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**ON PROGNOSTIC AND TREATMENT
PREDICTIVE FACTORS IN EARLY
STAGE BREAST CANCER**

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ABSTRACT

Breast cancer is the most common malignant tumor in Swedish women and the incidence is increasing with 1-2% per year in the Stockholm area. A subgroup of patients is women diagnosed with primary inoperable disease. This group constitutes about 15% of all women affected by breast cancer. Primary systemic (neoadjuvant) therapy either with chemotherapy or endocrine therapy is frequently used in these patients.

Prognostication is especially important in identifying patients whose prognosis is so favorable that adjuvant systemic therapy is unnecessary. Prognostic factors can also be useful in identifying patients with poor prognosis that warrants an aggressive approach. In the neoadjuvant setting the patients receive preoperative treatment and the behavior of the tumor during treatment may act as a biological model. Beside the earlier described prognostic factors additional factors such as clinical tumor response and early or late changes in biological markers during therapy may be of help in deciding the most beneficial therapy for the individual patient. However, the determination of a patient's clinical response to neoadjuvant therapy is sometimes difficult. An inaccurate response evaluation may prevent an early identification of non responders.

The conclusions from the thesis are:

1. Proliferation fraction (PF) (assessed in preoperative fine needle aspiration (FNA) biopsies) has a significant prognostic value which is independent of lymph node status, PgR status and tumor size. To our knowledge this is the first study demonstrating that PF can contribute prognostic information when analyzed in preoperative smears.
2. PAI-1 (assessed in surgical specimen) is a significant independent prognosticator independent of lymph node status.
3. A decrease in PF > 25% (assessed in FNA biopsies) during preoperative chemotherapy have a predictive value and may be of value in selecting postoperative adjuvant systemic treatment.
4. In elderly patients, a high initial PF (assessed in FNA biopsies) may predict a decreased probability of response. Moreover, a decrease in the percentage of ER positive cells > 50% after 3 months tamoxifen therapy significantly predicted a lower probability of a long-term clinical response
5. Cathepsin D (assessed in surgical specimen) may predict the benefit of tamoxifen amongst ER-positive patients.
6. There is a poor correlation between clinically and mammographically assessed tumor size. Menopausal status, BMI and use of HRT are factors that could influence the correlation between the two assessments. Clinical assessment may not be the optimal method for response evaluation of preoperative systemic therapy. Mammographic assessment contributes with changes in size as well as density and gives a reproducible information.

Key words: prognostic factors, predictive factors, PF, Cathepsin D, PAI-1, changes in biological markers, ER, FNA

LIST OF PAPERS

This thesis is based on following papers which are referred in the text by their Roman numerals:

- I Billgren A-M, Azavedo E, Svane G, Skoog L, Rutqvist LE:
Clinical Examination versus Mammography in Assessment of Tumor Size and Response to Preoperative Therapy in Breast Cancer.
Manuscript
- II Billgren A-M, Rutqvist LE, Tani E, Wilking N, Fornander T, Skoog L:
Proliferating Fraction During Neoadjuvant Chemotherapy of Primary Breast Cancer in Relation to Objective Local Response and Relapse-free Survival.
Acta Oncologica , 38 ,597-601, 1999
- III Billgren A-M, Tani E, Liedberg A, Skoog L, Rutqvist LE:
Prognostic significance of tumor cell proliferation analyzed in fine needle aspirates from primary breast cancer.
Breast Cancer Research and Treatment 71: 161-170, 2002
- IV Billgren A-M, Rutqvist LE, Johansson H, Hägerström T, Skoog L:
The Role of Cathepsin D and PAI-1 in Primary Invasive Breast Cancer as Prognosticators and Predictors of Treatment Benefit with Adjuvant Tamoxifen.
The European Journal of Cancer, 36,11, 1374-1380, 2000
- V Billgren A-M, Tani E, Skoog L, Wilking N, Rutqvist LE
Hormone Receptor Content and Cell Proliferation in Primary Breast Cancers During Tamoxifen Therapy.
Manuscript

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LIST OF ABBREVIATIONS

BMI	Body mass index
C.I.	Confidence interval
CR	Complete response
CT	Chemotherapy
DFS	Disease free survival
DRFI	Distant recurrence free interval
ER	Estrogen receptor
FNA	Fine needle aspirate
HR	Hazard ratio
HRT	Hormone replacement therapy
HT	Hormonal therapy
Ln	Lymph nodes
MIBI	[Tc-99m]-sestamibi
MRI	Magnetic Resonance Imaging
Neg.	Negative
No	Number
NS	Non significant
P	Probability
PAI-1	Plasminogen activator inhibitor-1
PD	Progressive disease
PET	Positron Emission Tomography
PF	Proliferating fraction
PgR	Progesterone receptor
Pos.	Positive
PR	Partial response
SD	Stable disease

INTRODUCTION

Breast cancer is the most common malignant tumor in Swedish women and the incidence is increasing with 1-2% per year in the Stockholm area. One out of nine women in Sweden will develop the disease during her lifetime. The primary treatment of breast cancer is surgery but most of the patients receive some kind of adjuvant treatment, either as radiotherapy, hormonal treatment or chemotherapy or a combination thereof. In spite of early diagnosis through mammography screening programs and more intense treatment schedules, about a third of the patients treated for breast cancer will experience a recurrence. These women are treated with systemic cytotoxic or hormonal therapy and in case of local recurrence also with surgery and radiotherapy. Breast cancer is still the single the most frequent cause of death in women of age 45-54 years in Sweden.

A subgroup of patients is women diagnosed with primary inoperable disease. This group constitutes about 15% of all women affected by breast cancer. Primary systemic (neoadjuvant) therapy either with chemotherapy or endocrine therapy is frequently used in these patients. The patients may become operable and in some cases tumor shrinkage may make a breast-conserving surgery possible.

Both with hormonal treatment as well as with chemotherapy it is often difficult to predict the response of the given therapy. This applies both to adjuvant treatment of primary disease and treatment of advanced disease. With hormonal treatment, a prerequisite is a hormone receptor positive tumor both in the adjuvant setting and in advanced disease. However, since some ER-positive tumors do not respond, there is a need for additional markers. Regarding chemotherapy tumors with a high proliferating rate have been claimed to benefit the most. The use of preoperative therapy in combination with the FNA technique has made it possible to study changes in biological markers during therapy. Such changes may be markers of sensitivity in relation to a given therapy and be of help to clinicians in deciding the most beneficial therapy in the individual case.

Well-established prognostic factors include lymph node involvement, tumor size, tumor stage and hormone receptor status. The value of proliferation rate measured with different methods has been suggested to be clinically relevant. However, its value as a prognosticator or predictor of the effect of systemic therapy in the adjuvant setting has not been demonstrated in large, prospective randomized trials. Other factors that have been suggested to be of clinical relevance include *bcl-2*, *c-erbB-2*, *p53*, cathepsin D and PAI-1.

BACKGROUND

CLINICAL ASPECTS

To the clinician, the role as advisor of the best individual treatment is intricate. In the adjuvant setting the clinician has knowledge of well-established prognostic factors such as tumor size, tumor stage, number of lymph node metastases, hormone receptor content(7,42,109,129). In addition, the surgical material may have been examined for other biological markers as proliferating rate, apoptosis marker bcl-2, presence of *c-erbB-2* and changes in p53. The recommended adjuvant treatment is based on statistical evidence of benefit for a subgroup of patients with the same established prognostic factors. Prognostication is especially important in identifying patients whose prognosis is so favorable that adjuvant systemic therapy is unnecessary. Prognostic factors can also be useful in identifying patients with poor prognosis that warrants an aggressive treatment approach.

In the neoadjuvant setting the patients receive preoperative treatment and the behavior of the tumor during treatment may act as a biological model. Beside the earlier described factors additional factors as clinical tumor response and early or late changes in biological markers during therapy may be of help in deciding the most beneficial therapy for the individual patient. However, the determination of a patient's clinical response to neoadjuvant therapy is sometimes difficult. An inaccurate response evaluation may prevent an early identification of non responders (80).

The increasing number of treatment options in invasive breast cancer underscores the importance of possible predictors of treatment effects as well as prognosticators.

CLINICAL RESPONSE EVALUATION

Primary systemic (neoadjuvant) therapy either with chemotherapy or endocrine therapy is frequently used in patients with locally advanced breast cancer. The patients may become operable and in some cases tumor shrinkage may make a breast-conserving procedure possible (8,86,116). Clinical assessment of tumor size, using criteria according to WHO, may be difficult to reproduce over time or between different clinicians as illustrated in previous studies (23,45,79,110,113).

Mammography may allow a more accurate measurement of tumor size. Mammograms permit a retrospective assessment and should, therefore, potentially be less influenced by inter-and intra-observer variability. The role of mammography in evaluation of response to therapy has been studied in previous reports, which suggest that the response rates were too high when the tumor was assessed clinically compared to mammographically using UICC criteria (17,23,40,80,85,110,113).

Clinical complete response after hormonal or cytostatic therapy has been suggested as a prognostic factor for disease free survival (36,39,40,58,61). In patients who later become candidates for surgery, the histopathological features of the residual tumor might give additional valuable information. The approach correlating clinical examination and mammographic assessments of tumor response to histo-pathological features has earlier been addressed (36,40,45,59,63,131).

Ultrasound (39,40,45,59), MRI, (2,104), MIBI-scint (75,79) and PET (121) have been tested in response evaluation. The results have suggested that all modalities add information to that of clinical examination but due to small numbers of patients their potential role has to be confirmed.

PROGNOSTIC FACTORS AND TREATMENT PREDICTIVE FACTORS

A prognostic factor for primary breast cancer may be defined as any assessable factor available at the time of diagnosis, that is associated with disease free survival (DFS) or overall survival (OS) in the absence of systemic adjuvant therapy (20).

Well-established prognostic factors includes lymph node involvement, tumor size, tumor grade and hormone receptor status(7,34,42,70).

A treatment predictive factor is here defined as any measurement associated with response or lack of response to a particular therapy or with better DFS or OS in the adjuvant setting. Well established treatment predictive factors are hormone receptor content for hormonal treatment and a tumor with high levels of *CerbB-2* for trastuzumab treatment.

DIFFERENT BIOLOGICAL APPROACHES

The rationale behind studying different mechanisms of action in cancer cells is to find prognostic and treatment predictive factors. In this study we have focused on three different approaches.

1. The significance of presence/absence of hormone receptors as an indicator of external influence of the tumor cell.
2. Proliferating rate as an indicator of internal tumor cell factors.
3. The significance of markers for invasive/metastatic potential of the tumor.

Other important mechanisms of action include apoptosis with bcl-2 as a marker and genetic alterations in cancer cells as reflected in different expression levels of p53 and *CerbB-2* (117,120), but they have not been addressed in this thesis.

HORMONE RECEPTORS

Estrogen receptors can be detected in 50-80% of the tumors from breast cancer patients. In the past, the receptor content was determined by a dextran-coated charcoal biochemical assay. More recently, most laboratories use an immunohistochemical assay (estrogen and progesterone receptor immunohistochemical assay, ERICA and PRICA). ERICA is preferable in that it does not require fresh tissue, allows correlation with histology, and can be performed even on very small tumors. The expression levels are related to patient age, with a lower incidence in younger premenopausal patients(19). Tumor ER status is also related to a variety of histologic characteristics of the tumor(43). Low grade tumors are nearly always ER-positive in contrast to tumors

demonstrating histologic evidence of poor tumor differentiation which are frequently ER-negative. However, no consistent associations with either tumor size, lymphatic or vascular invasion or axillary lymph node involvement has been reported. Though ER is required for estrogen-stimulated growth, many studies have correlated ER expression with the cell cycle kinetics of the tumor. In a study comprising more than 100 000 primary breast cancer tumors, it was conclusively demonstrated that a high proliferating rate measured as S-phase fraction was associated with hormone receptor negativity(130).

The antiestrogens such as tamoxifen acts through antagonizing ER-mediated transcriptional activation of genes required for tumor growth (93).

About 50 % of early breast cancers have PgR and of these most also are ER positive. Only <5% of the tumors have PgR without ER - an expected result if PgR is regulated by estrogen. This is in line with the fact that the amount of PgR in the tumor are related more to menopausal status than to age (19).

Tamoxifen is a non-steroidal antiestrogen. It antagonizes ER-mediated transcriptional activation of genes required for tumor growth. Tamoxifen was the first selective ER modulator (SERM) described. In spite of its anti-estrogen action in breast cancer, tamoxifen has estrogen agonistic effects in other organs such as endometrium, bone and the cardiovascular system. In bone and the cardiovascular system, this effect is beneficial, but long-term use of the drug results an increased risk of endometrial carcinoma (46). In the breast, tamoxifen has been claimed to upregulate PR indicating a partial estrogenic agonistic effect (62). Other putative functions of tamoxifen is suppression of IGF-1 levels and inhibition of IGF-1 stimulated growth possibly via interaction with growth hormone secretion (66,78).

Hormone receptor content as a prognostic factor

More than twenty years ago, ER status was first reported to correlate with prognosis (70). ER-positive patients were found to have longer DFS and OS compared to those with ER-negative tumors, suggesting that it was independent of treatment received. Numerous other studies have confirmed the prognostic value of ER-positivity. However, later studies with longer follow-up suggested that the improved prognosis in patients with ER-positive tumors may not be sustained (1,55). One report suggests an initially reduced risk for relapse in ER-positive patients, but this relative risk increases with time, so that with long follow-up, the prognostic significance disappears (60). In conclusion, the earlier demonstrated significance of ER positivity as a prognostic factor has been questioned, but it is still regarded as a significant but weak prognostic factor (93).

Hormone receptor content as a predictive factor

In the adjuvant setting

With hormonal treatment, a prerequisite is a hormone receptor positive tumor. This fact was early addressed in a large randomised study (109) and have later been confirmed in an overview of many randomised trials (52). Highly significant reductions in the annual rate both of recurrence and of death are produced by tamoxifen. Tamoxifen also significantly reduced the risk of contralateral breast cancer. The benefit of tamoxifen in

relation to recurrence is most pronounced in the first 5 years, but the mortality reduction is highly significant both in the first 5 years and the following 5 years, so the cumulative differences in survival are larger at 10 than at 5 years. Five years treatment is significantly more effective than shorter tamoxifen regimens.

Due to the fact that ER-negative tumors often have higher proliferating rate and are more likely to be amplified for *c-erbB-2* oncogen, one study has addressed the question if absence of ER could predict response to adjuvant chemotherapy (87).

In the neoadjuvant setting

Tamoxifen has been frequently used as primary treatment in elderly patients with breast cancer (3,5,48,50,88,118,119). A prerequisite for response to tamoxifen treatment is the presence of estrogen receptors in the tumor cells (3,15,26,32,52,95,109). Benefit from tamoxifen treatment has been shown even in tumors with a low proportion of positive cells (15,68), but tumors with a high proportion of positive cells have been reported to have a higher response rate (14,119). The level of ER for tumor responsiveness varies between different studies but several early studies suggested that more than 20% of the tumors should be positive in responsive tumors (26,48,118,119). Later studies in which both the percentage and intensity of receptor staining were taken into account, the threshold for response was reported at a score as low as 10 (on a scale with a maximum of 300) (15,68). These differences are most likely explained by technical differences in the evaluation of receptor positivity.

The rates of best clinical response in ER positive tumors have been reported to be between 65-80% (3,15,26,37,68,95). However, some tumors with high ER positivity fail to respond and some, which showed initial response relapse eventually, which demonstrates that additional predictors of long-term response to tamoxifen are of clinical importance.

Changes in hormone receptor content during hormonal treatment as a predictive factor

Several studies have focused on changes in ER and PgR during tamoxifen treatment (15,62,68,76,89,105,122). Most studies describe a decrease in ER content during tamoxifen treatment despite the interval between the two measurements were from 2 weeks to three months. The decrease in ER content could not in these studies significantly predict clinical response. In one study an increase in PgR content was demonstrated, in univariate analysis, to have a significant predictive value (15).

The results imply that changes in hormone receptor content during therapy may help to predict response.

PROLIFERATION RATE

Tumor cell proliferation has been extensively investigated as a potential prognostic marker, as well as a treatment predictive factor. The proliferation rate has been measured on surgical specimens in most studies, but also on cytological smears. The correlation between assessment on cytological smears and surgical specimen has been examined and showed a very good correlation (81,91).

The proliferation rate is in most studies measured with S-phase fraction (measured with flow cytometry or single cell techniques) (SPF), thymidine labelling index (TLI), mitotic index (MI) or with antibodies against Ki-67/MIB-1. The correlation between different methods is weak or non-existent in some studies ($r=0.22-0.42$) (49,72,106), but rather good in others ($r=0.72-0.91$) (11,12,33,83,102,126). One of the concerns in the use of these various measures of proliferation is ensuring standardization.

The rationale behind the use of Ki-67/MIB-1 analysis in our material, is the fact that it can be performed on both fresh tumor tissue as well as on paraffin embedded tissue with small amounts of tissue with absolute morphologic control. TLI on the other hand, requires fresh tumor tissue with metabolic intact cells, which severely limits the use of this technique. Analysis of S-phase fraction as measured by flow cytometry is accurate, but requires a large number of cells. The method is also expensive and does not allow morphologic control of the analyzed cells.

Proliferation rate as a prognostic factor

The value of proliferation rate measured with different methods has been suggested to be clinically relevant even though its value as a prognosticator is under discussion.

In many early large cohort studies, PF has been claimed to be a significant prognosticator. However, other studies have not confirmed these findings..

In some studies, however, the above definition of a prognostic factor was not fulfilled, due to intervening adjuvant systemic therapy or lack of information about given systemic therapy. For this reason it will be difficult to show the true prognostic value of PF in lymph node positive breast cancer patients, since most of these patients receive adjuvant systemic treatment. However, there are older studies including more than 100 patients, with both lymph node positive as well as lymph node negative patients, demonstrating an independent prognostic value of PF, in multivariate analysis (41,49,69,84,98-100,117,127,128). On the other hand, some other studies could not demonstrate an independent prognostic value for PF (54,73,132).

In lymph node negative patients the use of systemic therapy has been less frequent and PF has been demonstrated to be a significant independent prognosticator in multivariate analyses in some studies (12,13,18,22,64,77,82,96,107,114,115,124,133). Another study has not confirmed these findings (56). However, in this study an invasive marker PAI-1 was introduced in the multivariate analysis and this variable was significant but not PF. The above referred studies are listed in Table 1.

A high PF of the tumor in patients with lymph node negative cancers, is regarded as a prognostic factor in many breast cancer units. It is taken into account in addition to tumor size and potential lack of hormone receptors in deciding accurate adjuvant treatment.

Table 1: Studies with PF as an independent prognostic factor in multivariate analysis

Reference	Method	No	Group	Adj. Treatment	Multivariate	
					DFS	OS
Haerslev T, 1996	Ki-67	487	All	Unknown		NS
Railo M, 1993	Ki-67	327	All	Unknown	<0.02	
Molino A, 1997	Ki-67	322	All	N+preCT N+post HT	0.02	0.02
Pierga J-Y, 1996	Ki-67	127	All	11%CT 25%HT	0.03	
Lipponen P, 1992	MI	252	All	1% CT 2% HT	NS	
Pinder SE, 1995	MIB-1	177	All	No	-	<0.05
Fisher B, 1991	SPF	377	All	No	0.04	0.08
Sjögren S, 1998	SPF	270	All	11% CT 29% HT		<0.01
Toikkanen S, 1989	SPF	223	All	Unknown		0.02
Klintonberg C, 1986	SPF	210	All	11% CT 15% HT	0.04	
Gasparini G, 1994	SPF	168	All	N+pre CT N+post HT	<0.001	<0.001
Tubiana M, 1989	TLI	125	All	No CT	<0.05	0.05
Witzig TE, 1993	SPF	502	N+	86% CT	NS	NS
Brown RW, 1996	Ki67	618	N-	17% CT 12% HT	0.001	
Rudolph P, 1999	Ki-67	332	N-	23% HT	<0.001	<0.001
Harbeck N, 1999	Ki-67	125	N-	No	NS	
Clayton F, 1991	MI	378	N-	Unknown		<0.001
Merkel DE, 1993	SPF	280	N-	No	NS	0.05
Witzig TE, 1994	SPF	265	N-	No	0.05	NS
Sigurdsson H, 1990	SPF	250	N-	5% CT 23% HT	0.03	<0.001
Isola J, 1992	SPF	213	N-	No		<0.001
Stål O, 1993	SPF	152	N-	No	0.035	
Silvestrini R, 1997	TLI	3800	N-	No	0.08	0.02
Paradiso A, 1995	TLI	101	N-	No	<0.05	
Bryant J, 1998	SPF	1249	N-(ER+)	67% HT	<0.001	<0.001
Malmström P, 2001	SPF	204	N-(pre)	9% CT 3% HT	<0.001	
Clark GM, 1989	SPF	112	N-dipl.	Unknown	0.01	0.02

Proliferation rate as a predictive factor

In the adjuvant setting

The question of proliferation indices as predictors for response to polychemotherapy was introduced more than 10 years ago. Initially high PF was claimed to be a predictive factor in several small studies for all patients (9,47,101). In later studies the benefit was linked to only premenopausal patients (92,103). Others could not confirm the predictive value of PF (29). In the cohort of lymph node negative breast cancer patients, two randomised prospective trials have been conducted to find out if PF could identify the subgroup, which would benefit from adjuvant polychemotherapy (4,97). The results

from the studies support the use of PF as a predictive tool in selecting patients who will benefit most of adjuvant polychemotherapy in the subgroup of lymph node negative patients.

In the neoadjuvant setting

High PF has been claimed in several studies to predict clinical response to neoadjuvant chemotherapy (9,16,24,25,47,74,101).

There were early reports suggesting that proliferating rate was of importance in predicting response to antiestrogen therapy (94,95,118,119). Thus, slowly growing tumors were claimed to respond more often than those with a high growth rate. This result has been supported in one of two recent studies (15,68).

Changes in proliferation rate during therapy as a predictive factor

Changes in PF during neoadjuvant chemotherapy have been claimed to be a predictive factor of local clinical response (10,24,25,27,28).

Early reports suggested that a decrease in proliferating rate during tamoxifen treatment could predict clinical response (21,37,76,95,119). In recent studies, these findings have been supported, though the interval between the two measurements was short in some of the studies (15,31,68).

PROTEOLYTIC ENZYMES

The ability for cancer cells to invade surrounding tissue and metastasize is in some extent due to their proteolytic activity (30,111). Several proteolytic enzymes, regulated by a balance of proteases and their inhibitors, are involved and act by binding to cell surface receptors. Many of these proteases have several functions. Cathepsin D; stimulated by estrogen, is an acidic lysosomal protease acting directly on the cell membrane, as well as indirectly by activating Cathepsin B, which in its turn can regulate extracellular collagenases.

Plasminogen activator inhibitor-1 (PAI-1) is a serpin protease inhibitor that blocks the activity of urokinase-type plasminogen activator (uPA), which starts a cascade of events leading to the degradation of collagen and of the basement membrane proteins.

Proteolytic enzymes as a prognostic factor

Cathepsin D content as a prognosticator in breast cancer patients has been extensively studied but the results have been conflicting (38,57,65,71,123,125). Possible explanations of this discrepancy may be differences in methods and patients populations. Methods used are: monoclonal antibodies in tissue sections by immunohistochemistry and in tissue extracts by Western blotting, ELISA and IRMA assays. .

The prognostic independent impact of PAI-1 content has been demonstrated in multivariate analysis in breast cancer patients, as well as in the sub-group of node-negative patients (44,53,57,71). In these studies adjuvant tamoxifen were either not given, or only given to few patients. Some results suggest that the strength of PAI-1

content as a prognosticator could vary over time (112). In the subgroup of lymph node negative patients, PAI-1 has, in a recent published interim analysis of a randomised study, been shown to be a prognosticator, but longer follow-up is needed (67). The above referred studies are listed in Table 2.

Table 2: Studies with Cathepsin D and PAI-1 as an independent prognostic factor in multivariate analysis.

Reference	Factor	No	Group	Treatment	Multivariate	
					DFS	OS
Harbeck, 1999	Cath D	293	All	24% CT 39% HT	NS	NS
Fernö, 1994	Cath D	199	All	No	0.036	
Spyratos, 1989	Cath D	122	All	100% CT	<0.001	
Isola, 1993	Cath D	262	N-	No	<0.001	
Tandon, 1990	Cath D	188	N-	10% CT 9% HT	<0.001	<0.001
Kute, 1998	Cath D	90	N-	No	0.01	0.007
Gröndahl-Hansen, 1995	PAI-1	486	All	22% CT 4% HT	<0.001	NS
Jänicke, 2001	PAI-1	374	N-	No	0.007	
Harbeck, 1999	PAI-1	139	N-	15% CT / HT	<0.001	<0.001
Foekens, 1994	PAI-1	264	N-	No	<0.001	
Kute, 1998	PAI-1	90	N-	No	<0.001	NS
Foekens, 1994	PAI-1	354	N+	20% CT 5% HT	<0.001	

Proteolytic enzymes as a predictive factor

The predictive value of Cathepsin D for adjuvant tamoxifen treatment has previously been described in one study, which was based on a relatively small number of patients (38). In this study adjuvant tamoxifen significantly decreased the recurrence rate in lymph node positive patients with high Cathepsin D levels, whereas it had no effect in lymph node positive patient with low Cathepsin D levels. In lymph node negative patients no such difference was found.

AIMS OF THE STUDY

- To identify factors that may explain a difference between clinically and mammographically assessed tumor size, and compare mammographically assessed changes in tumor size and density with clinically assessed response to endocrine therapy or chemotherapy.
- To analyze if PF measured with Ki-67 antibody before and after 3 months of neoadjuvant cytotoxic treatment is a treatment predictive factor.
- To analyze if PF measured with Ki-67/MIB-1 antibody in preoperative FNA biopsies is a prognosticator of disease recurrence.
- To analyze the role of Cathepsin D and PAI-1 content as prognosticators of DFS and to explore the putative predictive value of Cathepsin D and PAI-1 content with respect to tamoxifen treatment.
- To assess the correlation between tumor hormone receptor content, rate of cell proliferating fraction and best clinical response, relate the changes in hormone receptor content during treatment and best clinical response and describe individual changes in receptor content in tumor cells during therapy

PATIENTS AND METHODS

PATIENTS

In patients with breast masses FNA biopsy is performed as a part of the routine diagnostic process at the Karolinska Hospital. In patients with breast cancer, hormone receptor content as well as PF are assessed on parts of the FNA material. Patients receiving primary systemic treatment are subjected to repeat FNA biopsy after approximately 3 and 6 months, respectively. All patients in paper I, II, III and V had a conclusive cytologic diagnosis of mammary cancer. In paper III the diagnosis was made on the surgical specimens. No patients had signs of distant metastases at diagnosis in paper I-IV. Seven patients in Paper V had stage IV disease at clinical presentation.

Paper I

A total of 225 patients were included in the study representing 3 cohorts of patients. The first cohort of patients was included in order to study possible differences between the two techniques of assessing tumor size as well as potential intervening factors. The two other cohorts were introduced to compare the two techniques according to response evaluation.

Cohort 1: By reviewing primary examination results from the mammography unit at Karolinska Hospital during the year 1999, we found 143 consecutive patients (median age 61 years) with mammographically distinct tumors.

Cohort 2: Fifty-two consecutive patients (median age 50 years) diagnosed during December 1995 to April 2000, who received neoadjuvant chemotherapy treatment and had mammographically distinct tumors at diagnosis as well as after 3 months of therapy.

Cohort 3: Thirty consecutive patients (median age 83 years) diagnosed January 1988 to August 1993, who received neoadjuvant endocrine therapy and had mammographically distinct tumors at diagnosis as well as after 3 months of therapy.

Paper II

During the period January 1990 – August 1993, 51 consecutive patients were diagnosed with large T2 or T3 breast cancers and received neoadjuvant cytotoxic treatment. The mean-follow-up time was 39 (17-72) months.

Paper III

A data file with 1239 patients with conclusive cytological diagnosis of breast cancer and assessment of PF with Ki-67/MIB-1, diagnosed during 1989-1994 at Karolinska Hospital, was matched with the Stockholm Breast Cancer Group data base at the Oncologic Centre in Stockholm. After excluding patients with bilateral cancers, patients undergoing primary treatment, not radically operated and patients with no information on excised lymph nodes, a total of 732 patients were included. The median follow-up time was 68 (14-124) months.

Paper IV

This study included 1851 consecutive patients with primary breast cancer diagnosed in the Stockholm and Gotland region during 1988-1992. Eligible for this study were patients with 1) no previous history of cancer, 2) primary radically excised tumors, 3) no signs of distant metastases and 4) sufficient remaining cytosol after hormone receptor analysis to determine Cathepsin D content. The median age was 62 years. During 1991-1992 PAI-1 content was also determined in the same cytosols as Cathepsin D content. Both Cathepsin D and PAI-1 content were measured in approximately 30% of all patients. The median follow-up time was 59 (39-88) months.

Paper V

During the period September 1987 to July 1994, 82 elderly patients with primary breast cancer were included in the study. The reason for primary treatment with tamoxifen was concomitant, debilitating disease (11), inoperable disease (24), advanced age making them unfit for surgery (22), and patients preference (25). Six patients were in clinical stage I, 43 in clinical stage II and 33 in clinical stage III. At diagnosis 21 patients had cytologically verified axillary lymph node metastases, and 7 patients had distant metastases. The median follow-up time was 68 (14-104) months.

TREATMENT

Both in paper I (cohort 2) and paper II, patients were treated as followed. Prior to surgery, all patients were treated with 3-4 courses of CEF (cyclophosphamide 600mg/m², epirubicin 60mg/m² and 5-fluorouracil 600mg/m² q.3 weeks). After 3-4 courses of chemotherapy all the patients were considered operable; 50 patients underwent a modified radical mastectomy and one patient had a partial excision of the breast with an axillary biopsy. Postoperative treatment was individualized depending of the clinical response to preoperative treatment.

In paper III and IV, patients were treated according to the Stockholm Breast Cancer Group protocol. Primary treatment was either modified radical mastectomy or breast conserving surgery including lymph node dissection. Patients with positive lymph nodes were treated with radiation therapy of 46 Gy given over a period of 4.5 weeks to the chest wall and regional lymph nodes. Premenopausal women with positive lymph nodes also received 6 months of CMF chemotherapy (cyclophosphamide 600mg/m², methotrexate 40mg/m² and 5-fluorouracil 600mg/m² iv on days 1 and 8, q. 4 weeks). Tamoxifen 40mg orally for 2 or 5 years was given according to the regional treatment practice guide-lines for breast cancer patients. All patients who underwent breast-conserving surgery received radiation therapy of 50 Gy over a period of 5 weeks to the remaining breast tissue.

In paper I cohort 3 as well as in paper V, patients were treated with tamoxifen 40 mg orally.

FNA BIOPSY

All fine needle aspiration (FNA) biopsies were performed by cytopathologists using 0.4-0.6 mm needles as described previously (134). Cytological evaluation was performed on air-dried May-Grünwald Giemsa stained smears.

IMMUNOHISTOCHEMISTRY

Analysis of hormone receptor content

In paper II and V the hormone receptor content was analyzed on cytological material. The smears were air-dried, fixed in buffered formalin and analyzed for estrogen and progesterone receptors by quantitative methods based on monoclonal antibodies (118). In paper III and IV a tumor tissue cytosol was prepared from each surgical specimen. The cytosols were prepared according to the method used for ER (134). Protein determination was obtained by the Bradford method (35). Estrogen and progesterone receptor content was determined by an immunoassay kit (Abbott Diagnostic, North Chicago, IL) according to the manufacturer's instructions, as previously described (6).

Analysis of PF

In paper II, III and V the smears for analysis of PF were air-dried, fixed in buffered formalin and analyzed for cell PF as previously outlined (51,118,134). The fraction of proliferating tumor cells was determined from parts of the specimen that was used for cytologic diagnosis.

Analysis of Cathepsin D and PAI-1

In Paper IV the antigen levels of Cathepsin D were determined in diluted cytosol fraction by ELISA (Cis-bio international, Gif-sur-Yvette, France). The PAI-1 antigen were determined in diluted cytosol fraction by ELISA (PAI-1 ELISA Kit # 821, American Diagnostica, Greenwich, CT, USA).

CLINICAL FOLLOW-UP AND ENDPOINTS

Follow-up visits were scheduled once every 3 months during the first 2 years, every 6 months during the 2-5 year period and yearly thereafter. These visits routinely included a physical examination and a yearly mammogram. Chest X-rays, bone scans, blood tests etc were only performed when signs or symptoms indicated a possible relapse. Distant disease recurrence was in the studies defined either as a cytologically verified lymph node or soft tissue recurrence outside the breast/chest wall, ipsilateral axilla or supraclavicular fossa or radiographical evidence of lung, liver or bone metastases.

In paper I, II and V clinical assessed tumor responses using UICC criteria were used.

In paper II disease free survival (DFS) and in paper III and IV distant recurrence free interval (DRFI) were used.

STATISTICAL METHODS

Paper I

To compare means of different assessments a paired t-test was performed, since the same tumor was assessed. Correlations between the two measurements was analyzed using simple regression analysis, which explains the value of a dependent value from one independent variable. The proportion of variability explained is the quadrate of the correlation coefficient.

Paper II

Fisher's exact test was used to compare proportions. Univariate and multivariate analyses of putative prognostic variables were done using Cox's proportional hazards model. The disease free survival plot was calculated according to Kaplan&Meier. Distributional comparisons were made with logrank test.

Paper III and IV

The association between cell PF, Cathepsin D content and PAI-1 content with various clinical parameters were examined using Spearman rank correlation, or with dichotomized parameters with the chi-square test of independence. Clinical parameters were dichotomized using the following groupings: age, <50 versus \geq 50 years; lymph node involvement, pN0 versus pN+; tumour size, <20 versus \geq 20 mm; estrogen receptor content, <0.05 versus \geq 0.05 fmol; progesterone receptor content, <0.05 versus \geq 0.05 fmol; adjuvant tamoxifen, treated versus not treated. Univariate survival curves were estimated using the method of Kaplan and Meier and differences between curves were tested using the logrank test. Due to missing or unknown data, 471 and 261 patients were available for the multivariate analysis of Ki-67 and MIB-1 values, respectively. Due to missing or unknown data, 1671 patients were available for the multivariate analysis of Cathepsin D and 491 patients in the multivariate analysis of PAI-1 content. Cox's proportional hazards regression model was used to assess the independent prognostic contribution of clinical parameters after adjusting for other factors. Wald chi-square statistics were used to estimate the significance of each factor. All reported p-values refer to two-sided test of significance. Prognostic effects were expressed as hazard rate ratios supplemented with 95% confidence intervals (C.I.). Test of interaction was performed by including product terms into the multivariate models.

Paper V

Fisher's exact test was used to compare proportions. Clinical parameters were dichotomized using the following groupings: age <89 versus \geq 89 years; lymph node involvement, pN0 versus pN+; tumour size, <20 versus \geq 20 mm; ER content, \leq 75% versus \geq 75% ; PgR content, \leq 50% versus >50%; PF \leq 7% versus >7% and decrease in ER content after 3 months \leq 50% versus >50%.

METHODOLOGICAL CONSIDERATIONS

Paper I

In this study most of the patients were examined by different clinicians. This raises the question how reproducible the clinical measurements actually were. Regarding assessments on mammograms they were performed by two doctors in this study, and may be reevaluated since the mammograms are still available.

Patients in the second cohort of paper I was a sub-population of the ones studied in paper II and patients in the third cohort of paper I were a sub-population of the ones studied in paper V. This fact was accepted though the studies involved had different aims.

Paper II

Most of the patients studied in paper II were also included in paper I, and this fact was accepted though the studies had different aims.

There was a clinically based assumption that a decrease in PF during therapy could be a sign of chemosensitivity. The choice of cut-off level for the decrease is crucial. A small decrease could be a variation in the estimation of PF. Although, this was a small study, the result was tested with different cut-off levels and the result was most significant for a decrease in PF of > 20%.

Paper III

We have only found one study which correlate the PF as measured by staining on aspirated cells and surgical material (91). A concordance of 70% was found. This fact may explain why the cut-off level in this study is different when comparing to other studies done on surgical specimens. In this study we chose to have the cut-off level close to the median level.

Another problem in comparisons of different series is the selected end-point. In this study distant recurrence free interval (DRFI) and not overall survival (OS) was chosen in order to minimize the effect of intercurrent deaths and effects of treatment due to distant disease recurrence.

Paper IV

One problem in making comparisons with other published studies is that different methods are used in measuring Cathepsin D and PAI-1 content. Cut-off levels also differ between investigators. In our study the ELISA method was used on remaining cytosols after hormone receptor analysis. Most of the studies have dichotomised the values according to the median value, which enables comparisons of high levels to low levels independent of which methods of measurement is used.

The end-point is different from other studies. We chose DRFI and not overall survival in order to minimise the effect of intercurrent deaths and effects of treatment due to disease recurrence. Disease free survival was not used due to possible interaction with surgical methods and postoperative radiotherapy.

Paper V

Most studies have an interval for the receptor measurement between 1-2 weeks, but in some recent reports the interval has also been longer: 1, 2 and 6 months, respectively. In line with clinical practice at the institution repeated FNA biopsies were performed at 3 months interval.

The endpoint was different from other studies (best clinical response compared to clinical response after 6 months therapy) leading to different response rates (78% compared to 39%). It should also be underlined that ER score and percentage of ER positive cells may not be directly comparable when evaluating ER status.

RESULTS

PAPER I

Correlation between clinically and mammographically assessed tumor size in 225 patients at time of diagnosis

There was a weak but significant positive correlation ($r^2= 0.59$) between tumor size as measured by palpation and mammography. The correlation was, however, only significant in postmenopausal patients. The correlation was stronger in patients with BMI \leq 25 and in patients not receiving HRT.

Comparisons of grouped data according to T-stage is shown in Table 2. Thus 27% of patients with clinical T1 tumors would have been up-staged to T2 or T3 tumors if staging had been based on mammography. Conversely, 22% of the clinical T2 tumors and 36% of the clinical T3 tumors would have been down-staged mammographically to T1 and T2 tumors, respectively.

Response evaluation

Fifty-two patients who received 4 courses of preoperative chemotherapy, were classified as responders or non responders by physical examination after 3 months. The effect of treatment was also evaluated from the mammograms. Clinical response was observed in 29 patients (56%), as compared to 17 patients (33%) with mammographic response. A decrease in tumor density after 3 months treatment was observed in mammograms from 21 patients (40%). This decrease in mammographic tumor density was recorded in 11 of 29 patients with clinical response. Only 12 of 29 patients with a clinical response measured according to the UICC criteria had a decrease in mammographic size of more than 50%. Sixteen of 29 patients with clinical response had either mammographic response in size or decreased tumor density after 3 months chemotherapy. In 5 of 17 patients with decrease in mammographic tumor area of more than 50%, no clinical response was recorded.

Thirty patients were given tamoxifen for 3 months after diagnosis. The treatment effect was assessed by physical examination and in 13 patients (43%) response was observed. However, in the same group of patients there were only 4 patients (13%) with mammographic assessed response after 3 months primary therapy.

A decrease in tumor density after 3 months of endocrine therapy was observed in mammograms from 11 patients (37%). A decrease in mammographic tumor density was recorded in 7 of 13 patients with clinical assessed response.

Only 3 of 13 patients with a clinical response measured according to the UICC criteria had a mammographic response. Seven of 13 patients with clinical response had either mammographic response in area size and/or decreased tumor density after 3 months endocrine therapy.

PAPER II

Predictive factors in 33 patients treated with preoperative chemotherapy

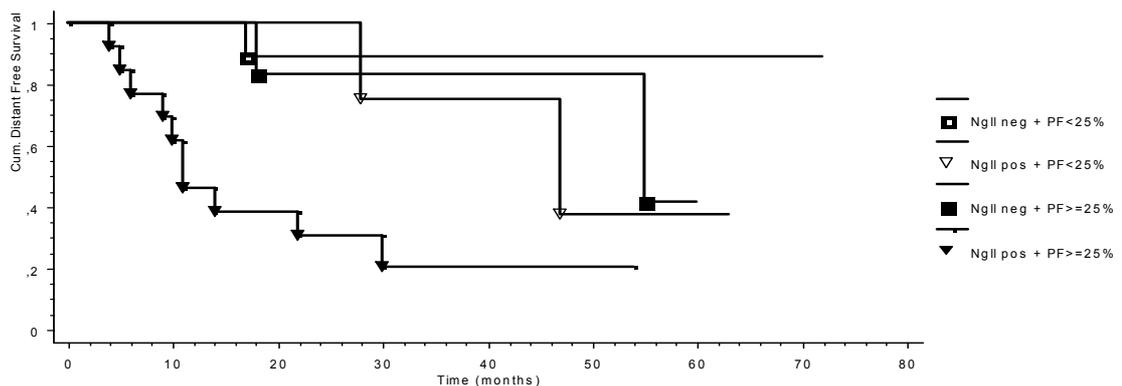
At follow-up 15 patients (45%) experienced disease recurrence. A univariate analysis revealed that clinically positive lymph nodes and a less or equal 25% decrease in PF during the first course of preoperative chemotherapy were significantly correlated with disease recurrence. According to multivariate analysis these factors were independent treatment predictive factors as can be seen in Table 3.

Table 3. Results of univariate and multivariate analysis on DFS

Factor	Cases (no)	Events (no)	Univariate relative hazard (95% C.I.)	P	Multivariate relative hazard (95% C.I.)	P
T2	16	5	1			
T3	17	10	1.76 (0.60-5.18)	0.31		
Neg. Ln.	15	3	1		1	
Pos. Ln.	18	12	5.45 (1.48-20.0)	0.01	4.95 (1.27-19.2)	0.02
ER>0	18	6	1			
ER=0	15	9	1.93 (0.68-5.44)	0.21		
PgR>0	17	6	1			
PgR=0	16	9	1.79 (0.64-5.05)	0.27		
PF<25%	13	6	1			
PF>=25%	20	9	0.78 (0.27-2.24)	0.64		
Clin. Resp.	19	10	1			
No clin. Resp.	14	5	1.37 (0.46-4.04)	0.57		
Decr. PF>25%	14	3	1		1	
Decr. PF< 25%	19	12	4.77 (1.33-17.1)	0.02	4.17 (1.13-15.5)	0.03

The corresponding DFS plot in each subgroup is displayed graphically in Figure 1.

Figure 1. Disease-free survival according to lymph nodes and a decrease >25% in PF



PAPER III

PF as prognosticator for DRFI in 732 patients

In univariate as well as multivariate analysis Ki-67/MIB-1 value was a strong ($p < 0.001$) significant prognosticator of DRFI, independent of lymph node status, progesterone receptor content and tumour size (Table 4).

PF as prognosticator for DRFI in 431 lymph node negative patients

In the subgroup analysis of 430 node-negative patients the distant recurrence-free rate after 5 years was 94.4 % in patients with low Ki-67/MIB-1 value compared to 88.7% in patients with high Ki-67/MIB-1 value. This difference was statistically significant ($p = 0.028$). Test of the interaction between tumor size and the value of PF revealed a p-value of 0.06. For node-negative patients with tumors ≥ 20 mm the distant recurrence-free rate after 5 years was 93.2% in patients with low Ki-67/MIB-1 compared to 80.7% in patients with high Ki-67/MIB-1 value. This difference was statistically significant ($p = 0.008$) and the corresponding Kaplan-Meier curves are shown in Figure 2. For patients with smaller tumors (< 20 mm) the Ki-67/MIB-1 value could not in this study add any prognostic information.

Figure 2. DRFI for lymph node negative patients according to PF and tumor size

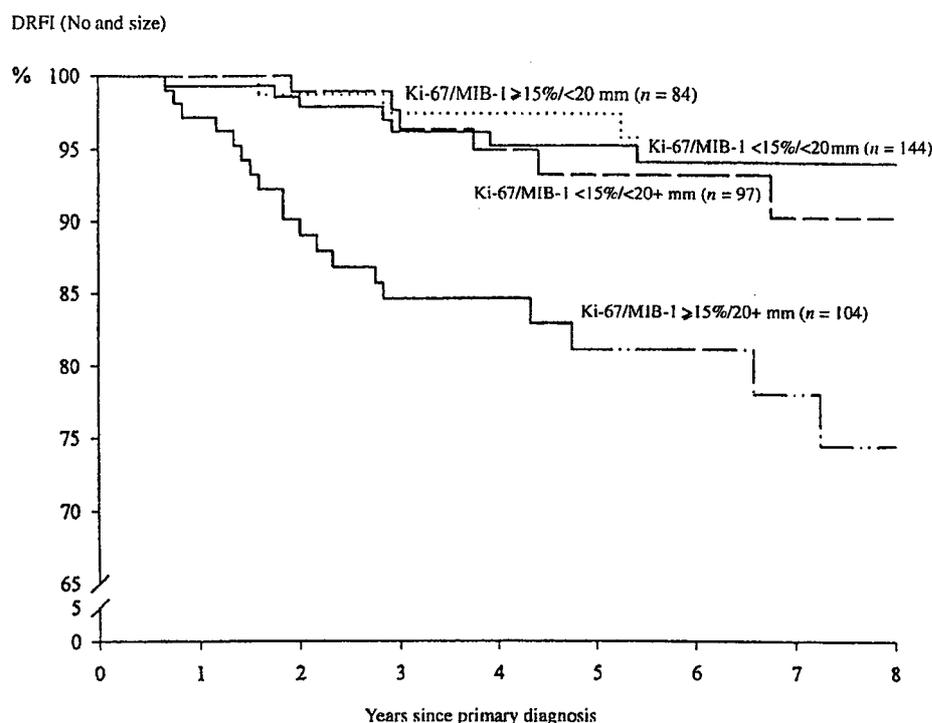


Table 4: Results of univariate and multivariate analysis on DRFI

Covariate	Univariate				Multivariate			
	No. Of events	No. of pats	HR (95% C.I.)	P-value	No. of events	No. Of pats	HR (95% C.I.)	P-value
Age years								
<50	43	190	1					
>= 50	83	542	0.7 (0.5-1.0)	0.058				
Nodal status								
pN0	39	430	1		32	358	1.0	
PN+	87	302	3.8 (2.6-5.6)	<0.001	78	261	3.7 (2.4-5.6)	<0.001
Tumor size								
<20 mm	34	320	1		32	269	1.0	
>=20 mm	91	408	2.6 (1.7-3.8)	<0.001	78	350	1.7 (1.1-2.6)	0.015
ER status								
<0.05 fmol	20	110	1					
>0.05 fmol	93	517	0.8 (0.5-1.3)	0.35				
PgR status								
<0.05 fmol	43	179	1		42	177	1.0	
>0.05 fmol	68	442	0.6 (0.4-0.8)	0.004	68	442	0.6 (0.4-0.8)	0.003
Ki-67/MIB-1								
<15%	38	375	1		32	314	1.0	
>=15%	88	357	2.7 (1.9-4.0)	<0.001	78	305	2.2 (1.4-3.4)	<0.001

PAPER IV

Cathepsin D content as prognosticator for DRFI in 1671 patients

In univariate analysis Cathepsin D content was a strong ($p < 0.001$) significant prognosticator. In multivariate analysis Cathepsin D was a weaker ($p = 0.020$), but still significant, prognosticator of DRFI independent of lymph node status.

Cathepsin D content as a predictor in 1671 patients of whom 1071 patients were treated with tamoxifen

The benefit of adjuvant tamoxifen treatment was studied in 4 subgroups by dichotomized estrogen receptor content and Cathepsin D content (Table 5). The level of Cathepsin D appeared to predict the benefit with adjuvant tamoxifen treatment in ER-positive patients although this result did not reach statistical significance ($p=0.09$). The interaction between Cathepsin D level and treatment among ER-positive patients was $p=0.17$.

Table 5. Effects of adjuvant tamoxifen on DRFI by ER and Cathepsin D level

	Adj tam	Cathepsin D <10 fmol		Cathepsin D \geq 10 fmol	
		No. Events / No. Patients	Hazard ratio (95% C.I.)	No. Events / No. Patients	Hazard ratio (95% C.I.)
ER<0.05 fmol	No	4 / 38	1.0	24 / 140	1.0
	Yes	5 / 40	1.3 (0.4-5.2)	23 / 110	1.3 (0.7-2.3)
ER \geq 0.05 fmol	No	2 / 83	1.0	50 / 339	1.0
	Yes	10 / 214	2.4 (0.5-12.3)	70 / 707	0.7 (0.5-1.1)

PAI-1 content as prognosticator for DRFI in 497 patients

Results of univariate and multivariate Cox-analysis of the effect of different covariates on DRFI are shown in Table 6. In univariate Cox-analysis PAI-1 content was a strong significant prognosticator ($p<0.001$) as well as lymph node involvement. In multivariate Cox-analysis lymph node involvement was the only strong significant prognosticator. Although PAI-1 was weaker in this respect, it was still an independent significant prognosticator ($p=0.003$).

PAI-1 content as prognosticator for DRFI in 348 lymph node negative patients

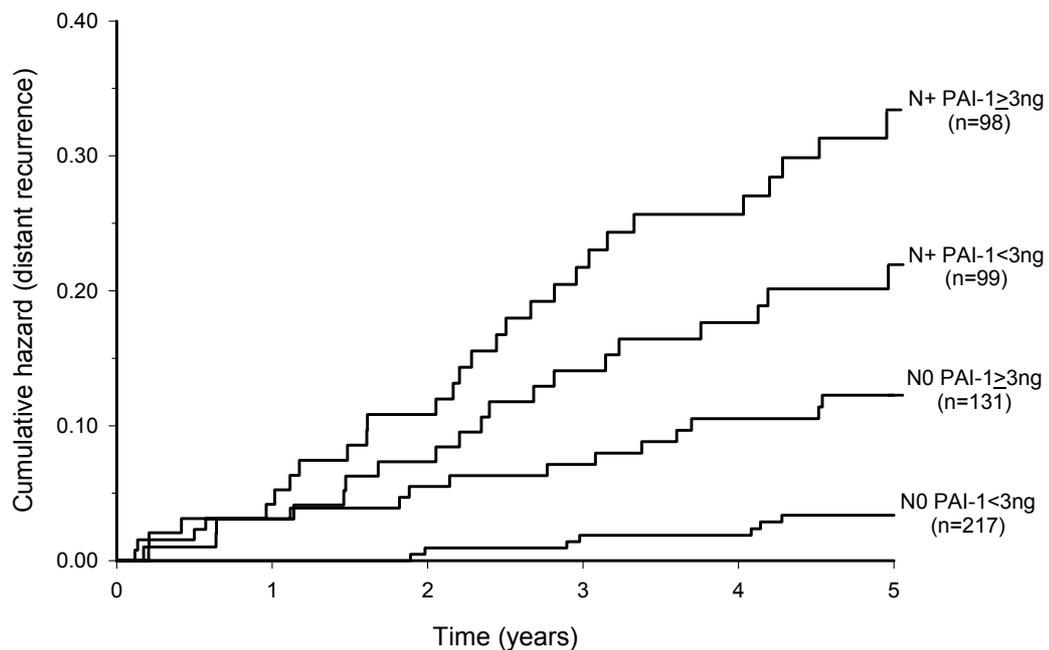
From the multivariate analysis the distant recurrence rate was 3.0% in patients with low PAI-1 as compared to 12.1% in patients with high PAI-1. This difference was statistically significant ($p=0.004$). The corresponding Kaplan-Meier curves based on all 348 node-negative patients with PAI-1 values, revealed a statistically significant difference ($p=0.002$) and are shown in Figure 3.

Table 6. Results of univariate and multivariate analysis on DRFI in 497 patients

Covariate	No. of events	No. of patients	Univariate		Multivariate	
			Hazard ratio (95% C.I.)	P-value	Hazard ratio (95% C.I.)	P-value
Age years						
<50	20	103	1.0		1.0	
>= 50	42	394	0.5 (0.3-0.9)	0.016	0.7 (0.4-1.2)	0.186
Nodal status						
pN0	20	315	1.0		1.0	
PN+	42	182	4.0 (2.4-6.9)	<0.001	3.1 (1.7-5.4)	<0.001
Tumour size						
<20 mm	24	295	1.0		1.0	
>=20 mm	38	02	2.4 (1.5-4.1)	0.001	1.6 (0.9-2.7)	0.099
ER status						
Negative	14	93	1.0		1.0	
Positive	48	404	0.7 (0.4-1.3)	0.299	1.0 (0.5-1.8)	0.887
PAI-1						
< 3 ng	22	289	1.0		1.0	
>=3 ng	40	208	2.7 (1.6-4.6)	<0.001	2.3 (1.3-3.9)	0.003

Adding Cathepsin D and/or adjuvant treatment did not show any significant difference.

Figure 3. The risk for distant recurrence over time according to lymph node status and level of PAI-1.



PAPER V

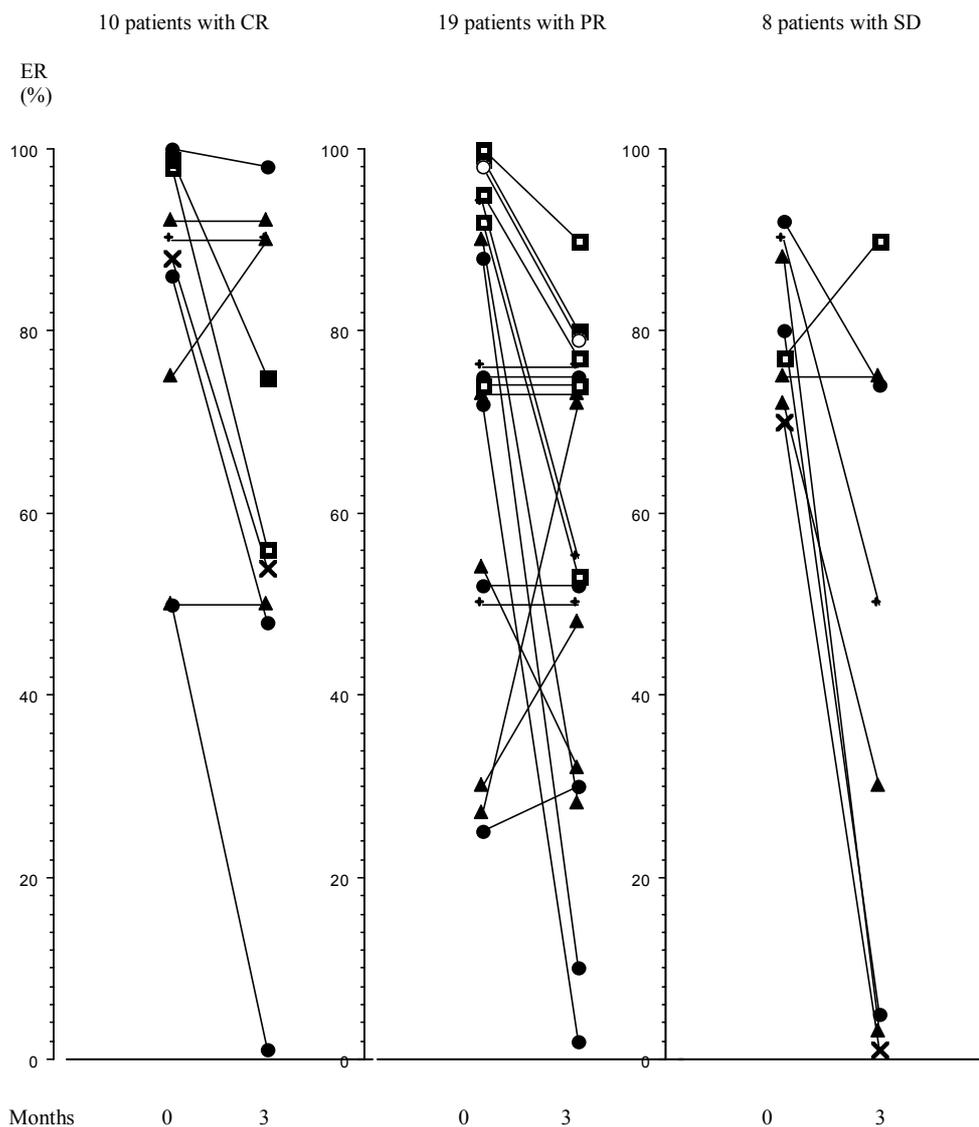
PF as a treatment predictive factor for best clinical response (CR+PR) in 40 ER positive elderly patients treated with tamoxifen

In the subgroup of 40 patients with known PF, it was observed that patients with a PF higher than 7% had a lower response rate than those with a lower PF. This difference was only of borderline significance ($p=0.05$).

A decrease >50% in ER positive cells as a treatment predictive factor for best clinical response in 37 elderly patients treated with tamoxifen.

The individual changes in ER content in the tumors between initiation of tamoxifen treatment and 3 months of therapy according to best clinical response are shown in Figure 6. There was a trend of a more rapid decrease of ER content in patients with SD compared to patients with PR and CR (Figure 4). A decrease in percentage of ER positive cells higher than 50% was found more often in less responsive tumors. This predictive result was statistically significant ($p<0.05$). The mean decrease in ER content was 41% in patients with SD compared to 19% and 17% in patients with CR and PR, respectively ($p=0.07$).

Figure 4: Individual changes in ER content after 3 months according to best response.



DISCUSSION

CLINICAL RESPONSE EVALUATION

In Paper I our data showed that clinical and mammographic assessment of tumor size at the time of diagnosis are poorly correlated. The discrepancies in tumor size were marked and clinical over- and under estimations were observed. There are several factors, which can explain a clinical overestimation. The main ones being a) different clinicians making the clinical evaluation b) the fact that tumor diameter include twofold the thickness of the skin and subcutaneous tissue. A correct deduction is difficult and seldom even attempted. This ought to make clinical measurement of tumor size being overestimated as compared to mammographic assessment, as has been reported previously (45,63,131). A clinical underestimation is more difficult to explain, but factors such as the size of the breast, the tumor depth in the breast, irregular consistency, and fibromatosis could all contribute. In line with the assumption that a reduction in volume and consistency of the surrounding breast tissue enables a more accurate determination of the tumor size, we found a better correlation between the two methods for postmenopausal women with BMI<25.

Our data showed a clinical assessed response rate of 56% in patients receiving chemotherapy, while the figure for mammographic response was 33%. These results are in line with earlier reports, which showed a higher clinical response rate with clinically compared to mammographically assessed response (17,23,40,85,113). The fact that only 43% of the patients with clinically assessed response had a mammographically assessed response could be interpreted in two ways. Primarily, mammographic response lags behind clinical response, as has been suggested by others (85). Secondly, clinically assessed response is only poorly correlated to true response. An even more marked discrepancy was observed in patients receiving endocrine therapy. In this group the clinical response rate was 43% whereas only 13% had a mammographically assessed response in tumor size. These results are in line with earlier reports for hormonal treatment which have shown lower mammographic response rates (80,85,110).

It seems likely that tumor response to chemotherapy or endocrine therapy also may induce changes in tumor density. Estimation of the density of a tumor is, however, difficult since factors such as the technical quality of each film, overall density of the breast and the density of the quadrant in which the tumor was located, have to be taken into account. In this study 40% of the patients treated with chemotherapy and 33% treated with endocrine therapy had a decrease of tumor density after 3 months. A volume reduction contribute by physics to a decrease in density, but this fact can not fully explain the amount and quality of the decreases assessed in this material. The decrease in density may to some extent be interpreted as a marker of treatment sensitivity though the biological explanation behind this decrease is unclear. This approach is in agreement with other authors claiming that a decrease in the density of the mass with little associated change in the dimensions, does not exclude an eventual good response to chemotherapy (113,131).

PROGNOSTIC FACTORS

In both paper III and IV we claim that PF, Cathepsin D and PAI-1, respectively, had a prognostic value. This information is not in accordance to our definition of a prognostic factor in this thesis, demanding absence of systemic adjuvant treatment given. In paper III, 20% of the patients received adjuvant chemotherapy and 66% of the patients received adjuvant hormonal treatment. Corresponding figures for the adjuvant treatment given was 11% chemotherapy and 60% hormonal treatment in paper IV. The confounding impact of this fact has to be considered in interpretation of our results. In the subgroup of lymph node negative patients no intervening adjuvant chemotherapy was given.

Proliferation rate

In paper III our results showed that the preoperative level of PF determined on FNA biopsy smears from primary breast carcinomas using KI-67/MIB-1 antibody, is a significant prognosticator of DRFI. This prognostic information was independent of lymph node status, progesterone receptor content and tumor size. Our results are in accordance with several other studies (84,98-100). However, in a Danish study with 487 patients and a 10 years follow-up, high PF was a significant parameter of poor prognosis in univariate analysis but in multivariate analysis, stratified by nodal status, it failed to be of prognostic significance (54).

FNA biopsy is an established technique for the diagnosis of breast cancer in clinical practice. The preoperative information about PF in the tumor is of value for clinicians in identifying subgroups of patients that need an aggressive treatment approach in order to cure. Equally important is to identify the subgroups of patients that may not have a substantial gain with adjuvant treatment.

Proteolytic enzymes

In paper IV our results showed that the level of Cathepsin D content, determined on cytosols using an immunoradiometric assay, is a significant prognosticator of DRFI in a multivariate analysis of 1671 patients. This was independent of lymph node status, tumour size and estrogen receptor content. Our result is in line with smaller previous studies (38,65,71,123,125), even though the end-points in these studies were different.

In paper IV, in multivariate analysis of 497 patients, the level of PAI-1 content was shown to be the only significant prognosticator of DRFI independent of lymph node involvement. Cathepsin D level did not, in this subgroup of patients, show any significance as a prognosticator. Our result is in line with two smaller studies (53,57). The observation that Cathepsin D loses its independent significance as prognosticator when PAI-1 content is introduced in the multivariate analysis has earlier been demonstrated in both these studies.

The clinical impact of this information is the possibility to identify a subgroup of patients who may need additional adjuvant treatment and equally important possibly identify patients who do not need adjuvant treatment.

PROGNOSTIC FACTORS IN LYMPH NODE NEGATIVE PATIENTS

Proliferation rate

In paper III, in the subgroup analysis of 432 node-negative patients, the statistically significant prognostic value for PF defined as different DRFI was maintained ($p=0.01$). Our results are in line with studies comprising 618 and 332 patients, respectively, where PF was a significant independent predictor of DFS (12,108). PF (assessed with other methods) in lymph node negative patients has been demonstrated to be a significant independent prognosticator in multivariate analyses (13,22,64,77,82,96,114,115,124,133). Still, another study has not confirmed these findings (56). However, in this study an invasive marker PAI-1 was introduced in the multivariate analysis and this variable was the only significant one.

Taking into account tumor size in this subgroup, the prognostic value of Ki-67/MIB-1 was only statistically significant among the tumors $>20\text{mm}$ ($p=0.001$). One interpretation of this result is that DRFI for patients with smaller tumors is so good that, due to small numbers of events, a difference in DRFI is difficult to demonstrate.

In lymph node negative patients, a combination of a high PF, lack of hormone receptors and a large tumor size will be taken into account in deciding accurate adjuvant treatment in many institutions.

Proteolytic enzymes

In paper IV, in the subgroup analysis of 1072 node-negative patients, the statistically significant difference between DRFI was maintained ($p=0.031$) for the two Cathepsin D levels. This result is also in line with previous smaller studies in node-negative patients (65,71), even though the studied end-points were different.

In the subgroup analysis of 315 node-negative patients from the multivariate analysis the distant recurrence rate was 3.0% in patients with low PAI-1 compared to 12.1% in patients with high PAI-1. This difference was statistically significant ($p=0.004$). The same results were demonstrated in a smaller study from Germany (57).

The studies mentioned above made clinical impact in Germany. Thus, lymph-node negative patients have been randomized based on PAI-1 level to adjuvant chemotherapy. In a recent published interim analysis of this study, PAI-1 has been shown to be a prognosticator, but longer follow-up is needed (67).

According to the results from the two German studies in lymph node negative patients, assessment of PAI-1 may be a more valuable tool than PF, in defining the subgroup of patients, who need more aggressive adjuvant treatment.

TREATMENT PREDICTIVE FACTORS IN THE ADJUVANT SETTING

Proteolytic enzymes

In Paper IV the level of Cathepsin D appeared to predict the benefit of tamoxifen in estrogen receptor positive patients, although this result did not reach significance ($p=0.09$). A significant result of Cathepsin D as a predictor in lymph node positive patients has been previously suggested (38).

The observed correlation between Cathepsin D-content and the benefit with adjuvant tamoxifen could potentially be of predictive value, but today there is insufficient documentation to support such a correlation. Therefore it is not recommended to withhold adjuvant tamoxifen in ER positive patients on the basis of a Cathepsin D-assay.

TREATMENT PREDICTIVE FACTORS FOR LOCAL CLINICAL RESPONSE

Changes in hormone receptor content

In our study, there was a tendency towards a more rapid decrease in ER content after 3 months of tamoxifen therapy in tumors from patients with SD, as compared to patients with PR and CR. Thus, a decrease in the number of ER positive cells of more than 50% significantly ($p<0.05$) predicted a lower, or perhaps slower response rate. The mean decrease also appeared to be larger in patients with SD. One early report presents results in agreement with our findings of a more pronounced decrease in percentage of ER positive cells during tamoxifen therapy in non responding tumors (76). However, a recent report demonstrated a larger decrease in ER-score in patients with CR and PR compared to SD and PD (68). One explanation for the different results could be the different endpoints (best clinical response compared to clinical response after 6 months therapy) leading to different response rates (78% compared to 39%). Another explanation could be the different interval between the two assessments. It should also be underlined that ER score and percentage of ER positive cells may not be directly comparable when evaluating ER status.

The relevance of changes in hormone receptor content during tamoxifen therapy have to be more thoroughly investigated and reproduced in larger studies before taken into clinical practice, since the biology behind the decrease is yet unclear.

Proliferation rate

In paper V we report that in the subgroup of 40 patients with known PF, a low level of PF appeared to predict response, although this result was only of borderline statistical significance ($p=0.05$). Our results are, however, in line with a study comprising 52 patients which showed that a high PF (assessed with TLI) significantly predicted a lower response rate as compared to a low PF (94). Similar results were also demonstrated in another study comprising 118 patients where PF in the tumor was assessed with Ki-67 antibody (90).

Knowledge of the level of PF in the tumor ought to give valuable information in deciding optimal treatment in elderly women with ER-positive tumors.

Changes in proliferation rate

In paper II we showed that suppression of proliferation $>25\%$ in the tumor 3 weeks after the first course of preoperative chemotherapy, was demonstrated (in multivariate analysis) to be a significant predictive factor for recurrence-free survival. This observation could be interpreted as a sign of sensitivity to chemotherapy, since the decrease in PF was independent of lymph node status. This observation is also reported from other small studies, where reduced proliferation during neoadjuvant chemotherapy leads to improved prognosis (27,47).

A decrease in PF after 3 weeks of therapy as a surrogate marker of sensitivity to a certain chemotherapy regimen could be of great clinical relevance. An early identification of non responding patients would give these an opportunity to change to a more effective treatment. The above result have to be confirmed in larger studies and the FNA biopsy technique offers the possibility to introduce other markers as bcl-2 in the investigation.

GENERAL CONCLUSIONS

Clinical response evaluation

- There is a poor correlation between clinically and mammographically assessed tumor size. Menopausal status, BMI and use of HRT are factors that could influence the correlation between the two assessments. Clinical assessment may not be the optimal method for response evaluation of preoperative systemic therapy. Mammographic assessment contributes with changes in size as well as density and gives a reproducible information.

Prognostic Factors

- Proliferating fraction (PF) (assessed in preoperative FNA biopsies) has a significant prognostic value which is independent of lymph node status, PgR status and tumor size. To our knowledge this is the first study demonstrating that PF can contribute prognostic information when analyzed in preoperative smears.
- Cathepsin D (assessed in surgical specimen) is a significant independent prognosticator.
- PAI-1 (assessed in surgical specimen) is a significant independent prognosticator independent of lymph node status. Cathepsin D loses its significant independent prognostic value when PAI-1 is introduced in multivariate analysis.

Treatment predictive factors in the adjuvant setting

- Cathepsin D (assessed in surgical specimen) may predict the benefit of tamoxifen amongst ER-positive patients.

Treatment predictive factors for local clinical response

- A decrease in PF $> 25\%$ (assessed in FNA biopsies) during preoperative chemotherapy has a predictive value and may be of value in selecting postoperative adjuvant systemic treatment.
- In elderly patients, a high initial PF (assessed in FNA biopsies) may predict a decreased probability of response. Moreover, a decrease in the percentage of ER positive cells $> 50\%$ after 3 months tamoxifen therapy predicted a lower probability of a long-term clinical response

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