from the sentinel node procedure might decrease with an accumulation of such risk factors.

In the case of breast cancer larger than 3 cm, paper III confirms findings from earlier publications, showing a false negative rate and detection rate comparable to those of early validation studies limited to smaller tumours. However, patients with postoperatively diagnosed tumour multifocality revealed a higher false negative rate than the remaining patients with unifocal disease. Even though the study had not been designed for such a subgroup analysis, these findings warrant further investigation. There is some controversy about the validity of the sentinel node biopsy in multifocality. While some studies found multifocality to be a risk factor for a false negative biopsy, other groups have reported the contrary. Multifocality and multicentricity are still widely regarded as contraindications for the sentinel node procedure, a recommendation that might have to be further evaluated.

One problem when dealing with tumour multifocality is the wide variety or absence of its definition in publications on the subject. Additionally, the common practice of putting the diameter of the largest focus on a par with overall tumour size might underestimate the real situation considerably. However, even the use of tumour surface area or tumour volume may not fully account for the increased prevalence of axillary metastases in multifocal cancer, and altered biological tumour properties have been suggested as a possible explanation. Especially in larger tumours, the effect of multifocality on the false negative rate is little studied, due to the exclusion of larger tumours from most early validation studies. Therefore, it seems appropriate to consider a completion axillary clearance in those cases where large tumour size and multifocality coincide, until stronger evidence is available.
Immunological aspects of the sentinel node biopsy

The findings described in papers I and II confirm earlier observations of decreased expression of T-cell co-stimulatory and signalling molecules in patients with malignancies. The most profound down-regulation of CD3-ζ and CD28 was found in germinal centres and in the main T-cell activation area, the paracortex. In the latter, the agreement between both parameters was strongest.

The first encounter between antigen-presenting dendritic cells (DCs) and naïve T-cells is assumed to take place in the paracortex of lymph nodes regional to neoplasms. This is supported by the observation that antigen-specific T-cells first accumulate in the paracortex after subcutaneous antigen injection, thereafter moving on to lymph node follicles. Most studies investigating the immunology of lymph nodes regional to malignant melanoma and breast cancer derive from Cochran et al., who described the strongest evidence of immunological perturbation in the paracortical area of sentinel lymph nodes. In breast cancer patients, the relative portion of the lymph node occupied by paracortex is significantly smaller in sentinel (SLN) compared to non-sentinel lymph nodes (NSLN), and the number, density and maturity of DCs in these areas is decreased. These findings suggest that the immunology of regional lymph nodes is influenced by their position relative to the primary tumour. Interestingly, those lymph nodes located closest and those furthest away from the tumour show less immunological reactivity than “intermediate” nodes. It was hypothesised that the latter nodes were too far from the tumour to be immunosuppressed, but close enough to be exposed to immunostimulatory tumour antigens.

The apparently distance-related impact on host immunoreactivity both within the nodal architecture and the regional network of lymph nodes suggests the existence of tumour-derived signals modulating the immune response. Findings in patients with colorectal cancer support this theory. In ovarian cancer, a circulating 16kD protein isolated from ascites fluid was found responsible for the nearly complete selective suppression of CD3-ζ. Otsuji et al. found that tumour-derived macrophages in mice exerted oxidative stress on T-cells, resulting in suppression of CD3-ζ. This effect could be imitated by oxidative agents like hydrogen peroxide and diamide, prevented by treatment with the antioxidant N-acetylcysteine, and reversed by macrophage deletion. A second murine study reported that sustained cell-contact with activated macrophages was necessary to induce CD3-ζ suppression. Further possible pathways of host immunosuppression include the presence of IL-4, TGF-β and soluble HLA class I molecules.

It has been suggested that the CD4+ and CD8+ T-cell subsets react differently to co-stimulatory signals via the CD28/B7 pathway. The T-cell response to B7.1-dependent co-stimulation is prolonged in the CD4+ subset, featuring IL-2 secretion and clonal expansion. In the CD8+ subset, however,
the helper-independent response is short-lived. Additionally, CD8+ T-cells require higher levels of B7.1 for an activation equivalent to CD4+ T-cells. Thus, the degree of expression of B7.1 on the antigen-presenting cell might influence the development of helper or cytotoxic T-cell responses. In papers I and II, the degree of CD28 down-regulation was far more pronounced in CD4+ than in CD8+ T-cells. Similar observations were reported from metastases to malignant melanoma after biochemotherapy: both CD4+ and CD8+ T-cells showed a marked loss of CD28 in areas of tumour regression while CD28 expression was hardly decreased close to unaffected tumour growth, and the down-regulation of CD28 was generally more emphasised in CD4+ than in CD8+ T-cells. This appears contrary to the differential expression of CD28 on peripheral blood T-cells in healthy individuals. In papers I and II, the vast majority of T-cells in the area of strongest immunological perturbation, the paracortex, were CD4+, and thus, the loss of both CD28 and CD3-ȗ might mainly concern the CD4+ subset. Progressive loss of CD28 has been described in CD4+ and CD8+ T-cells in chronic inflammation and replicative senescence. It has also been hypothesised that this situation might be similar in cancer patients, where long-term exposure to tumour antigens leads to chronic stimulation – which may lead our thoughts back to the theory of cancer immunoediting and its three phases described in the introduction of this thesis.

There is evidence for close interactions between CD28 and CD3-ȗ. The tyrosine phosphorylation of CD3-ȗ and ZAP-70, as well as their association, is one of the earliest signalling events after TCR engagement, and is strongly inhibited in the absence of a co-stimulatory CD28/B7-mediated signal. ZAP-70 activation, on the other hand, might lead to phosphorylation of the cytoplasmic CD28_pYMXM motif which would in turn allow the binding of phosphatidylinositol 3-kinase, thereby augmenting the second signal provided by CD28/B7 interaction. The apparent link between CD28 and CD3-ȗ functions seems to be reflected in the findings of papers I and II, where the agreement between both molecules was strongest in areas of the most marked down-regulation.

Although it is undisputed that CD28 engagement delivers potent positive co-stimulatory signals for T-cell activation, it has been shown that this second signal is not always obligatory. Interestingly, especially in the context of the differences of CD28 expression in CD4+ and CD8+ T-cells, the latter subset was reported to be less dependent on CD28 co-stimulation (reviewed in ). Furthermore, the requirement of CD28 signalling appears to be dependent on the strength and the duration of antigen presentation. Another striking feature of CD28 co-stimulation is the CD28-dependent up-regulation of the inhibitory molecule CTLA-4 (CD152) on the cell surface, which binds the same ligands as CD28 albeit with a much higher affinity, and antagonises most major CD28-mediated events. While the absence of a co-stimulatory signal from CD28/B7 may lead to T-cell anergy, clonal
deletion and the differentiation of T-cells into a Th2 phenotype, the lack of a negative signal from CTLA-4/B7 results in unregulated T-cell expansion. Hence, the CTLA-4/B7 pathway has emerged as a pivotal regulator mechanism in the induction and maintenance of peripheral tolerance in autoimmune disease (reviewed in 155).

Truly successful treatment against cancer implies the deletion of the very last remaining malignant cell and necessitates a functioning host immune response. However, cytotoxic drugs are unlikely to enhance natural defence mechanisms. There is an abundance of ongoing research evaluating different approaches to immunological treatment regimens in breast cancer (reviewed in 156-159). One already established successful agent is the humanised monoclonal antibody trastuzumab (Herceptin®) for breast cancer overexpressing HER2. However, there remains a large proportion of patients who will not respond to immunological therapies. Based on the heterogeneous immunological patterns observed in papers I and II and other reports, one might hypothesise the existence of distinct tumour types differing in the immune responses they evoke. While a high expression of CD28 or CD3-ζ might reflect a non-immunogenic tumour, not recognised by the host immune system, or else an immunogenic, but non-immunosuppressive tumour, a significant down-regulation of immunoreactivity might be due to immunosuppressive tumour characteristics. As metastases were found both in strongly and in less down-regulated lymph nodes, this could be explained by either non-immunogenic tumours surpassing the immune system without being recognised, or immunosuppressive tumours “overriding” the immunological defence. Thus, the differential information available from the immunological analysis of the sentinel lymph node might play an important role for the selection of patients likely to benefit from immunological treatment approaches.
Conclusions

In this thesis, clinical and immunological aspects of the sentinel node biopsy (SNB) were studied.

In breast cancer larger than 3 cm, the SNB technique was shown to represent the status of the axillary lymph nodes with a false negative rate (FNR) and detection rate that are comparable to early validation studies including smaller tumours. However, when large tumour size coincided with tumour multifocality, the FNR was increased. The SNB can thus be safely performed in unifocal breast cancer larger than 3 cm. If tumour multifocality is diagnosed in these patients, however, a completion axillary dissection should be considered until further data on SNB in multifocal disease are available.

The omission of a subsequent axillary dissection in sentinel node-negative breast cancer patients yields overall axillary recurrence rates ranging from 0% to 3.6% internationally. In the Swedish multicentre cohort trial, the isolated axillary recurrence rate was 0.6% (13/2246) after a median follow-up of 37 months. These results encourage the use of the SNB as a single staging procedure even in a heterogeneous multicentre setting. However, long-term data will have to be awaited before the definitive failure rate of the SNB procedure can be determined.

The sentinel lymph node (SLN) is not only the first node on the lymph drainage pathway to harbour metastatic cancer cells, but also the showplace of immunological changes reflecting host immunoreactivity against the tumour. The expression of the $\gamma$ chain of the T-cell receptor and of the costimulatory molecule CD28 were found to be down-regulated to a varying extent. The most pronounced loss of both T-cell parameters, and the strongest agreement between their respective degrees of down-regulation, were observed in the main T-cell activation area, the paracortex. These findings might have prognostic implications and aid the selection of patients who are likely to benefit from immunological treatment approaches.
Future perspectives

Even though seminal advances in breast cancer research have been made during the last century, in terms of understanding its nature, developing less invasive albeit no less accurate axillary staging methods, broadening the treatment repertoire and improving survival, a substantial number of patients will still die of their disease, despite all therapeutic efforts. In metastatic disease, surgery is hardly likely to provide survival benefits. However, as cytotoxic drugs act according to the first order of kinetics, they will not be able to extinguish the very last cancer cell and thus cure the patient. Therefore, immunological treatment approaches are sought for, and one of the first successful results of this search is the monoclonal antibody trastuzumab for HER2 positive breast cancer. Further drug development is underway. However, there are still a substantial number of patients who will not respond to immunotherapy. To understand the mechanisms by which a tumour can escape the host immune system, and thus also evade the effects of immunotherapy, is one of the greatest challenges in cancer immunology today.

The first two papers of this thesis aimed to add to the understanding of the regional immunodeficiency frequently observed in cancer. Further research should involve the opposite side of the immunological synapse, namely the antigen-presenting dendritic cells. While the morphology and distribution of those cells is well documented in the sentinel lymph nodes from breast cancer patients, their functional properties are not yet sufficiently understood. The analysis of activation parameters and co-stimulatory receptors will help to shed light on the intricate interactions taking place in the regional lymph nodes. Furthermore, understanding the immunological impact of the lymph nodes’ position relative to the primary tumour will contribute with new information about the mechanisms of cancer-related immunodeficiency.

The adoption of the sentinel node concept has clearly decreased postoperative morbidity and improved the quality of life for node-negative breast cancer patients. However, numerous questions remain to be addressed in clinical trials. The third and fourth paper of this thesis aimed to make a contribution to the safety issues regarding the staging method. While we could show that its use appears safe in tumours larger than 3 cm, we also found an increased false negative rate in large multifocal tumours. As this trial had not been designed to address the question of the sentinel node biopsy’s applicability in multifocal disease, further studies are needed to clarify this issue. Concerning the use of the sentinel node biopsy as a single staging procedure
in early breast cancer, the results presented in this thesis are encouraging. However, breast cancer is a slowly growing disease, and the median follow-up time today is too short to determine the true failure rate of the method. Therefore, long-term follow-up data will be analysed with regard to the development of axillary recurrences after a negative biopsy, and their impact on breast cancer survival.

It seems interesting to speculate about another potential effect of the establishment of the sentinel node biopsy procedure. Before its development, only one or two sections of each retrieved axillary lymph node would be evaluated by the pathologist. This routine pathology has now been complemented by the analysis of multiple or even serial sections of the sentinel lymph node(s). As the sentinel node is the most likely location of nodal metastasis, this may lead to an improved staging accuracy. It is thus possible that breast cancer survival will improve due to stage migration and the improved recognition of sentinel node-positive patients who would have been node-negative by traditional axillary lymph node dissection.
Sammanfattning på svenska

Bakgrund och syfte
Bröstcancer är den vanligaste cancerformen hos kvinnor världen över. Förekomst och antal av lymfkörtelmetastaser i armhålan (axillen) är den starkaste prognostiska faktorn för recidivrisk och överlevnad. Därför grundas planeringen av adjuvanta behandlingar till en stor del just på axillstatus.


Avhandlingen syftar till att utvärdera sentinel node tekniken från ett kliniskt och ett immunologiskt perspektiv. Kliniskt belyses dess användning för tumörer större än 3 cm (delarbete 3) samt dess säkerhet som enda staging metod i axillen vid negativ biopsi (delarbete 4). ”Portvaktskörteln” läge betyder också att den är skådeplats för den första kontakten mellan antigenpresenterande celler och naiva T-celler, och erbjuder därmed en unik möjlighet att studera det immunologiska svaret mot tumören (delarbeten 1 och 2).

Delarbete 1.
T-cell receptorns viktiga funktionella beståndsdel ζ-kedja (CD3-ζ) har visats vara nedreglerad vid olika cancersjukdomar och korrelerar med cancerstadium. Dess förlust leder till försämrad T-cell funktion. CD3-ζ undersöktes i sentinel node biopsier från 24 bröstcancerpatienter med hjälp av immunhistokemiska dubbelfärgningar. Graden av CD3-ζ nedregleringen varierade mellan olika patienter men också mellan de olika delar av körteln som un-
dersöktes: primära folliklar, germinalcentra och paracortex. Den kraftigaste
nedsättningen i uttrycket sågs i paracortex, det viktigaste T-cell aktiverings-
området.

Delarbete 2.
CD28 är en receptor på T-celler som levererar en nödvändig co-stimulerande
signal i samband med T-cellens kontakt med antigen-presenterande celler
(APC). Avsaknad av denna signal leder till T-cellsanergi, tolerans eller cell-
död. Den är nedreglerad i ett antal olika cancersjukdomar. I samma sentinel
node biopsier som i delstudie 1 (varav 21 kunde analyseras) studerades ut-
trycket av CD28 på såväl CD4+ som CD8+ T-celler med hjälp av immunhis-
tokemiska dubbelfärgningar. Resultaten kunde sedan jämföras med uttrycket
av CD3-ζ. CD28 nedregleringen var klart mer utpräglad på CD4+ än på
CD8+ T-celler. Uttrycket av CD28 var lägst i paracortex, och här var också
överensstämmelsen med CD3-ζ starkast.

Delarbete 3.
Från den svenska nationella databasen identifierades 109 patienter med
bröstcancer större än 3 cm som var planerade för både sentinel node biopsi
och en kompletterande axillutrymning, oavsett biopsins resultat. Sentinel
node biopsin lyckades i 103 fall (103/109, detection rate 94.5%). Den falskt
negativa kvoten, definierad som andelen patienter med negativa sentinel
node biopsier av alla de patienter där axillutrymningen visat körtelmeta-
sar, var 8/64 (12.5%). Trots att multifokalitet hade varit ett preoperativt ex-
klusionskriterium, kunde man identifiera 16 multifokala tumörer först vid
den postoperativa patologiska undersökningen. Den falskt negativa kvoten
var 30.8% (4/13) för patienter med multifokala tumörer gentemot 7.8%
(4/51) för patienter med unifokal cancer. Denna skillnad var statistiskt signi-
fikant (p = 0.012).

Delarbete 4.
Den svenska multicenter cohortstudien har inkluderat 3534 patienter med
kliniskt körtelnegativ, unifokal bröstcancer ≤ 3 cm mellan 2000 och 2004. I
de fall där sentinel node biopsin utföll negativ planerades ingen komplette-
rande axillutrymning. Ur hela studiepopulationen selekterades 2246 patienter
som inte hade genomgått någon ytterligare axillkirurgi efter en negativ sen-
tinel node biopsi. Primär endpoint var uppkomst av axillrecidiv. Efter en
median uppföljningstid på 37 månader kunde 27 axillrecidiv identifieras
(1.2%). Bland dessa var axillen den första isolerade recidivlokalisisationen
i 13 fall (0.6%); i 7 fall var axillrecidivet kombinerat med ett lokalrecidiv i det
opererade bröstet, och i ytterligare 7 fall med en generalisering av sjukdomen.

Konklusioner

Sentinel node biopsin visar en tydlig immunologisk påverkan i form av nedreglering av viktiga funktionella T-cellsparametrar. Kännedom om den immunologiska situationen i körteln kan få betydelse som prognostisk faktor men också tjäna till att identifiera de patienter som kan ha nytta av immunoterapi.

Sentinel node tekniken kan anses vara en tillförlitlig metod även för bröstcancer större än 3 cm med undantag för multifokala tumörer där resultaten tyder på lägre säkerhet. Därför rekommenderas en kompletterande axillutrymning för denna subgrupp tills ytterligare data finns tillgängliga.

Sentinel node biopsin som enda axillingrepp har hittills lett till ett begränsat antal axillrecidiv. Resultaten från den svenska multicenterstudien stämmer väl överens med data som rapporterats från andra internationella undersökningar. Detta styrker sentinel node biopsins roll som staging instrument, men långtidsuppföljning måste avvaktas innan säkra slutsatser kan dras.
Acknowlegements

I wish to express my gratitude to all those people who have, in one way or another, supported me during the work on this thesis. Especially, I want to thank the following:

My dear mentor and friend **Leif Bergkvist**, for being the most inspiring, reliable and knowledgeable guide through the world of surgical science I could have hoped for. For offering me your calm support in any situation. For always being at hand in spite of your usually packed schedule. And for breaking the speed limit reading and commenting my manuscript drafts!

My co-tutor **Annika Håkansson** and her husband **Leif Håkansson**, for sharing fascinating insights into the field of tumour immunology. For excellent technical and scientific knowledge and invaluable inspiration for *papers I and II*.

All those **breast cancer patients** from all over Sweden who agreed to participate in these studies, and enabled us to make our research ideas come true.

Breast nurses **Eva Alm**, **Elisabeth Rados**, **Marica Hallsten-Larsson**, and the wonderful staff of the **Breast Unit** and **Ward 7** in Västerås, for maintaining order in my papers and lists despite the threat of chaos, for reminding forgetful doctors to consider patients for inclusion into our studies, and for always being ready with a warm smile. Without you these trials would never have been realisable!

**Jerzy Leppert**, head of the Centre for Clinical Research in Västerås, for his stunning ability to make impossible things possible. For providing the time and resources that made my research possible. And for your innovative attitude that took us all the way to the Camargue!

**Marie-Louise Engström Walker**, for her instalment and maintenance of the sentinel node database at the Centre for Clinical Research. For putting up with my endless scribbled lists during data validation and actualisation for *paper IV*!
Co-authors Jan Frisell, Christian Ingvar, Göran Liljegren and Per-Ebbe Jönsson, for sharing invaluable knowledge, inspiration and experience with me during the preparation of papers III and IV.

Bertil Gustafsson, for his expert pathological assessment of the immunohistological sections and his co-authorship in papers I and II, and Karin Helander and Catharina Tranaeus-Röckert for excellent technical help in performing the immunohistochemical staining, all at the Department of Pathology and Cytology, University of Linköping.

John Öhrvik, for meticulous and knowledgeable statistical support and invaluable contributions throughout this thesis.

The colleagues and staff at the Centre for Clinical Research, for your friendly support, administrative help, and great company. Vive la France!

Another hint at reminding forgetful surgeons...the great staff at the Operation Clinic and Department of Pathology at the Central Hospital in Västerås for your reliable help collecting and processing tissue samples.

Colleagues and breast surgeons all over the country, for contributing with patient accrual and follow-up data collection. Those helpful breast nurses, oncology nurses and secretaries who guided my way through heaps of patient files during visits to various Swedish hospitals, and who provided constant support and updates when chasing missing data for paper IV, especially Kerstin Candell (Falun), Christina Lindström (Norrköping) and Gun-Britt Engholm (Örebro). You have an admirable patience!

My dear parents, for most likely adding spoonfuls of curiosity to my baby food and providing me with a generous amount of genetic stubbornness. A very useful quality in scientific research!

My friends who must have wondered where I had disappeared to when I yet again dived into another project. I owe you quite some time…

And what would I be without you, my dearest David? I still wonder how you could put up with the huge piles of papers drowning our flat during the last months…through which you would make your way with a lovely smile, bringing me yet another cup of your expert cappuccino.
References

75. Chiba T, Ohtani H, Mizoi T, et al. Intraepithelial CD8+ T-cell-count becomes a prognostic factor after a longer follow-up period in

76. Rosen PP, Groshen S, Saigo PE, Kinne DW, Hellman S. Pathological prognostic factors in stage I (T1N0M0) and stage II (T1N1M0) breast carcinoma: a study of 644 patients with median follow-up of 18 years. J Clin Oncol 1989;7(9):1239-51.


90. Rabinowich H, Reichert TE, Kashii Y, Gastman BR, Bell MC, Whiteside TL. Lymphocyte apoptosis induced by Fas ligand-expressing


117. Nieweg OE, Estourgie SH. What is a sentinel node and what is a false-negative sentinel node? Ann Surg Oncol 2004;11(3 Suppl):169S-73S.

146. Maccalli C, Pisarra P, Vegetti C, Sensi M, Parmiani G, Anichini A. Differential Loss of T Cell Signaling Molecules in Metastatic Mela-


Acta Universitatis Upsaliensis

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 259

Editor: The Dean of the Faculty of Medicine

A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)