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Studies on Prediction of Axillary Lymph Node Status in Invasive Breast Cancer

BY

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ABSTRACT

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Breast cancer is the most common malignancy among females in Sweden. Axillary lymph-node dissection is a standard procedure in the management of breast cancer, aiming at obtaining prognostic information for adjuvant therapy decisions. Axillary dissection entails considerable morbidity. The aims of this study were to establish more selective surgical approaches and to investigate angiogenesis, a potential predictor for lymph-node metastases and prognosis.

Clinical nodal status, tumour size and S-phase were associated with nodal metastases in cohort of 1145 women. The proportion of nodal metastases was 13% in the subgroup with the lowest risk.

In a study from two registries, 675 and 1035 breast cancers ≤ 10 mm diagnosed by screening mammography had nodal metastases in 6,5% and 7%, respectively. Clinically detected cancers had a risk of 16% and 14%, respectively.

In a study on 415 women, a 5-node biopsy of the axilla had a sensitivity of 97,3% and a false negative rate of 2,7% in comparison with axillary dissection.

Six sections from 21 breast cancers were analysed for microvessel density (MVD). The inter-section variation contributed more to the total variance than inter-tumour variation, 45,0% and 37,3%, respectively.

In a cohort of 315 women, breast cancers with high MVD more frequently had p53 mutations (27,1%) compared with cases with low MVD (18,4%). This difference was not statistically significant (p=0,075). p53 mutations were associated with a worse outcome, whereas MVD was not.

In conclusion, women with screening detected ≤10 mm breast cancers have a low risk of lymph node metastases and some may not need axillary dissection in the future. The 5-node biopsy could be an alternative to axillary dissection. MVD is associated with methodological weaknesses and routine use is not recommended.

Key words: Breast cancer, lymph node metastases, prognostic factors, predictive factors, axillary surgery, angiogenesis, immunohistochemistry.

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

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II Arnesson LG, Ahlgren J. Omitting axillary surgery for low-risk breast cancer patients. A Swedish prospective cohort study. Acta Oncol 2000;39:291-4.

III Ahlgren J, Holmberg L, Bergh J, Liljegren G. Five node biopsy of the axilla. An alternative to axillarydissection of level I-II in operable breast cancer. Eur J Surg Oncol 2001; (Accepted).

IV Ahlgren J, Risberg B, Villman K, Bergh J. Angiogenesis in inasive breast carcinoma – A prospective study of tumour heterogeneity. Eur J Cancer 2001; (In press).

V Ahlgren J, Lindgren A, Lindahl T, Klaar S, Bergh J. Angiogenesis by Chalkley counting in primary breast cancer – Relationship with p53 mutations. 2002 (Submitted).

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Abbreviations

BCS	Breast conserving surgery
BCSS	Breast cancer specific survival
CI	Confidence interval
CMF	Cyclophosphamide, methotrexate, 5-fluorouracil
CV	Coefficient of variation
ER	Oestrogen receptors
FVIIIRag	Factor VII related antigen
H&E	Hematoxilin and Eosin
HPF	High power field
OS	Overall survival
PR	Progesterone receptors
IHC	Immunohistochemistry
LR	Likelihood ratio
MRM	Modified radical mastectomy
MVD	Microvessel density
RH	Relative hazard
RFS	Recurrence-free survival
ROC	Regional Oncologic Centre, Uppsala/Örebro
SD	Standard deviation
SPF	S-phase fraction

Introduction

Breast cancer is the most common female malignancy in the western societies. The incidence of breast cancer in Sweden has increased steadily by 1-1,5% annually during the recent decades and more than 6300 women contracted the disease in 1999 [1]. The good news are that major improvements have been achieved in the management of breast cancer during recent decades. Breast cancer surgery has been refined, the cosmetic results have been improved by the widespread use of breast conserving surgery (BCS), in combination with radiotherapy, that to a large extent has replaced the more traumatic mastectomy without compromising survival rates [2].
The use of radiotherapy as an adjunct to surgery reduces the local and regional relapse rates by about two thirds whereas the impact on survival is small but statistically significant [2, 3]. The increasing use of adjuvant polychemotherapy, to eradicate micro metastases, gives a relative reduction of the mortality of about 25% [4]. The use of adjuvant tamoxifen to oestrogen receptor (ER) positive subsets results in similar benefits [5].

A great deal of the laboratory-based research has been focused on finding new prognostic- and predictive factors as well us expanding the understanding of the biology of breast cancer. Important results from this research field is the increased knowledge on the c-erbB-2 oncogene [6] and the tumour suppressor gene p53 [7]. The application of microarray technique, which enables the mapping of thousands of different genes in a tumour, represents the most recent progress in this research area [8, 9]. The development of molecular biology has also resulted in the description of two breast cancer susceptibility genes (BRCA1 and BRCA2) [10, 11]. The achievements of molecular biology will almost certainly improve the medical management of increasing numbers of women with or at risk for breast cancer in the near future.

Although a multitude of new prognostic factors has been proposed, the strongest one is still axillary lymph-node status [12]. Nodal status is obtained by histopathological examination of lymph nodes that are retrieved by the means of axillary surgery. The down side of this procedure is the morbidity associated with axillary surgery such as arm oedema, numbness, pain and weakness of the arm [13, 14]. The introduction of mammography screening throughout Sweden has led to earlier diagnosis of breast cancer. Thereby the proportion of

women with small tumours without lymph node metastases has increased. In the Swedish Two-County study the proportion of lymph node positivity was 27% in the group that was invited to mammography and 42% in the control group [15]. This means that axillary surgery is over-treatment in the vast majority of women with breast cancer.

Establishing alternatives to axillary clearence is therefore of great importance for the majority of women with breast cancer. One potential alternative may be the extended use of prognostic factors. One such factor that deserves further evaluation is angiogenesis, i.e. a tumour's ability to induce the formation of new blood vessels. This process is a necessary step in tumour progression [16] that can be estimated in the primary tumour [17, 18].

Background

Incidence

In Sweden a total of 6311 women were diagnosed with breast cancer during 1999 according to the Swedish Cancer Registry [1]. The age corrected incidence of breast cancer has increased from 65-70/100.000 women at the beginning of the 1960ies to 120-125 at the end of the 1990ies [1]. This corresponds to an annual increase of 1-1,5% during the recent decades. The number of deaths attributable to breast cancer was 1485 in 1999 [19]. The mortality seems to have decreased slightly during the last decade, in the years 1987-98 the average death rate was 32,9/100.000 women compared with an average of 29,8/100.000 during 1997-99 [19]. In USA and the UK the breast cancer mortality in year 2000, at ages 20-69, is predicted to have decreased by 25-30% compared with the mid-eighties [20]. The most likely explanation for this positive trend is the broad application of adjuvant systemic therapy and earlier diagnosis due to screening mammography.

The reasons for the increase in incidence are not completely understood but it seems safe to postulate that breast cancer is a disease with multifactorial etiology. The risk of contracting breast cancer is positively correlated with increasing age, being a rare disease during the first four decades of life. In 1999 only 3,5% of all new breast cancers in Sweden were diagnosed in women under the age of 40, and 40% were diagnosed at age 40-59 [1]. Endogenous and exogenous hormonal factors also influence the risk of developing breast cancer. For example

early menarche, late menopause, obesity among postmenopausal women and hormone replacement therapy are factors associated with an increased exposure of the breast parenchyma to estrogens and thereby increasing the breast cancer risk [21]. Ionising radiation to the breast parenchyma is another established risk factor for breast cancer [22].

A large number of studies have been focused on dietary factors and the risk of breast cancer. Especially fat intake has attracted much attention in this context. However, results from studies on total fat intake have been equivocal [23-25]. Restrictions on total energy intake during childhood and adolescence has been hypothesised to be a protective factor for breast cancer [26]. Among other dietary constituents alcohol has consistently showed a correlation with increased breast cancer risk. In a meta-analysis of 12 case-control studies [27] a consumption of two drinks per day was estimated to induce a relative risk of 1,4 compared to non-drinkers. There is also some evidence that vitamin A may protect against breast cancer [23].

In recent years the breakthrough in molecular biology has resulted in increased knowledge on hereditary forms of breast cancer. It has been estimated that 5–10% of all breast cancers are caused by inherited mutations [28]. The majority of breast cancers caused by genetic predisposition are caused by mutations in BRCA1 or BRCA2, two breast cancer susceptibility genes that have been identified [10, 11]. Individuals with a family history of breast cancer can nowadays undergo genetic testing with the aim of detecting mutations in either of these two genes. This has opened up possibilities to offer preventive measures to bearers of these genetic abnormalities and.

Diagnostic procedures

A palpable lump, tenderness, oedema or secretion from the mamilla are signs and symptoms that often lead a woman with breast cancer to a physician. The recommended work-up in this situation is: 1) physical examination of the breasts and regional lymph-nodes, 2) mammography and 3) morphological diagnosis by the means of fine needle aspiration cytology or a core needle biopsy for histopathological examination.

A large proportion of all breast cancers in Sweden are detected by screening mammography, which was implemented during the last years of the 1980ies and in the beginning of the

1990ies in most Swedish counties. In the Uppsala-Örebro health care region with 1,9 million residents, 38,6% of 10.777 invasive breast cancers diagnosed 1992-2001 were detected by screening mammography [29]. Controlled studies on screening mammography indicate a reduction of breast cancer mortality with about 25% [30, 31]. Earlier detection by screening has also led to a change of the stage-distribution. The tumours are smaller and the proportion of patients with lymph-node metastases in the axilla has decreased. In the Swedish Two-County study the proportion of lymph node positivity was 27% and 42% in the invited group and the control group respectively [15]. A current estimate from the Uppsala-Örebro health care region, based on data from 8389 axillary dissections, is a node positivity rate of 35,4% [29]. Screening mammography has thus created a challenge to physicians to adopt the treatment strategies to the changed spectrum of breast cancer.

Staging of breast cancer

The TNM staging [32] is derived from the size of the primary tumour (T), lymph node status (N) and distant metastases (M). Clinical staging (cTNM) is based on the clinical findings prior to surgery whereas pathological staging according to the TNM system (pTNM) includes information from the histopathological examination of the resected breast and axillary tissue.

The primary tumour (T) is classified as follows: Tx, primary tumour cannot be assessed; T0, no evidence of primary tumour; Tis, carcinoma in situ or Paget disease of the nipple; T1, tumour 20 mm or less; T2, tumour more than 20 mm but not more than 50 mm; T3, tumour more than 50 mm. T4, tumour of any size with direct extension to the chest wall or the skin or tumour with oedema of the breast or inflammatory breast cancer.

The regional lymph nodes (N) are classified as follows: Nx, regional lymph nodes cannot be assessed; N0, no regional lymph node metastases; N1, metastasis to one or more movable ipsilateral axillary node; N2, metastasis to one or more ipsilateral axillary node fixed to one another or to other structures; N3, metastases to ipsilateral internal mammary lymph node(s).

Distant metastasis (M) is classified as follows: Mx, presence of distant metastasis cannot be assessed; M0, no distant metastasis; M1, distant metastasis including metastases to one or more ipsilateral supraclavicular node.

Stage grouping is based on the three parameters T, N and M (Table1).

Table 1.

Table 1	TNM stage grouping
Stage 0	Tis N0 M0
Stage I	T1 N0 M0
Stage IIA	T0-1 N1 M0
	T2 N0 M0
Stage IIB	T2 N1 M0
	T3 N0 M0
Stage IIIA	T0-2 N2 M0
	T3 N1-2 M0
Stage IIIB	T4 N0-3 M0
	T0-3 N3 M0
Stage IV	Any T Any N M1

Prognostic and predictive factors in clinical use

Several prognostic and predictive factors are used in the routine management of operable breast cancer. They are used for the evaluation of recurrence risk and for the selection of adjuvant therapies. There is a difference between a prognostic and predictive factor.
A prognostic factor gives information on the risk of recurrence in the absence of adjuvant therapy, i.e. a prognostic factor can be used to predict the natural history of a tumour.
A predictive factor gives information on the likelihood that a tumour will respond to a specific treatment.

Lymph node status and tumour size

For women with operable breast cancer the presence of lymph node metastases is the strongest prognostic factor [12]. The pN staging for operable breast cancer is a qualitative parameter. By counting the number of involved nodes the prognostic power of nodal status can be further increased. This has been demonstrated clearly in a large American study on more than 24.000

women [33]. In order to correctly establish pN status at least 10 lymph nodes should be examined [34]. The number of examined lymph nodes, i.e. the quality of the procedure, is dependent on both the surgeon and the pathologist [35].

The size of the primary tumour is also a powerful prognostic factor and readily available in almost all cases. The risk of recurrence is positively correlated to tumour size [33, 36, 37].

Tumour grade

The grading system currently used in Sweden was first described by Bloom and Richardson [38] and later modified by Elston and Ellis [39]. It is based on three components: tubule formation, nuclear pleomorphism and mitotic index, each scored on a scale from 1-3. The added scores determine the grade according to the following:

Added score	Tumour grade	Degree of differentiation
3-5	Grade I	Well differentiated
6-7	Grade II	Moderately differentiated
8-9	Grade III	Poorly differentiated

Grade according to Elston and Ellis is an established prognostic factor for recurrence free survival (RFS) and overall survival (OS) [39, 40]. Compared with other prognostic parameters, the evaluation of histologic grade is cheap and can be done in virtually in all cases of breast cancer. One possible disadvantage is that the evaluation may vary between different observers. Boiesen and co-writers [41] investigated grading of 93 breast cancers done by 7 different pathologic departments, which resulted in an overall mean kappa of 0,54, indicating a moderate reproducibility.

DNA-ploidy

DNA-ploidy is a measurement of the DNA content in the nucleus of cells. Normal somatic cells are referred to as diploid or euploid. Due to genetic instability many tumour cells have a DNA content that is more or less than what is referred to as diploid. This is called aneuploidy and is associated with a worse prognosis [42].

S-phase fraction (SPF)

The S-phase is the phase of DNA-synthesis that takes place before cell division and thereby a measure of cell proliferation. The fraction of cells in S-phase can be estimated with flow cytometry. The estimate is a percentage that usually is dichotomised. High SPF correlates with increased risk of recurrence and death, and is regarded as a useful prognostic factor [43]. A high S-phase fraction is defined as > 7% in diploid tumours and >12% in non-diploid tumours at the department of pathology at Akademiska Sjukhuset Uppsala. At the University hospital in Linköping a cut-off value of 10% is used for all breast cancers, irrespective of DNA-ploidy.

Estrogen- and progesterone receptor status

Oestrogen receptors (ER) and progesterone receptors (PR) are expressed in a majority of breast cancer tumours. The first report on the prognostic value of ER was published more than 20 years ago [44]. However, with longer follow-up time ER and PR are not strong prognostic factors, although women with receptor positive cancers have a somewhat better prognosis during the first years after diagnosis [45]. The greatest utility of ER and PR is as predictive factors and this was demonstrated in the late 1970ies [46]. Tumours expressing both ER and PR are the most likely to benefit from endocrine therapy but those who express either ER or PR still have significant responses [45]. About 50-60% of women with receptor positive advanced breast cancer will benefit from endocrine therapy while responses among receptor negative women are rare [45]. The predictive value of receptor status has also been established in the adjuvant setting [5].

In recent years, the biochemical analyses of ER and PR, that requires fresh frozen tissue, have to a large extent been replaced by immunohistochemical (IHC) methods employed on paraffin sections. Immunohistochemistry is more suitable for small tumours and possibly gives superior prognostic information [47-49].

New prognostic and predictive factors

The prognostic- and predictive markers of today aim at giving information on prognosis and therapy selection. The implications of these markers are known on group levels but they are not sufficient to identify the individual woman at risk. A prognostic factor gives information on the outcome, at best completely unrelated to different therapies. Ideally, a predictive factor enables the rational use of drugs, i.e., treatment only of those who benefit from a selected drug

or a combination of drugs. The sex-hormone receptor status (ER and PR) is so far the only established predictive factor for the management of women with breast cancer. Therefore, there has been a demand for further markers, especially prognostic- and predictive factors working at the individual level. Indeed, numerous potential prognostic factors identifying different risk groups have been reported [12]. Three factors with special interest will be discussed below: the c-erbB-2 oncogene, the p53 tumour suppressor gene and angiogenesis. These three factors have all been claimed to have prognostic properties. One of them, c-erbB-2, is already a routinely used predictive factor, whereas p53 and angiogenesis in the future may be of value in optimising the decision making in breast cancer therapy.

c-erbB-2

c-erbB-2 (ERBB2,HER2/neu) is an oncogene located on the long arm of chromosome 17 [50]. C-erbB-2 encodes a transmembrane tyrosine growth factor receptor belonging to the epidermal growth factor receptor (EGFR) family .[51]. In a Swedish population based study, including screening detected tumours, the prevalence of overexpression of c-erbB-2 was 19% [52]. Overexpression of this gene has been associated with a poor prognosis in breast cancer [6, 52, 53], although not all studies have shown an independent prognostic value [54]. Furthermore, it has been suggested that overexpression of c-erbB-2 is associated with decreased sensitivity to tamoxifen and CMF-like chemotherapy [55]. The most interesting aspect of c-erbB-2 is the possibility to target the gene product with trastuzumab, a monoclonal antibody. Trastuzumab in combination with chemotherapy results in prolonged survival compared with chemotherapy alone in women with advanced breast cancer overexpressing c-erbB-2 [56]. As a consequence, determination of c-erbB-2 status with IHC, to be confirmed by fluorescence in situ hybridisation (FISH) on paraffin embedded tumour sections is a routine analysis for women with recurrent breast cancer.

p53

p53 is a tumour supressor gene located on the short arm of chromosome 17 [57]. Due to its central role in cell cycle control, execution of programmed cell death (apoptosis) and defence mechanisms after DNA damage, p53 has been called “the guardian of the genome” [58]. Mutation of p53 is found in 20-25% of breast cancers [7, 59]. The most common method of assessing p53 status is immunohistochemistry, which is done on paraffin sections. This method detects intracellular accumulation of altered p53 protein, or enhanced levels of normal

p53 protein, that is interpreted as an evidence for an altered p53 function. Although IHC is a fast method with low costs, the prognostic and predictive value is inferior to p53 status obtained by gene sequencing [60, 61]. The potential use of p53 in the future seems to be as a predictive factor. p53 mutations have been associated with increased resistance to FEC chemotherapy [62] and tamoxifen [63], while taxane based chemotherapy seems to have increased efficacy in women with p53 mutated breast cancers [62]. Thus, if these findings can be reproduced in large randomised controlled trials, determination of p53 status has the potential to substantially improve the clinical management of breast cancer.

Angiogenesis

Angiogenesis, i.e. the ability of a tumour to induce formation of new microvessels, was reported to be of importance for tumour growth more than 30 years ago [64]. Angiogenesis stimulates progression of both primary and metastatic tumours by several mechamisms. Firstly, the growth of tumours beyond the size of 1-2 mm^3 is dependent on angiogenesis [65]. Without access to vasculature tumour, their growth is limited by diffusion of nutrients and accumulation of waste products. Secondly, the presence of microvessels facilitates tumour cell access to the blood circulation [66, 67], a prerequisite for the establishment of distant metastases. Thirdly, endothelial cells in the microvasculature release growth factors that can stimulate tumour growth [68]. Moreover, angiogenesis seems to be involved in the production of proteolytic enzymes [69], which is of importance in tumour invasiveness.

Angiogenesis is regulated by numerous angiogenic factors, one of the most important being vascular endothelial growth factor (VEGF) [70], which is a potent mitogen for endothelial cells. High levels of VEGF correlate with a poor prognosis in breast cancer [71-73]. Interestingly, the tumour suppressor gene p53 putatively exerts a negative effect on angiogenesis, wild-type p53 protein seems to down regulate VEGF [74]. This finding is corroborated by the findings of Linderholm and co-workers, showing that mutation of p53 correlates with increased levels of VEGF [75, 76].

Another link between p53 status and angiogenesis that has been postulated is that wild-type p53 protein induces the formation of thrombospondin 1, which is a potent inhibitor of angiogenesis [77]. This pathway is affected when mutant p53 protein is present, leading to decreased formation of thrombospondin 1 and consequently increased angiogenesis [78, 79].

Estimation of tumour angiogenesis by a histological grading system and that a high grade of neovascularisation correlates with tumour aggressiveness was described 3 decades ago [80]. The presently most widely used method for estimation of tumour angiogenesis was developed by Weidner and co-workers [17]. They showed that tumour blood vessels can be visualised with immunohistochemical staining of tumour sections, using a monoclonal antibody to an antigen expressed by endothelial cells. The microvessel density (MVD) is then estimated by counting these highlighted microvessels within the areas with the most intense neovascularisation (also called hotspots). Several studies have demonstrated that MVD independently can predict poor prognosis in operable breast cancer [18, 81-85] including lymph-node negative patients [86-92]. In contrast, other authors could not find any prognostic value of MVD [93-99]. One important reason for the contradictory results may well be methodological problems.

Despite contradictory results regarding MVD and prognosis in breast cancer, several authors have reported a correlation between high MVD and lymph-node metastases [17, 18, 82, 83]. Although some authors report a lack of relationship between MVD and nodal status [100-102], this putative relationship is of interest since factors associated with lymph-node metastases could potentially be used for prediction of axillary lymph-node metastases. A predictive factor for nodal metastases could be used for identifying women with high risk of node positivity, and inversely, also help to define a subgroup of women with a low enough risk of lymph-node metastases to allow omission of axillary surgery.

Finally, there have been some reports on the predictive value of angiogenesis [102-104] but most of the interest, in the therapeutical aspect of angiogenesis, has been focused on the development of specific anti-angiogenic drugs [105, 106]. Though, the clinical breakthrough of this conceptually appealing quest for a new class of anticancer drugs is still to be awaited.

Treatment

Surgery

Until the 1980ies mastectomy was considered to be the operation of choice for almost all patients. The increasing numbers of patients with small tumours created a need for less

extensive surgery and breast conserving operations became increasingly more common at the end of the 1980ies. No survival differences have been demonstrated in comparisons between BCS combined with radiotherapy and larger operations where the whole breast is removed [2]. The type of BCS used in Sweden is sector resection [107]. In the Uppsala-Örebro health care region, registry data from 1992 –2001 on 9912 breast cancers treated with primary surgery, shows that sector resection constitutes 60,0% of the operations [29].

Surgical management of the axilla

The axilla is anatomically a triangle shaped area with the axillary vein forming the superior margin and the latissimus dorsi and serratus anterior muscles forming the posterior and medial borders. This anatomic region is divided into three levels: level I - lateral and below the lateral margin of the minor pectoral muscle; level II - the lymph nodes under the minor pectoral muscle; and level III - the lymph nodes medial and above the medial margin of the minor pectoral muscle. The extent of axillary surgery is usually defined according to these three levels of the axilla.

The radical mastectomy, originally described by Halsted [108], included not only removal of the whole breast and the pectoral muscles but also clearance of the axillary lymph nodes of level I-III and removal of the intrapectoral lymph nodes. The underlying hypothesis of the radical mastectomy was that breast cancer is a localised disease that disseminates first to the regional lymph nodes and then to distant organs in an orderly fashion, thus, by extensive surgery the chances for cure would be maximised. This view was challenged by results from randomised studies showing that the extent of lymph node removal did not seem to affect survival [109, 110]. The interpretation of these data was that failure after surgery usually is because of the systemic dissemination of cancer cells before surgery, rather than an inadequate operative technique. Since the impact on survival by axillary surgery is unclear, the main reasons for this part of the operation is to ensure a correct staging of the disease and to achieve local tumour control. The second reason for axillary surgery is to ensure local control of the disease in the axilla. After level I-III clearance of the axilla, isolated axillary recurrences are rare (1-1,4%) despite no further treatment [109].

The recommended type of axillary surgery is dissection of level I-II [111] since this procedure meets the requirements of accurately staging the patient and ensuring local control. Axelsson

and colleagues found in a large study [34] comprising 7145 lymph node negative women, that at least 10 lymph nodes should be removed in order to correctly establish node negativity. Women with lymph node negative tumours and less than 10 removed lymph nodes had a worse prognosis compared with those who had at least 10 nodes removed. The obvious reason for this is that a subset of women with <10 removed nodes were wrongly classified as being node negative. The Danish group [34] also reported a low local relapse rate of 3% after level I-II dissection without further treatment in node negative patients if at least 5 lymph nodes were removed. Recht and co-writers reported data from 1624 patients that underwent level I-II dissection [112]. The local relapse rate was 2,2% in women not given radiation to the axilla. Other studies have shown that the frequency of "skip" metastases to level III when levels I-II are negative is about 1% [113, 114], thus, the risk of leaving metastases behind in level III is very low.

Complications to present axillary staging procedures

Axillary surgery is in the long run one of the major sources of treatment associated morbidity. Lymphoedema of the arm, the most widely recognised complication of axillary dissection, is commonly defined as an increase of arm circumference of >2 cm or increase of arm volume >200 ml. The incidence of lymphoedema is 10-25% [115]. The extent of axillary surgery correlates with the risk of lymphoedema [14, 116, 117]. Other complications are also common. In a report on 126 patients after axillary dissection without radiotherapy [13], 70% of the women complained of numbness, 33% of pain and 25% experienced weakness of the arm. Although these complications mostly were described as mild, 39% of the women experienced an effect upon their daily lives [13].

Less extensive surgical procedures

Due to complications associated with axillary dissection, efforts have been made to reduce the extent of axillary surgery. In a Scottish study [118] patients were randomised between axillary clearance and lower axillary sampling. The latter method consisted of surgical removal of 4-5 nodes near the lateral border of the breast. In this study, the sampling procedure was followed by axillary clearence during the same operation in a subset of 67 patients. This experiment resulted in a sensitivity of the sampling procedure of 100%. Later, the same group published a report from the whole randomised study including 417 women [119]. Patients that underwent sampling were treated with radiation therapy in case of node positivity. The long-term results

with reference to survival were identical with the two methods. A second study from the same research group included 466 women treated with BCT [120]. Once again, no survival difference could be detected when sampling and clearence were compared. In contrast, Kissin and co-authors [121] showed that 24% were erroneously staged with sampling compared with results obtained with axillary dissection. Moreover, data from the large Danish registry study [34] question the use of sampling. According to their results at least 10 lymph nodes should be removed to eliminate the risk of misclassification.

The sentinel node biopsy is a new method for minimally invasive axillary surgery. By the means of peritumoural injections of blue dye and/or a radiolabeled colloid a few hours before surgery the lymph node that first receives the drainage from the breast can be identified visually and/or by a gamma probe. The false negative rate in one of the largest series from a single institution was 6,7% [122], whereas the corresponding figure in two multicenter studies was 11% [123, 124]. Data from randomised comparisons between axillary dissection and the sentinel node procedure alone, with reference to local control and preferably survival, should be awaited before this promising method can be adopted in clinical practise.

Adjuvant radiotherapy

The use of ionising radiation given as an adjunct to surgery reduces the risk of local recurrence by two thirds [125]. Radiotherapy also reduces breast cancer mortality with an absolute reduction of about 5% at 20 years of follow-up [125]. This positive effect is counter balanced by an absolute survival reduction due to deaths from other diseases than breast cancer of about 4% at 20 years. The resulting absolute increase of overall survival at 20 years is about 1% [125]. However, the total survival benefit is greater among young women and those with high risk of recurrence [125]. Recent studies on post-mastectomy radiotherapy given in combination with chemotherapy indicate that the survival gains might be significantly larger [126-128] if the radiotherapy is given with a more modern technique. The view that the survival benefit of adjuvant radiotherapy at most is marginal is now being challenged [3].

Radiotherapy to the axilla increases the risk of sequelae such as lymphoedema and impaired mobility of the arm [129]. The highest risk of arm-lymphoedema is seen among women that are treated with both axillary surgery and radiotherapy to the axilla [130]. The radiotherapy

technique is of outmost importance for avoiding toxicity. This should include adequate dose planning, preferably based on computerised tomography, and modern fractionation schedules. The standard treatment is 2Gy/fraction to a total dose of 50-54Gy over 5-5,5 weeks to the breast after BCS and 2Gy/fraction to a total dose of 50Gy over 5 weeks to the chest wall after mastectomy. In lymph node positive women the axilla, supraclacivular fossa and the internal mammary chain are usually treated with 2Gy/fraction to a total dose of 50Gy over 5 weeks.

Adjuvant systemic therapy

The rationale for using adjuvant polychemotherapy is that systemic treatment early in the course of breast cancer can eliminate micrometastases and thereby increase the chance of cure. Meta analyses have shown that polychemotherapy gives a relative risk reduction for death by about 25 % [4]. Recent data show that chemotherapy regimens including an anthracycline (doxorubicin or epirubicin) are superior (relative risk reduction for death 11%) to older regimens based on alkylating agents [4]. Hence, the standard treatment currently in Sweden is a combination of 5-fluorouracil, epirubicin and cyclophosphamide (FEC regimen) given in seven courses. Tamoxifen is a selective estrogen receptor modifier (SERM) with activity against ER or PR positive breast cancer. The use of this drug in the adjuvant setting gives a relative risk reduction for recurrence by almost 50% and a relative risk reduction for death by about 25% when taken daily for five years [5]. The combination of polychemotherapy and tamoxifen to receptor positive women produces additional benefit [4]. Thus, the relative risk reduction for death for most women treated with chemo-endocrine therapy clearly must be considerably greater than 25%.

Treatment of recurrent breast cancer

Once distant metastases are clinically detected the disease is incurable and expected median survival in this situation is typically in the range of 2-3 years [21]. Women with metastatic disease are treated with chemotherapy and/or hormonal therapy which can alleviate symptoms and also improve median survival [131-134]. Recently the addition of the monoclonal antibody trastuzumab to chemotherapy has been demonstrated to prolong survival for women with c-erbB-2 positive advanced breast cancer [56]. Palliative radiotherapy, adequate analgesics, bisphosphonates in case of bone metastases, erythropoeitin, and psychological support are other important means of good palliation in the treatment of metastatic breast cancer [21].

Aims of the study

The overall aim of the study is to investigate different strategies to decrease the need for axillary dissection in women with breast cancer. The study consists of two parts where papers I-III are aiming at a more selective surgical approach. Papers IV-V investigate angiogenesis, a potential predictor not only for prognosis but also for lymph-node metastases.

Specific aims

Paper I:

To delineate a subgroup of women with breast cancer, from a population with ongoing screening, with a low enough risk of lymph node metastases to allow omission of axillary surgery.

Paper II:

To define a subgroup of women by, the use of small tumour size and detection by screening, with a low enough risk of lymph node metastases to allow omission of axillary surgery within the framework of a prospective cohort study.

Paper III:

To test whether a biopsy of five lymph nodes is as informative on histopathological lymph node status as a level I-II dissection of the axilla in operable breast cancer.

Paper IV:

To investigate if intra tumoural heterogeneity of angiogenesis demonstrates a significant variation which may influence the results of this parameter in primary breast cancer.

Paper V:

To investigate the relationship between MVD and cDNA sequenced determined p53 mutations for exons 2-11 in breast cancer and to analyse the correlation between MVD and recurrence free survival, breast cancer corrected survival and overall survival.

Materials and methods

Paper I

A database in which all new cases of breast cancer in the South-East Sweden Health Care Region are to be reported was used. We analysed data from 1145 women with primary breast cancer retrospectively in order to define a subgroup in which axillary dissection could be omitted. Women with tumours larger than 50 mm and/or with less than 5 examined lymph nodes were excluded. We used the clinical and pathological features of the tumours that are used in clinical practice: clinical nodal status, age, tumour size, hormonal receptors, DNA ploidy and SPF and that had been prospectively reported to the database.

Paper II

The second paper is based on two registry studies that were made as a part of the preparations for a multicentric Swedish prospective cohort-study for omission of axillary surgery in women with low risk of having lymph-node metastases. Data from the breast cancer registries from the South-East Swedish Region and the Uppsala-Örebro Region was used. All newly diagnosed breast cancers within the two health care regions are to be reported to their respective registry. No restrictions were made on the number of removed lymph-nodes.

Paper III

Four hundred and fifteen females from Örebro and Uppsala were entered in a prospective study on a 5-node biopsy of the axilla. Women with clinical stage $T_{0\text{-}3}N_{0\text{-}1}M_0$ breast cancer were eligible. They were included in the study after informed consent. All patients were operated by the same surgeon using a standardised technique. Each patient underwent sector resection or mastectomy. The axillary surgery consisted of a 5-node biopsy followed by a dissection of the remaining axillary tissue in level I-II in the same operation.

The 5-node biopsy was begun with dissection at the axillary tail of the breast until five lymph nodes had been removed. Each of these lymph nodes were submitted to the pathologist in separate boxes and labelled lymph node 1 to 5. After the first five lymph nodes had been retrieved, the dissection of the axilla continued until the axillary fat in level I-II had been excised. This material was also submitted in a separate box to the pathologist. The aim of the five-node biopsy and the level I-II dissection was to remove

a total of at least 10 axillary lymph nodes for histopathological examination. In this way the experimental method could be compared with the gold standard procedure in each patient.

All the removed tissue was fixed in formaldehyde and stained with van Gieson or H&E. The axillary level I-II specimens were carefully palpated in order to find as many lymph nodes as possible. Sections of all nodes were examined with routine pathology. Immunohistochemical staining was not used in the examination of the nodes. The breast tumours were also stained with van Gieson or H&E and examined with routine pathology.

Paper IV

Twenty-one consecutive invasive breast cancers were recieved as fresh specimens at the Department of Pathology in Örebro from May to October 1994. No preoperative treatment of breast cancer was allowed. The whole tumour was sectioned in 5mm slices. Fixation was run overnight in 4% buffered formaldehyde solution. After dehydration all tumour tissue was embedded in paraffin. H&E staining of all tumours was done for routine assessment of type and grade. Six 4µ sections were cut from each tumour. In all cases sections were separated as widely and evenly as possible within the tumour. The position of all sections within the tumour was registered and labelled A-F. The sections (n=126) were stained immunohistochemically with a CD31 monoclonal antibody (JC70). In each section the areas with the most intense neovascularisation (hotspots) were identified and the MVD was obtained by counting vessels in 200 X fields (0,72mm^2) in three such hotspots. All slides were assessed simultaneously by two observers.

Statistical methods

We used Statistica software (Statsoft OK, USA) for calculation of standard deviation (SD), confidence intervals, and the independent two-sided t-test for comparisons between groups. Variability was expressed by the coefficient of variation (CV) which is defined as 100*SD divided by the mean. A nested ANOVA (Statistica software) was used to analyse the proportion of the total variance that each sampling level contributed to. For this analysis we considered cases, intersection and intrasection as three levels in a hierarchic model.

Paper V

Tumour material was analysed from 315 consecutive women with primary breast cancer who underwent surgery in Uppsala County, Sweden, during the period January 1st 1987 to December 31st 1989 and from whom fresh frozen material was saved, which was the routine procedure. Routine histopathological examination and determination of ER, PR and S-phase fraction was performed.

Regular follow-up visits were performed during 5-10 years. In November 1999 the patient records were re-examined and updated. Survival information on the women by the 1st of November 1999 was retrieved from the Swedish Population Registry. To get information on death causes we used data both in medical files and from death certificates. The follow-up of recurrences was obtained by reviewing the patient records. When women had been referred to another hospital or to a GP for further follow-up, we retrieved information about the date of the latest check-up and status concerning signs of recurrence.

MVD analysis

From 305 tumours we were able to retrieve fresh frozen tumour material. A piece of the frozen tumour tissue was thawed and then fixed. After dehydration the specimens were embedded in paraffin. Sections with a diameter of 4-5 mm were thus obtained and mounted on slides. We used a monoclonal antibody to CD31 (JC70A, Dako AS, Glostrup, Denmark) in a 1:10 dilution. The sections were predigested with protease. The quality of the intratumoural staining was judged using blood vessels in adjacent benign breast tissue as internal positive control. The most vascularised areas of the tumour tissue were located at low magnification (10X oculars with 4X and 10X objectives). Thereafter a 25-point Chalkley eyepiece graticule was employed [135] over the same tumour region and orientated so that the maximum number of points at 200X (0,95 mm^2) were on or within areas of stained microvessels. Thus the highest graticule count was recorded for each tumour.

p53 analysis

The p53 status in tumours was analysed by sequencing with cDNA for exons 2 to 11 on homogenised fresh frozen tumour samples. The sequencing products generated were analysed with an automated laser fluorescence sequencer. The sequence was finally compared with the wild-type p53 sequence. Nucleotide alterations that had an impact on he protein were

considered mutations. Immunohistochemical determination of p53 status was performed on paraffin sections using the monoclonal mouse antibody Pab 1801.

Statistical methods

For non-parametric comparisons between groups we used the χ^2-test. For estimation of overall survival (OS), breast cancer specific survival (BCSS) and recurrence free survival (RFS) we used life table analyses and the Kaplan Meier method. The log-rank test was employed for analysis of differences between groups. For OS all deaths were counted as events. For BCSS only deaths from breast cancer were considered to be events. Events for estimation of RFS were all breast cancer relapses or death from breast cancer. Relative hazards (RH) for OS and BCSS were estimated by Cox's proportional hazards method in univariate and multivariate models. We used Statistica Software (StatSoft Inc., Tulsa, OK) for all analyses except for Cox's proportional hazards analyses for which the PHREG procedure in SAS for PC (Armonk, NY) was used.

Results

Paper I

Both clinical nodal status and tumour size were strongly correlated with pathological nodal status. Also SPF >10% was strongly correlated with node positivity in univariate analysis (Table 2).

In multivariate analysis there was a correlation between high SPF and nodal metastases among tumours up to 20 mm but not with tumours greater than 20 mm. Women with clinically negative nodal status and tumour size <=20 mm and <=10 mm had pathologically positive nodes in 25% and 13%, respectively (in the original publication the latter estimate was miscalculated, the correct estimate is 13%, not 15%). If tumours with high SPF were excluded, the corresponding estimates were 24% and 14%.

Table 2.

Characteristics of 1145 patients included in analysis according to pathological nodal status.

	Number of patients (%)					
	All	pN-		PN+		Chi-2
Clinical nodal status						
CN_0	997	695	(70)	302	(30)	
CN_{1a}	48	23	(48)	25	(52)	
CN_{1b}	100	19	(19)	81	(81)	P<0.0001
Tumour size (mm)						
<=10	284	247	(87)	37	(13)	
11-20	522	341	(65)	181	(35)	
21-30	244	114	(47)	130	(53)	
>30	95	35	(37)	60	(63)	P<0.0001
Age (years)						
<50	230	139	(60)	91	(40)	
>=50	915	598	(65)	317	(35)	p=0.16
ER-status[1]						
ER+	672	363	(61)	266	(39)	
ER-	184	97	(57)	81	(43)	p=0.33
PR-status[1]						
PR+	564	347	(62)	219	(38)	
PR-	292	168	(58)	124	(42)	p=0.26
S-phase fraction[2]						
<10%	471	286	(65)	185	(35)	
>=10%	220	103	(46)	117	(54)	P<0.0001
DNA ploidy[1]						
Diploid	346	228	(66)	118	(34)	
Non-diploid	510	287	(56)	223	(44)	P=0.0048

pN- denotes node negative and pN+ node positive patients. [1]Estimated in 856 cases. [2]Estimated in 730 cases.

Paper II

From the South-East Swedish Region 2325 tumours ≤15 mm were included. The proportion of lymph-node metastases was 11% among tumours ≤10 mm and 24% for tumours 11-15 mm. The lowest risk of having lymph-node metastases was 7% and this was found among screening detected tumours ≤10 mm. The corresponding figure for clinically detected tumours ≤10 mm was 14%.

The figure for screening detected tumours ≤10 mm from the Uppsala-Örebro Region was 6,5% (30/464), whereas the proportion of lymph node positivity among patients with clinically detected breast cancers ≤10 mm was 14% (34/211).

Results from extended analysis made in December 2001

It is reasonable to believe that the subgroup of clinically detected tumours has a greater mean tumour size compared with tumours detected by screening mammography. Moreover, since the screening detected subgroup consists of women aged 40-70, the clinically detected subgroup will have a different age distribution. In December 2001 we performed a multivariate analysis on the relation between positive lymph node status and detection mode adjusted for tumour size and age using data from the registry at the Regional Oncologic Centre of Uppsala/Örebro. Data from 803 tumours with a maximum diameter of 10 mm diagnosed from September 1992 to December 1996 was selected. Detection mode, tumour size and age were entered in a logistic regression model with lymph node status as response variable (Table 3). The results from this analysis show that the mode of detection is the strongest predictor of lymph node metastases, clinically detected tumours being more likely to have positive lymph nodes. Both tumour size and age are also independent risk factors for nodal metastases, whereas decreasing tumour size as expected shows a correlation with decreasing risk for metastases, age have a non-linear relationship. Age 50-54 years was the only age group with an odds ratio that was statistically significant.

Table 3.

Odds ratios for having lymph node metastases dependent on tumour size, detection mode and age. Logistic regression analysis based on 804 cancers with maximum tumour size 10 mm. Detection mode is diagnosis by screening mammography versus clinical detection. Odds for women aged 55-59 years were used as reference for the age categories.

Variable	Odds Ratio	95%CI	p-value
Tumour size	0,89	0,83-0,95	0,0006
Detection mode	0,32	0,20-0,50	0,0001
Age 40-44 years	1,16	0,52-2,61	0,72
Age 45-49 years	0,57	0,28-1,16	0,12
Age 50-54	0,45	0,22-0,92	0,029
Age 60-64	0,61	0,31-1,22	0,16
Age 65-70	0,50	0,25-1,00	0,051

Paper III

Th number of women with positive lymph node status was 149/415 (36%). The number of lymph node negative axillae was 266/415. The number of lymph node positive axillae that were missed in the 5-node biopsy was 4/149, thus, according to the 5-node biopsy 270 were classified as lymph node negative which gives a false negative rate of 1,5%. The distribution in different subgroups of the 4 erroneously classified cases is given in Table 4. No risk factor for erroneous classification can be observed due to the low number of such cases. The clinical and pathological features of the four cases with a false negative 5-node biopsy are given in Table 5.

The 5-node biopsy had a sensitivity of 97,3% (CI 97,1-97,5) and a negative predictive value of 98,5 (CI 98,4-98,6). Among women with detection by screening (n=204) the sensitivity was 95,8% (CI 95,7-96,0) and the negative predictive value was 98,7 (CI 98,7-98,8). The corresponding estimates for clinically detected cases (n=197) were 97,9% (CI 97,9-98,0) and 98,0 (CI 98,0-98,1). The –LR was 0,027 for all women, for those with screening and clinically detected tumours the –LR was 0,042 and 0,021 respectively.

As expected, the sensitivity of the 5-node biopsy increased for each lymph node examined but the difference with reference to negative predictive value and sensitivity was only marginally increased when the performance of 4 nodes was compared with 5 nodes (Table 6).

Table 4.
Clinical and pathological characteristics of study population in relation to lymph-node positivity and numbers of cases with false negative 5-node biopsy

Variable	Grouping		Node positive		Number of node pos cases missed in 5 node biopsy
		%	Numbers	%	
All patients (n=415)		100	149/415	36	4
Age (n=415)	<=50	24	41/9742		0
	>50	76	108/318	34	4
Menopause, (n=412)	pre	27	46/109	42	0
	post	73	101/303	33	4
Presentation (n=401)	screening	50	48/204	24	2
	clinically	48	97/197	49	2
cN status (n=415)	cN_0	83	89/344	26	4
	cN_1	17	60/71	84	0
Surgery (n=415)	BCS	67	69/280	24	3
	MRM	32	80/135	59	1
T-size (n=412)	0-10 mm	27	13/111	12	1
	11-20 mm	40	47/168	28	1
	21-30 mm	19	46/78	59	1
	>30mm	13	40/55	73	1
Tumour type, (n=411)	ductal	85	129/350	37	3
	lobular	11	16/47	34	1
	other	3	2/14	14	0
ER status (n=415)	positive	60	102/249	41	4
	negative	23	34/96	35	0
	unknown	17	13/70	19	0
PR status (n=415)	positive	56	95/234	41	4
	negative	27	40/110	36	0
	unknown	17	14/71	20	0

cN status denotes clinical nodal status. BCS denotes breastconserving surgery.
MRM denotes modified radical mastectomy.

Table 5.

The clinical and pathological features of the four cases with a false negative 5-node biopsy.

Age	Type of surgery	Tumour size (mm)	Involved nodes	Histological type	ER/PR	SPF
55	BCS	25	3/24	Lobular	+/+	Low
70	BCS	12	1/8	Ductal	+/+	Low
82	MRM	60	1/16	Ductal	+/+	High
73	BCS	9	1/9	Ductal	+/+	Low

BCS denotes breast conserving surgery. MRM denotes modified radical mastectomy. Involved nodes is number of metastatic nodes/number of examined nodes.

Table 6. Number of patients with and without histopathologically involved lymph nodes, negative predictive value, sensitivity and negative likelihood ratio related to number of examined lymph nodes (all patients) n=415. Lymph node 1-5 + level I-II is the reference.

Number of excised lymph nodes	Negative predictive value (95% CI)	Sensitivity(95% CI)	Negative likelihood ratio (-LR)
1 lymph node	83.6% (83.5-83.9)	65.1% (64.1-66.1)	0.349
2 lymph nodes	91.1% (90.9-91.3)	82.6% (82.0-83.2)	0.174
3 lymph nodes	95.3% (95.3-95.5)	91.3% (90.9-91.7)	0.087
4 lymph nodes	97.8% (97.7-97.8)	96.0% (95.7-96.3)	0.04
5 lymph nodes	98.5% (98.4-98.6)	97.3% (97.1-97.5)	0.027

Paper IV

Median age of the patients was 69 years (range 35 - 88). The median size of the 21 tumours was 20 mm (range 10 - 40 mm). Lymph-node status was positive in 8 cases and negative in 11. Nodal status was not assessed in two elderly women.

In six patients the quality of immunostaining was not judged as satisfactory in a proportion of the six sections (Table 7). All 8 sections with questionable staining were stained a second time with a highly vascularised tumour-section as positive control, none of them turned to be assessable by this procedure.

Measures of variation

The mean of all MVDs (n=345) was 82,5/200XHPF (median 75, range 21 - 196). The mean of the highest scores from each section (n=115) was 93,3 (median 86,5, range 40-196) The mean of the highest score from each tumour (n=21) was 128,4 (median 120, range 87–196) (Table 7). The highest MVD from each section was plotted in order to visualise the intratumoural heterogeneity (Figure 1.). The CV was analysed in different subgroups with reference to tumour size (<= 20 mm vs. > 21 mm) and number of blocks taken (2-5 blocks vs. >5). The independent two-sided t-test was used in this analysis but no differences could be found.

A nested ANOVA of variance components was performed in order to assess the contribution of the three sampling levels to the total variance. The highest level of the hierarchic model, the different tumours, contributed with 37,3% of the total variance. The corresponding figures for the methodological levels, the intersection and intrasection levels, were 45,0% and 17,7% respectively. Thus, variation between different sections of the tumours contributed more to the total variance than did variation between different tumours.

Legend to figure 1.

The highest MVDs from all assessable sections (n=115) are plotted. Each tick mark on the X-axis represents one tumour. The tumours are ordered by increasing Mean MVD.

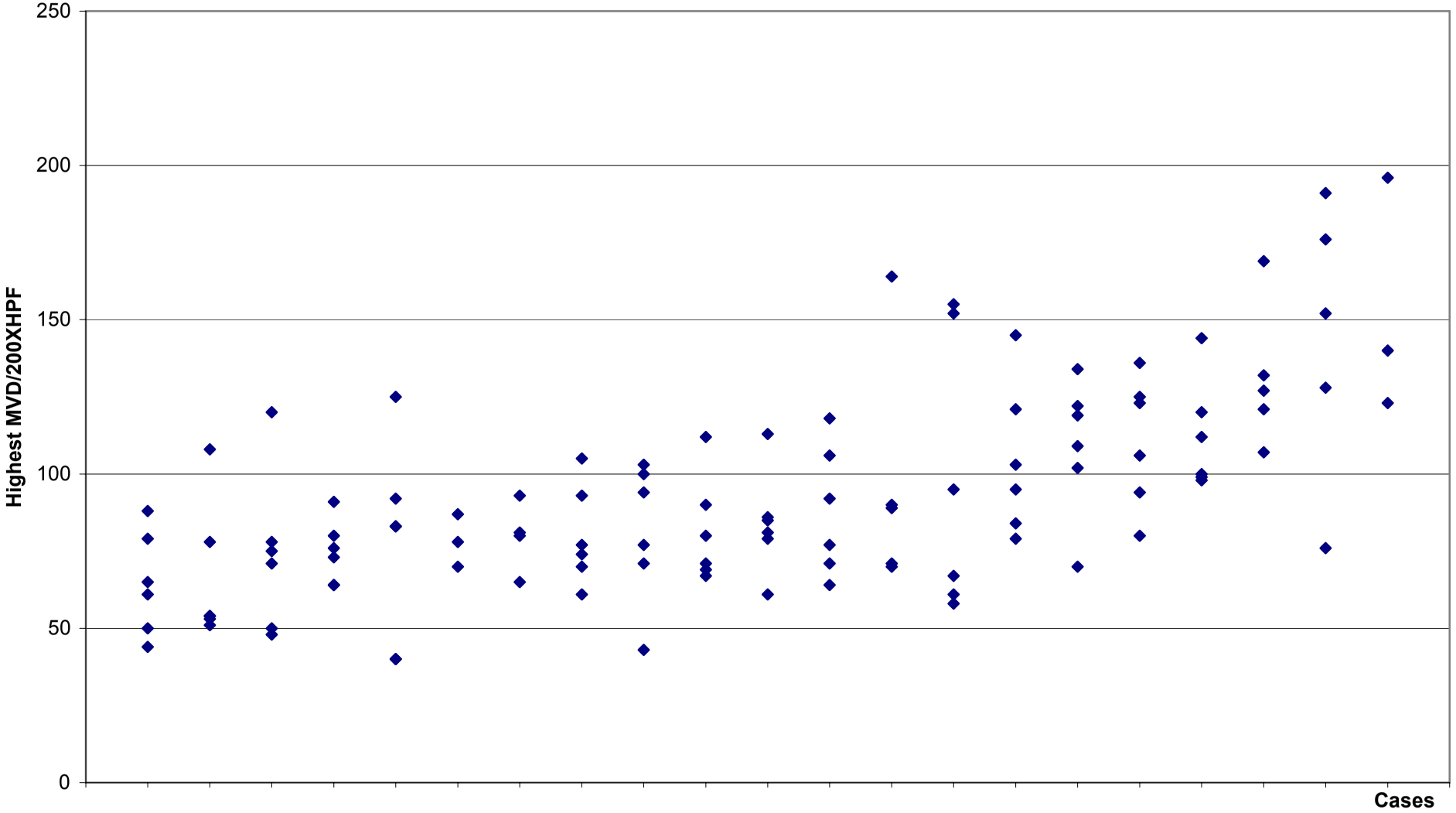
Table 7.

The highest MVD in all 115 assessable sections. The tumours are ordered from lowest mean MVD (top) to highest (bottom)

		Sections, highest of three counts							
T-size (mm)	Blocks #	A	B	C	D	E	F	Mean (A-F)	CV%
15	4	44	50	88	65	79	61	64,5	26,0
10	2	51	53	54	54	78	108	66,3	34,3
22	5	48	71	75	78	120	50	73,7	35,4
19	5	91	76	73	64	80	64	74,7	13,8
15	3	83	40	83	92	125	40	77,2	42,3
10	3	-[2]	-[2]	70	87	-[1]	78	78,3	10,9
22	7	-[1]	-[1]	65	81	93	80	79,8	14,4
28	6	61	77	105	93	70	74	80,0	20,2
20	3	100	43	71	94	103	77	81,3	27,9
16	5	69	67	71	80	90	112	81,5	21,1
11	2	79	113	61	86	81	85	84,2	19,9
17	7	64	77	106	92	118	71	88,0	24,0
29	7	70	89	71	90	-[1]	164	96,8	40,0
35	7	152	95	155	61	58	67	98,0	45,9
30	4	84	145	121	95	79	103	104,5	23,7
11	5	134	122	70	102	109	119	109,3	20,3
22	4	94	80	106	125	123	136	110,7	19,1
21	5	120	98	100	112	144	99	112,2	15,9
15	3	169	-[2]	127	132	121	107	131,2	17,6
30	15	76	176	191	128	152	-[1]	144,6	31,3
40	10	-[1]	-[1]	123	-[1]	140	196	153,0	25,0

Mean (A-F) is mean value of highest scores. [1] Section not adequately stained. [2] No invasive cancer within section.

Figure 1.



Application of potential cut-offs.

In this analysis we chose the median of all the highest scores (86,5/200XHPF) from each section (n=115) as a tentative cut-off level, which was applied to the scores from each sectioning level (A-F). In this way we had 6 sets of dichotomised scores, each representing a different part of the tumours. We then compared the results from one set of sections with a second set of sections (A-B; A-C; A-D; A-E; A-F; B-C; etc.) and calculated the proportion of tumours for which both results of a pair were concordant with reference to the cut-off level. Fifteen comparisons were thus made for each of the 21 tumours. In these paired comparisons the mean proportion of concordant results was 59,0% (95% CI (55,3:62,8)). The result was similar if the upper tertile (101,3/200XHPF) was chosen as a cut-off level, 64,7% (95% CI (60,0:69,5)). This example shows that more than one third of the dichotomised estimates will change from high to low or from low to high if the analysis is made on a second section from another part of the tumour.

Paper V

The median follow-up for survival and recurrence was 122 and 73 months, respectively. The number of women that had died was 137, of these 74 died of breast cancer, 61 died of unrelated causes and in 2 cases the cause of death was unknown. Recurrence was observed in 116 women with one individual lost to follow-up.

Two hundred ninety-six of the 315 tumours were assessable for MVD, in 10 cases there was no frozen tissue and in another 9 there was no invasive cancer in the new section. The median of all Chalkley counts was 3 with a range from 1-10. The relationship between MVD as a dichotomous variable (the median was used as cut-off level) and clinical and pathological parameters was investigated (Table 8). A Chalkley count above median was associated with nodal involvement and high SPF.

The cDNA-based sequencing method for analysis of p53 mutations was successful in all but 4 tumours. The number of tumours with a mutation was 69. Thus, in the whole material the proportion of tumours with a p53 mutation was 21,9%. Among the 311 sequenced tumours 19 were missing data on MVD. That left 292 cases for comparisons between p53 status and

MVD. Among tumours with MVD above the median the proportion of mutations was 27,1%. The corresponding figure for those with low MVD was 18,4%. This difference was near the level of statistical significance (p=0,075). We also found that there was a statistically significant correlation between high MVD and p53 mutations (p=0,037), if tumours (n10) with a mutation in the evolutionary conserved regions 2 or 5 were excluded. This was due to the fact that 6/10 tumours with mutation in region 2 or 5 had a low MVD. We then analysed MVD with reference to p53 IHC but no correlation could be established.

Table 8.

Relationship between MVD and other clinical and pathological features

Variable	**Grouping**	**% high MVD**	**χ^2 test**
Age	≤50 vs ≥51	50,0 vs 47,2	p=0,71
Tumor size	T_1 vs T_{2-3}	44,4 vs 51,7	p=0,21
Lymph-node status	N_0 vs N_1	43,2 vs 56,5	p=0,036
Estrogen receptors	pos vs neg	45,5 vs 58,6	p=0,073
Progesterone receptors	pos vs neg	45,9 vs 60,0	p=0,082
S-phase fraction	low vs high	45,7 vs 60,0	p=0,049
Vascular invasion	neg vs pos	44,7 vs 57,4	p=0,094
p53-mutation	neg vs pos	45,1 vs 57,6	p=0,075
p53 IHC	neg vs pos	46,2 vs 54,4	p=0,26

Analyses of OS, BCSS and RFS

There was no statistically significant correlation between MVD and outcome when analysis was done on all patients or on subgroups of node negative and node positive patients. Tumours with a mutation of the p53 gene entailed a statistically significantly worse prognosis with reference to OS, BCSS and RFS (Table 9). A possible prognostic value of MVD among tumours without p53 mutation was not consistent, a worse outcome in terms of OS (p=0,032) was reduced to a trend when BCSS (p=0,13) was analysed and the RFS did not differ (p=0,86) (Table 9).

Multivariate analysis showed that p53 was an independent risk factor for OS and BCSS with RHs of 1,91 (1,23-2,97) and 1,76 (1,01-3,08), respectively (Table 10). The RH of MVD did not reach the level of significance. Nodal status was the most powerful risk factor with a RH of 2,72 (CI 1,79-4,12) and 3,35 (1,98-5,67) for OS and BCSS, respectively. The relative hazards for MVD and p53 were stable through the models (Table 10), indicating that they did

not mutually confound each other or were confounded by tumour stage. However, due to the findings in Table 9 indicating a different effect of p53 mutation depending on MVD status, we allowed for an interaction between MVD and p53 mutation in one model. The interaction term was not statistically significant regardless if MVD was treated as a continuous or dichotomous variable, but the RH for p53 mutation was shifted upwards to a RH of 2,5-3,5 when the interaction term was introduced in the model (data not shown).

Table 9.

Life table analyses on overall survival (OS), breast cancer specific survival (BCSS) and recurrence free survival (RFS) at 10 years in relation to MVD and p53 mutation. p-values are from the log rank test.

		10 Years Cumulative Survival		
		OS % (SE)	BCSS % (SE)	RFS % (SE)
All patients	n 315	61,4 (2,7)	75,8 (2,6)	54,2 (3,6)
MVD low	n 154	64,8 (3,9)	77,8 (3,5)	54,1 (5,1)
MVD high	n 142	57,6 (4,2)	71,0 (4,1)	50,6 (5,4)
		p = 0,12	p = 0,17	p = 0,86
p53 wt	n 242	64,7 (3,1)	78,8 (2,8)	56,1 (4,1)
p53 mut	n 69	47,8 (6,0)	61,2 (6,4)	42,6 (7,9)
		p = 0,017	p = 0,024	p = 0,022
p53 wt, MVD low	n 124	70,0 (4,1)	80,6 (3,7)	56,1 (4,1)
p53 wt, MVD high	n 102	59,7 (4,9)	73,6 (4,6)	52,6 (6,6)
		p = 0,032	p = 0,13	p = 0,86
p53 mut, MVD low	n 28	36,1 (9,0)	49,5 (10,4)	33,5 (12,2)
p53 mut, MVD high	n 38	47,4 (8,1)	57,7 (8,7)	33,8 (9,6)
		p = 0,39	p = 0,73	p = 0,72
MVD low, p53 wt	n 124	70,0 (4,1)	80,6 (3,7)	56,1 (4,1)
MVD low, p53 mut	n 28	36,1 (9,0)	49,5 (10,4)	33,5 (12,2)
		p = 0,0059	p = 0,043	p = 0,27
MVD high, p53 wt	n 102	59,7 (4,9)	73,6 (4,6)	52,6 (6,6)
MVD high, p53 mut	n 38	47,4 (8,1)	57,7 (8,7)	33,8 (9,6)
		p = 0,54	p = 0,31	p = 0,057

MVD low denotes microvessel density ≤ 3. MVD high denotes microvessel density ≥ 4.

p53 mut denotes p53 mutation. p53 wt denotes p53 wild-type. SE denotes standard error.

Table 10.
Multivariate survival analyses showing relative hazards (RH). All analyses corrected for age.

Overall Survival

	RH (95%CI)		
Variable	univariate	model 1	model 2
MVD (continuous)	1.1 (0.98-1.3)	1.1 (0.96-1.2)	1.1 (0.92-1.2)
p53 mut (yes vs no)	1.9 (1.3-2.8)	2.0 (1.3-3.0)	1.9 (1.2-3.0)
T size (mm)	-	-	1.0 (0.99-1.0)
N+ (yes vs no)	-	-	2.7 (1.8-4.1)
S-phase (high vs low)	-	-	1.6 (1.0-2.4)

Breast Cancer Specific Survival

	RH(95%CI)		
Variable	univariate	model 1	model 2
MVD (continuous)	1.2 (0.99-1.4)	1.1 (0.96-1.3)	1.1 (0.89-1.3)
p53 mut (yes vs no)	1.8 (1.1-3.1)	1.8 (1.1-3.09)	1.8 (1.0-3.1)
T size (mm)	-	-	1.0 (0.99-1.0)
N+ (yes vs no)	-	-	3.4 (2.0-5.7)
S-phase (high vs low)	-	-	1.2 (0.67-2.2)

MVD denotes microvessel density. Mut denotes mutation T size is tumour size. N+ is axillary lymph-node positive. High S-phase: Diploid tumours >7%; non-diploid tumours >12%. CI denotes confidence interval.

General Discussion

Paper I-III

Clinical palpation as the only means of deciding whether a patient must undergo axillary surgery or not is inadequate. Fisher and co-workers [136] reported a false negative rate of 39% and a false positive rate of 27%. The corresponding figures in our study (I) were 31% and 19%. These somewhat lower figures are probably partly due to a lower prevalence of nodal metastases in a screened population. Clinical node negativity can not be considered sufficient for allowing omission of axillary surgery. However, the current praxis that all women with preoperatively palpable lymph nodes are recommended axillary operation is justifiable, given a proportion of positive nodes of 52% for cN1a, and 81% for cN1b (Paper I).

Larger tumour size was strongly correlated with the presence of lymph node metastases in our study (Paper I). This correlation has been reported several times before [33, 34]. However, tumour size alone cannot be considered as a sufficient means to delineate a low risk group since the proportion with lymph node metastases was 13% among women with cN0 status and tumour size ≤10 mm (Paper I). This risk is too high to allow omission of axillary surgery.

The third factor that correlated with lymph node metastases was SPF (Paper I). The correlation was limited to tumours ≤20mm. Stål and co-writers [42] reviewed 16 articles regarding the relation between SPF and nodal status, 13 did not show a statistically significant correlation whereas 3 did. The majority of cases in our study (70%) had a tumour size of 20 mm or less, most likely because of screening, and this could explain why most other investigators did not find a correlation. Data from 605 women was entered into a logistic regression analysis of correlation between high SPF, adjusted for tumour size among cN0 tumours (Paper I). The frequency of pN_1 in the ≤10mm subgroup was 14% and 21% in the low SPF and high SPF groups respectively. The clinical consequence of this finding is that one should not consider omitting axillary surgery in women with high SPF tumours. On the other hand, low SPF is not useful as a low risk criterion for a tentative subgroup in which axillary surgery could be omitted. The reason for this is that the correlation between high SPF and pN_1 is not strong enough and that small tumours with high SPF are rare.

Several studies with a similar design as in our study (Paper I) have been published [137-151]. The main findings regarding the most common risk factors of 15 such studies are summarised in Table 11. From Table 11 one can conclude that tumour size and peritumoural vascular invasion are the histopathological factors that are most consistently associated with nodal metastases with positive correlation in 12/15 [137-151] and 9/10 studies respectively.

Table 11.

Reference	Number of cases	Inclusion criteria	% pN1	T size (larger)	Young age	High grade	Clinical detection/non palpable	Vascular invasion
Chadha 139	263	pT1; cN0	27	+	NS	NS^{N}	ND	+
Ravdin 148	5963[1]	Complete data on risk factors	39	+	+	ND	ND	ND
Barth 138	918	pT1	23	+	NS	+^{N}	+	+
Fein 141	1598	pT1-2; ≥10N	28	+	NS	NS^{T}	+	+
Mustafa 146	1641	pT<=10mm	16	NS	+	+^{N}	ND	ND
Cutuli 140	893	cT<=30mm	25	NS	NS	+^{T}	+^{4}	ND
Velanovich 151	851	-	ND	+	ND	NS^{N}	ND	ND
Olivotto 147	4312	<90 years	32	+	+	+$^{N+T}$	+^{3}	+
Gann 143	14993	≥6N; <79 years	36	+	+	+^{T}	ND	ND
Gajdos 142	850	pT1	25	+	+	NS^{T}	ND	+
Gonzales-Vela 144	102	≥10N	53	+	ND	NS^{T}	ND	+
Shoup 150	204	pT1	25	+^{2}	NS	+$^{2, N}$	ND	+^{2}
Anan 137	1003	pT1	25	NS	+	ND	NS	+
Rivadeneira 149	919	pT;<=10 mm	18	+	+	+^{T}	ND	NS
Guarnieri 145	547	pT1; ≥5N	29	+	NS	NS^{T}	ND	+

[1]An additional 6001 cases in a validatin set. [2] Univariate analysis only. [3] Palpable tumour and/or palpable lymph nodes. [4] T0 vs. T1 vs. T2. [N] Nuclear grade. [T] Tumour grade. + denotes statistically significant correlation with lymph node status. NS = not statistically significant. ND = No data reported. N =lymph nodes.

As one can see from Table 11 the size of the cohorts varies largely and the selection of cases is also quite different between different studies. The prevalence of lymph-node metastases ranges from 16-53%. Two studies included only cancers with size ≤ 10mm [146, 149], while stage I-III cancers were included in another [147]. The fifteen studies [137-151] all tried to define a group with low risk for lymph-node metastases. The risk for having lymph-node metastases in these subgroups ranged from 0-17% (data not shown in table).

Since increasing tumour size is the most consistent risk factor for lymph node metastasis, our second study (Paper II) was focused on women with a tumour size of 10 mm or less. The use of detection mode (screening mammography vs. clinical) was based on findings in a study by Arnesson and co-workers [152] who reported that 9% of 229 screening detected cancers were lymph node positive compared to 20% of 89 clinically detected tumours. Our study (Paper II), based on data from two large breast cancer registries, showed that detection by screening mammography is associated with a considerable lower risk of positive lymph nodes when compared with clinically detected tumours. This finding was later confirmed with a multivariate analysis (Table 3), in which detection mode was adjusted for age and tumour size. This is in accordance with a study reported by Fein and co-writers [141], who also identified mammographical detection as a predictor of low risk for nodal metastases. The correlation between mode of detection and node positivity was retained in multivariate analysis [141]. The proportion of lymph node metastases was 15,8% among 487 women with mammographically detected tumours whereas 32,7% had lymph node metastases if the tumour was detected by physical examination. Fein and colleagues reported a 0% risk of lymph node metastases among mammographically detected tumours ≤ 5 mm and a 5-10% risk for mammographically detected tumours with histopathologic size 6-10 mm and age > 40. Other groups [138, 147] have used palpable versus non-palpable breast tumour as a potential predictor for nodal metastases. Both these studies showed that palpability is an independent risk factor for axillary metastases. A French study [140] reported that clinical tumour size (T0 vs. T1 vs. T2) was independently correlated with pN status.

Thus, the reason for mammographical detection being a predictor for node negativity seems to be something more than a mere matter of tumour size. This phenomenon is probably partly due to the tendency for mammography to detect biologically less aggressive cancers. Duffy and co-workers found that interval cancers and cancers among non-attenders had a significantly higher proportion of grade 3 compared with incident cases detected with mammography [153]. Hakama and co-writers [154] found that the proportion of diploid tumours was higher among cancers detected in incident screens compared with interval cases and cancers among non-attenders.

Based on the findings in our study (Paper II), we proposed a prospective Swedish multicentric cohort-study. After careful ethical and medical considerations, the study started accrual of patients in 1997. In this study axillary dissection is omitted in screening detected tumours with size ≤10 mm and grade I-II according to Elston and Ellis and/or low SPF. Women with multifocality or a prior history of cancer are excluded. The primary end point is axillary recurrence and a total of 1500 women are to be recruited. The accrual is estimated to stop early 2002. No results are yet available from this study.

The five node biopsy

Although there is hope that it will be possible to forgo axillary surgery in a low risk group, this subset comprises only a minority of newly diagnosed breast cancers. Thus, in order to improve the management of the axilla for the vast majority of women with breast cancer other strategies are needed. One alternative is lymph-node sampling of the axilla, a method that is less extensive than axillary dissection. The Scottish trial on sampling versus axillary clearance showed a sensitivity of 100% of a four-node biopsy from the axilla [118]. However, the estimation of sensitivity, which is a key parameter, was based on only 67 women. Moreover, the Scottish trial included patients with on average larger tumours than currently are seen in areas with well functioning screening. In contrast, our study of the 5-node biopsy (Paper III) included a large number of women (n 415) and half of them had screening detected cancers. The false negative rate in our study was 2,7% (Paper III), this estimate is encouraging since several studies of the sentinel node procedure have shown false negative rates of 6,7-11,4% [122-124]. The negative predictive value in our series was 98,5%, the corresponding estimate reported from the sentinel node procedure is 93-96%. The reproducibility of our study can be questioned, since one surgeon made all operations. However, for a specialised team a training period with 30-50 operations with the same technique as in this trial would be easily accomplished. The recommendations for the sentinel node procedure are similar.

The only direct comparison between a sampling procedure and the sentinel node technique that has been published [155] included 200 women. Ten out of 60 node positive patients were not detected with the sentinel node procedure. The corresponding figure for 4-node sampling was 1/60. The value of this study has been questioned [156] because of the use of suboptimal

amounts of radiocolloid and the lack of an established reference method for comparison, i.e. no axillary dissection was performed.

An interesting aspect is that serial sectioning and/or immunohistochemical staining of the sentinel node(-s) often are employed [122, 124], whereas only routine pathological examination was performed in our study (Paper III). In a recent review [157] it is estimated that the use of IHC and/or serial sectioning produces a conversion to node positivity in 9-33% of tumours initially classified as node negative due to detection of micrometastases. It is reasonable to believe that the use of more sensitive pathological methods is an important part of the sentinel node procedure, it is even tempting to hypothesise that the sensitivity could be higher with the sentinel node procedure in comparison with level I-II dissection. A possible argument against this is that micrometastatic sentinel nodes sometimes are accompanied with macrometastases in non-sentinel nodes [158]. Serial sectioning and IHC are feasible when used in conjunction with the sentinel node procedure but have been considered too unpractical to be used in routine handling of specimens from level I-II dissections. The 5-node biopsy represents something in between. One could speculate that the performance of the 5-node biopsy could be improved by using these refined histopathological techniques. Although there seems to be a worse outcome for women with lymph node micrometastases compared with those without [157], the long-term prognostic value of IHC-positive sentinel nodes is still unknown.

In contrast to most studies on the sentinel node procedure we reported the –LR. This measure of a test's performance is not affected by differences in prevalence between populations and it can be used to calculate the post-test probability if the pre-test probability is known. In this context the pre-test probability is the prevalence of lymph node metastases in a population with breast cancer. An LR of 1 means that the post-test probability is exactly the same as the pre-test probability. Likelihood ratios greater than 10 or less than 0,1 generate large and often conclusive changes from pre-test to post-test probability [159]. The –LR of 0,027 for the 5-node biopsy thus supports the view that this procedure can be a valuable diagnostic tool.

Although axillary surgery per se not seems to affect survival [109] the risk of withholding adjuvant treatment for the women that were falsely defined as node negative must be considered. This problem has decreased during recent years since most breast cancer patients

nowadays will be given adjuvant tamoxifen and even chemotherapy regardless of lymph-node status [160]. In our series 3 out of 4 women with lymph node metastases not detected in the 5-node biopsy would have been treated with tamoxifen according to current guidelines. The fourth woman would not have been offered adjuvant systemic treatment if her nodal status had been based on the 5-node biopsy only. This potential risk of under-treatment must be balanced with the benefits of a less traumatic surgical procedure in the rest of the patients.

Even though we do not know the true incidence of arm symptoms after a 5-node biopsy, the Scottish group has reported less morbidity of 4-node sampling compared with axillary clearance of level I-III [120, 161]. Moreover, several studies indicate that increasing number of removed nodes causes increased incidence of arm morbidity [14, 116, 117]. It is also most likely that the sentinel node procedure causes less arm morbidity than a 5-node biopsy.

Although the sentinel node procedure probably is superior, in terms of less associated morbidity, the 5-node biopsy seems safe to use as an alternative to level I-II dissection in women not suitable for a sentinel node procedure. Ideally, the 5-node biopsy should be compared with the sentinel node procedure in a randomised study.

Paper IV-V

Estimation of angiogenesis, methodological considerations

A large number of studies have reported that prognostic information can be obtained from estimation of MVD [18, 81-85, 87-92], but several studies question the use of MVD as a prognostic factor [93-99]. The reason for these conflicting results is probably methodological problems, maybe related to the immunohistochemical technique, inter observer variability and/or intra tumoural heterogeneity. Our methodological study (Paper IV) focuses on the latter. Present methodological recommendations [162, 163] advocate determination of MVD in the areas of highest vascular density (hotspots). The underlying hypothesis for the hotspot method is that systemic dissemination of cancer cells is more likely in the areas of highest vessel density [18]. Since identification of hotspots is of great importance for the method, the chance of identifying the “hottest-spots” would most likely be influenced by the potential variation of vascular density between different parts of a tumour.

Our prospective study, which employed a predefined and systematic handling of all tumour material, demonstrates that there is a marked intra tumoural variation of MVD (Paper IV). In the nested ANOVA analysis the variation between different sections contributed more to the total variance than the variation between different tumours, 37,3% and 45,0% of the total variance, respectively. This means that there is a risk that a MVD score is more influenced by the sampling procedure and less by the true angiogenic capacity of the tumour. The influence of intra tumoural heterogeneity on the results has been addressed in previous studies but, as can be noticed below, there has been no standardised way of designing or reporting studies on heterogeneity. In a Dutch study [164], a design similar to ours was used. They analysed all available blocks (2 - 4) from 10 breast cancers. Their data showed that the variation between blocks contributed more to the total variation than the variation between tumours, which is identical to what we found. The CVs for different blocks from the same tumour in the Dutch study ranged from 5,7 - 54,9%. Although the sampling technique was not identical in our study, the corresponding range of CVs in our material, based on twice as many cases, is quite similar (9,4 - 44,1%) (Paper IV). The Dutch group concluded that sections from multiple blocks should be analysed to identify the "hottest-spot" for each tumour, a handling, which in routine use would be unpractical. Bosari and co-workers [82] retrospectively examined all available blocks (on average three) from 120 breast cancers. In 14% of the cases the difference between the average and the maximum score was more than 20%. However, it is unclear if this moderate variation predominantly was the result of intra- or intersection heterogeneity.

The variation within each section constituted 17,7% of the total variance in our study. The difference between average score and maximum score exceeded 20% in 19% of our 115 sections (Paper IV). The intra section variation was much more pronounced in a study reported by Axelsson and colleagues [95] with a greater than 20% difference in 49% of 220 breast cancers. They could not find any prognostic value of MVD and concluded that this was mainly due to variability from field to field within the same section. Van Hoef and co-writers [94] retrospectively analysed MVD in 93 breast cancers, without finding any independent prognostic information. In 41 of the tumours, MVD was assessed in two sections from two different blocks. This comparison showed a concordance with reference to MVD categories of 71 - 78%, which could introduce a substantial error in the method according to the authors.

Some studies have claimed that intra tumoural heterogeneity is of less importance [165, 166]. The study by Martin and co-workers [165] comprised 20 breast cancers and included an analysis of three different blocks from each tumour. Eighty-five percent of the patients were correctly assigned to the high MVD and low MVD groups regardless if one or three sections were taken into account, they drew the conclusion that the MVD measured in one section is representative of whole tumour vascularity. The Danish study [166] mainly dealt with the reproducibility of different counting procedures but they also concluded that intra tumoural variation does not substantially affect the results. The relevance of this conclusion must be questioned since they selected 40 archival cancers with the object of obtaining different degrees of MVD. This could lead to overestimation of the inter tumoural variation in relation to the intra tumoural variation.

It has been argued that the probability of finding the hotspots can be increased by counting the ten apparent highest fields [84]. In another study, counting 4 hotspots gave better prognostic information than 10 [91]. The suggestion to count several hotspots seems to be supported by our findings. The problem with this approach according to our data, is that if one counts a large number of apparent hotspots it is likely that one will end up with a high score in most tumours. In our study (Paper IV), all tumours had at least one score above the median MVD if all scores from each tumour were taken into account.

We used an antibody to CD31 in both our studies on angiogenesis (Paper IV-V). Anti-CD31 has been recommended by the international consensus on the methodology and criteria for quantification of angiogenesis as being the most useful antibody, followed by anti-CD34 and anti-FVIII-Rag [163]. One advantage with anti-CD31 is that it does not react with lympho-endothelial cells [163]. Martin and co-authors found anti-CD31 less reliable with up to 13% failed stainings [84] compared with 2% for anti-CD34 and 1% for anti-FVIIIRag. In our studies, 6% (Paper IV) and 0% (Paper V) were judged as inadequate.

Interobserver variability is not a negligible problem, variable reproducibility between different observers has been reported ($0,45 < r < 0,99$)[166]. By following the consensus recommendations [163], and having two observers that simultaneously assessed the slides (Paper IV), we tried to reduce the impact of interobserver variability. In the other study (Paper V) we had only one observer but a Chalkley eyepiece graticule was used for the assessment of

MVD. Hansen and co-workers [166] have shown that the interobserver variability is lower for the Chalkley method compared with classical counting of microvessels. The Chalkley method is probably more robust since no decisions have to be made on whether adjacent stained structures are separate countable microvessels or not. Furthermore, the Chalkley graticule method makes the procedure less time consuming [135].

To conclude the methodological discussion, it seems quite clear that intratumoural heterogeneity can influence the results and that this represents a major problem for the method. Intratumoural variation seems to be one main reason for the conflicting data on the usefulness of MVD as a prognostic factor in breast cancer. Interobserver variability and immunohistochemical technique also are likely contributors to diverging results.

The relation between angiogenesis and p53

Although there are methodological problems associated with MVD, we chose to continue our studies on MVD (paper V) due to the following reasons: *Firstly,* the important role of angiogenesis in the malignant progression of solid tumours is undisputed. *Secondly,* mutations of the tumor-suppressor gene p53 has been demonstrated to regulate not only apoptosis and proliferation but also angiogenesis [78, 79], which gives a molecular rationale for a relationship between MVD and mutations of p53. *Thirdly,* the opportunity to analyse the potential relationship between MVD and cDNA determined p53 mutations for exons 2-11, which has not been investigated before, to the best f our knowledge. *Fourthly,* the opportunity to study this relationship on a large population based cohort of women with complete and long-term follow-up.

The main finding of our study is that high MVD, assessed by the Graticule counting method, seems to correlate with mutations in the p53 tumour suppressor gene. The proportion of p53 mutation was 27,1% among women whose tumour had a high MVD, the corresponding figure for those with a low MVD was 18,4%. The correlation between high MVD and p53 mutation reached only borderline statistical significance (χ^2-test, p=0,075). However, we found that there was a statistically significant correlation between high MVD and p53 mutations (χ^2-test, p=0,037) when tumours (n10) with a mutation in the evolutionary conserved regions 2 or 5 were excluded. In fact, 6/10 tumours with mutation in region 2 or 5 had a low MVD. We do not know if this is a reflection of a more complicated relationship between angiogenesis and

p53 status or if it is merely a play of chance due to the relatively low number of women with a p53 mutated tumour. Therefore, this retrospective subanalysis must be interpreted cautiously.

Our study could not show any correlation between MVD and p53 analysis by IHC and this finding is in agreement with several previously published studies [102, 167-170]. Thus, studies on angiogenesis and p53 IHC fail to support the thrombospondin-1 based mechanism. The reason for this could be that immunohistochemistry is inferior to sequenced based determination of p53 status [60, 61, 171]. The false negative and false positive rate with p53 IHC was 33% and 30%, respectively, compared with sequenced based p53 status [60].

MVD and lymph-node status

Among standard prognostic factors, high MVD was associated with positive lymph-node status (p=0,036) and high S-phase (p=0,049). The proportion of lymph-node positive women with high MVD was 56,5%, i.e., more than 40% of lymph-node positive women had primary tumour with a low MVD. Thus, MVD is unlikely to be of practical value for predicting the axillary lymph-node status. In a simple predictive model consisting of only a few criteria, that ideally should be easy to adopt in the routine management of breast cancer, there is a need of powerful predictive factors like tumour size and detection mode (Paper I-II). Moreover, the methodological aspects of MVD are problematic as have been outlined above.

MVD and p53 in relation to prognosis

No prognostic information could be obtained from MVD status in our study. Women whose tumours had a mutation of the p53 gene had a statistically significantly worse prognosis with reference to OS, BCSS and RFS according to univariate analysis and this effect was retained in multivariate modelling. Women with tumours with low MVD and mutation seemed to do worse with reference to OS and BCCS compared with women with high MVD and p53 mutation. Although this difference was not statistically significant, it is a bit surprising since the difference was in the wrong direction compared with what one would expect. When we explored the possibility of a more complicated relationship between MVD status and p53 mutation in terms of prognosis and introduced an interaction term in the multivariate modelling, our findings were not convincing for an interaction. Although the number of women in these subgroups were limited and the data must be interpreted with caution, we

found the pattern interesting enough to be worth studying further when we accumulate more events.

The lack of prognostic power of MVD in our study is not surprising. It has been mentioned before that several studies have reported negative findings [93-99]. In order to avoid methodological pitfalls as much as possible we followed the recommendations that has been published [163] with the exception that the readings of the scores were done by one person. We also used the Chalkley eye-piece graticule counting method which is time saving [135] and possibly superior from a methodological standpoint [166]. One limitation of our study was the use of relatively small tumour sections. This could lead to underestimation of MVD, especially since the intratumoural heterogeneity of this parameter is pronounced. Nevertheless, the lack of prognostic information from MVD in this rather large cohort of women with complete and long-term follow-up is not supportive for the use of MVD as a prognostic factor in breast cancer.

General conclusions

- Positive clinical lymph node status, increasing tumour size and high S-phase fraction are all associated with increased risk of axillary lymph-node metastases in breast cancer. However, even if these factors are used in combination, they can not define a subgroup of women with a sufficiently low risk of having lymph node metastases to allow omission of axillary surgery.

- Breast cancers diagnosed with screening mammography have a lower risk of having axillary lymph node metastases than clinically detected tumours. This association is independent of the woman's age and the size of the tumour. Diagnosis by screening mammography and tumour size ≤ 10 mm are criteria, combined with other low risk features that may be useful for characterisation o a subgroup of women in which axillary surgery may be omitted as a part of a prospective study.

- Five-node biopsy of the axilla has a high sensitivity and a low false negative rate compared with axillary dissection of level I-II in women with breast cancer. The 5-node biopsy is an alternative to axillary dissection and compares very favourably with results reported in the literature from the sentinel node biopsy procedure.

- Angiogenesis estimated with MVD shows a marked variation between different sections from the same tumour. Intra tumoural hetereogeneity in breast cancer makes the use of MVD questionable.

- There seems to be a relationship between angiogenesis (MVD) and cDNA sequenced determined p53 mutations for exons 2-11 in primary breast cancer. Tumours with high MVD more often had p53 mutations. p53 status had independent prognostic value in a multivariate analysis whereas MVD had not.

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Studies on Prediction of Axillary Lymph Node Status in Invasive Breast Cancer

Errata:

Page 28, first paragraph: ”…false negative rate of 1,5%…” is wrong
”…false negative rate of 2,7%…” is corrrect