TO PREDICT RESULTS OF BREAST CANCER THERAPY

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To my family

To predict Results of Breast Cancer Therapy

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ABSTRACT

We have used the almost complete national Swedish Cancer register, regional quality management register and one randomized adjuvant endocrine trial to study, the effect of radiotherapy in breast cancer with 1-3 positive nodes (study 1), the effect of tamoxifen in estrogen receptor (ER)-positive tumors depending on expression of the progesterone receptor (PR) (studies 2 and 4) and the development of the survival rates of breast cancer in Sweden 1989-2013 (study 3).

Study 1. We compared relative breast cancer survival in two Swedish health care regions that between 1989 and 2006 had different guidelines for postoperative radiotherapy. Patients with 1–3 positive lymph nodes in the western region received radiotherapy of the remaining parts of the breast only, while patients in the south eastern region also received therapy of regional lymph nodes. Other aspects of the guidelines were very similar including those for screening mammography, surgery and adjuvant medical treatment. **Results:** The 10-year relative survival for patients with 1–3 positive lymph nodes was 78% in the western region and 77% in the southeastern region (p=0.12). **Conclusions:** There was little or no influence of addition of lymph node radiotherapy on survival in patients with 1–3 positive lymph nodes in a population with screening mammography and modern systemic treatment.

Studies 2 and 4 We investigated the independent predictive value of progesterone receptor (PR) determined with immunohistochemistry (IHC) in estrogen receptor (ER) positive tumors from patients participating in the Stockholm trial of adjuvant tamoxifen. **Methods** We evaluated patients without lymph node metastasis for whom PR in study 2 was determined by IHC in *tissue micro arrays* (thin cores of tumor tissue). In study 4, PR was scored by gene expression and by IHC of *entire tumor sections* and separate analyses of patients with luminal A tumors were performed. **Conclusions** PR positivity determined by IHC or gene expression is a marker indicating long-term benefit from adjuvant tamoxifen. We observed a very marked benefit for patients with PR positive luminal A tumors.

Study 3 During the recent decades, breast cancer survival has gradually improved but there is limited knowledge on the improvement in population-based studies of patients diagnosed with different stages of disease and in different age groups. **Patients and methods.** In two Swedish health care regions a total of 42 220 female breast cancer patients below 90 years of age were diagnosed between 1989 and 2013. **Results.** Using patients diagnosed 1989-1993 as a reference the relative risk of 5 year mortality decreased with 49% (ci95% 45 – 58) for patients diagnosed in the end of the observation period. **Conclusions**. Improvements were seen in all age groups but was unevenly distributed between stages and age groups pointing to the need for further improvements for younger and elderly patients.

Conclusions in summary: In a population invited to mammography, regional radiotherapy in patients with breast cancer and 1-3 positive nodes seems to result in little or no influence in survival. Expression of PR seems to indicate better long-term effect of tamoxifen in ER-positive tumors. The 5-year mortality in breast cancer has been halved in southeast and western Sweden between 1989-2013.

Keywords: Breast cancer, survival, endocrine therapy

POPULÄRVETENSKAPLIG SAMMANFATTNING

ARBETE 1

Syfte Att jämföra överlevnaden i bröstcancer i två regioner i Sverige med olika strålbehandling av patienter med en till tre dottersvulster i armhålan. **Metoder** Mellan 1989 och 2006 behandlades patienterna mycket lika i den västra och sydöstra sjukvårdsregionen, förutom gällande strålbehandling. I den sydöstra regionen erhöll nästan alla patienter strålbehandling av operationsområdet och närliggande körtelområde efter bortoperation av bröstet medan ett fåtal av patienterna i den västra sjukvårdsregionen strålades. De patienter som erhöll bröstbevarande kirurgi fick mer omfattande strålbehandling i sydöstra regionen. **Resultat** 77% av patienterna i sydöstra regionen överlevde i 10 år jämfört med 78% i västra regionen. **Slutsats** Strålbehandling av lymfkörtlar och operationsområde påverkade inte överlevnaden hos patienter med en till tre metastaser i armhålan.

ARBETE 2 OCH 4

Syfte Att undersöka om tumörernas innehåll av progesteronreceptor kan förutsäga nyttan av förebyggande behandling med antiöstrogentabletter. **Metoder** En studie som genomfördes mellan 1976 och 1990 jämförde förebyggande antiöstrogenbehandling med kontroll. Från denna studie utvärderade vi 618 patienter med bröstcancer utan dottersvulster i armhålan vars cancer innehöll receptor för östrogen. I millimetertjocka kolvar från patienternas bortopererade tumörer synliggjordes i mikroskop med hjälp av antikroppar tumörcellernas innehåll av progesteronreceptor. I arbete 4 valde vi 582 patienter och studerade progesteronreceptorn i hela tvärsnitt av tumörerna. Vi mätte även uttryck av genen för receptorn samt utförde separata analyser av den undergrupp av bröstcancer som kallas luminal A. **Resultat** Patienter vilka hade progesteronreceptor i tumörerna eller uttryckte genen för receptorn hade mångårig nytta av antiöstrogenbehandling, medan nyttan var mer begränsad då progesteronreceptor inte kunde påvisas. Våra resultat visar också på mycket god effekt av antiöstrogenbehandling på luminal A tumörer med båda receptorerna. **Slutsats** Närvaro av progesteronreceptor eller dess genuttryck i bröstcancer visar på långvarig nytta av antiöstrogenbehandling.

ARBETE 3

Syfte Att undersöka om senare års förbättringar av överlevnaden bland bröstcancerpatienter gäller för patienter i alla åldrar och sjukdomsstadier. **Metoder** Vi studerade 42 220 kvinnliga bröstcancerpatienter som insjuknat mellan 1989 och 2013 i sydöstra respektive västra sjukhusområdet. De behandlades i enlighet med nationella och regionala riktlinjer.

Resultat och slutsats Under tidsperioden 1989-2013 minskade risken för bröstcancerdöd med 49%. Förbättringar sågs i alla åldersgrupper men var ojämnt fördelad mellan olika stadier och åldersgrupper. Vi uppmärksammar behovet av ytterligare förbättringar främst för de yngsta och äldsta patienterna.

LIST OF PUBLICATIONS

I.   Nordenskjöld A.E, Fohlin H , Albertsson P, Arnesson L-G, Chamalidou C, Einbeigi Z, Holmberg E, Nordenskjöld B, Karlsson P, 2015, No clear effect of postoperative radiotherapy on survival of breast cancer patients with one to three positive nodes: a population-based study
Annals of Oncology 2015; 26: 1149-1154

II.   Nordenskjöld A.E, Fohlin H, Fornander T, Löfdahl B, Skoog L, Stål O, 2016 Progesterone receptor positivity is a predictor of long-term benefit from adjuvant tamoxifen treatment of estrogen receptor positive breast cancer.
Breast Cancer Research and Treatment 2016; 160(2): 313–322

III.   Nordenskjöld A.E, Fohlin H, Arnesson L-G, Einbeigi Z, Holmberg E, Albertsson P, Karlsson P, 2018, Breast cancer survival trends in different stages and age groups – a population based study 1989-2013.
Acta Oncologica, 2018 Dec 4:1-7. DOI: 10.1080/0284186X.2018.1532601

IV.   Nordenskjöld A.E, Dar H, Albertsson P, Fornander T, Karlsson P, Skoog L, Stål O, Lindström L. Progesterone receptor gene expression and immunohistochemistry as predictors of adjuvant tamoxifen response in estrogen receptor positive breast cancer
Manuscript

CONTENTS

ABBREVIATIONS

AIs=aromatase inhibitors

ALNI= axillary lymph node involvement

BCS=breast conserving surgery

BCM=breast cancer mortality

CI=confidence interval

CMF=cyclophosphamide, methotrexate, fluorouracil

CYP450 enzymes=cytochrome P 450 enzymes

DCIS=ductal cancer in situ

DFS=disease free survival

DMFS=distant metastasis-free survival

EBCTCG= Early Breast Cancer Trialists' Collaborative Group

ER=estrogen receptor

FFPE=formalin fixed paraffin embedded

GnRH=gonadotropin releasing hormone

HER2=human epidermal growth factor receptor 2

HG=histologic grade

HR=hazard ratio

IHC= immunohistochemistry

ISH=in situ hybridization

Ki 67=proliferation associated antigen

LBA=ligand-binding assay

mAB= monoclonal antibody

NHG=Nottingham histologic grade

OFS= ovarian function suppression

OS=overall survival

PR= progesterone receptor

RFS=recurrence free survival

RR= recurrence rate

SCR=Swedish cancer register

SERM=selective estrogen receptor modulator

SNOMED= systematized nomenclature of medicine

STEPP= subpopulation treatment effect pattern plots

TMA=tissue microarray

TNM=tumor node metastasis

TNBC=triple negative breast cancer

1   BREAST CANCER- BACKGROUND

1.1   EPIDEMIOLOGY

Breast cancer is the most common cancer in women and the second most common cancer overall with 1.67 million new breast cancer cases globally in 2012[1,2]. It has been estimated that about one in ten women will be affected by breast cancer. The incidence of breast cancer standardized by age has gradually increased. In Sweden, the increase for the last twenty years is 1.2 per cent per year[3].

However, while the incidence has gradually increased in the Nordic countries, mortality has as shown in figure 1 slowly decreased. The reasons for the marked decrease in the proportion of fatal breast cancer patients are several including earlier diagnosis and improved therapy.
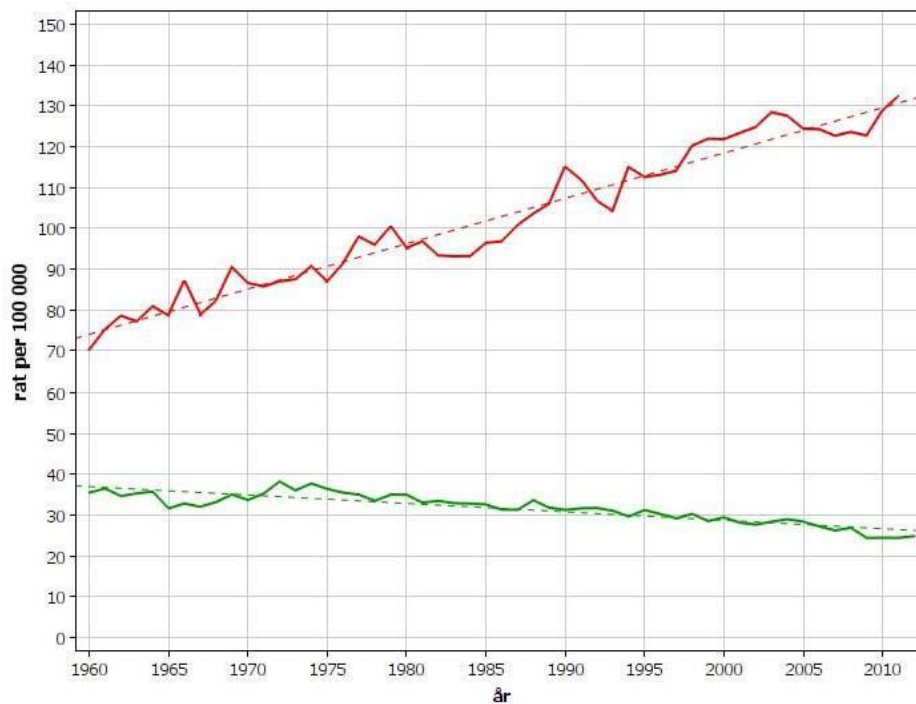
Figure 1 Breast cancer incidence (red) and mortality (green). Age standardization according to the nordic standardpopulation of the year 2000: NORDCAN[4] .

1.2 DIAGNOSIS

Patients seek help from the health care system with a lump or other symptoms such as secretion, redness or deformation of the breast. Any such symptoms from the breast should be referred to a specialized unit with no delay. There the breast should be investigated with a triple diagnostic procedure, including palpation, mammography (often combined with ultrasound and sometimes breast magnetic resonance imaging) and fine needle cytology or core biopsy. Patients may also be referred to specialized breast units for triple diagnostics following a suspicious finding at mammography screening.

The radiological examinations should be performed before the biopsy. A lobular cancer can be difficult to detect with mammography and cytology. Therefore, a diagnostic resection should be considered when radiology and cytology do not correlate with symptoms and the findings from physical examination.

Finally, the diagnosis is established with microscopy of either a fine needle aspirate or a histologic specimen.

In Sweden, it has been compulsory since 1958 for both the treating physician and the pathologist/cytologist to independently notify the Swedish cancer register (SCR) of all new incidences of cancer. The SCR receives reports containing the ICD code of the malignancy, the histological systematized nomenclature of medicine (SNOMED) code, the TNM stage of the disease, the date of diagnosis, the date of birth for the patient, and the personal identification number unique to each individual in Sweden. The completeness of the national register is about 96%[5]. The majority of the patients are also regionally registered in a quality-management register. These registers contain more detailed information of incident tumor characteristics and primary treatment. For breast cancer, >95% of the patients are treated according to the management programs and registered in the regional quality-management register databases associated with the programs[6].

1.3 SCREENING

During the nineteen seventies several randomized trials were initiated in Sweden to investigate the ability of mammography screening to reduce mortality from breast cancer. The results demonstrated a significant reduction of breast cancer mortality in persons randomized to mammography[7]. Therefore, nationwide mammography screening was introduced in the late eighties and the National Board of Health and Welfare (Socialstyrelsen) now recommends women between 40 and 74 years to participate. All Nordic countries have now introduced mammography screening. Denmark was last to introduce it in 2009 partly as an effort to improve breast cancer survival rates that since many years were inferior to those reported from other Nordic countries and similar to corresponding unfavorable data from Great Britain[8].

With the introduction of screening, the distribution of the biologic subtypes of breast cancer

shifted, and overall incidence increased by approximately 10%[9]. Decades later, this increased age-adjusted incidence of breast cancer has remained high[10]. The bulk of this increase has been the early stage breast cancers, suggesting that screening contributes disproportionately to the diagnosis of biologically more indolent forms of breast cancer[11,12]. While screening is associated with a relative mortality reduction of 20%[7,13], it has increased the diagnosis of low risk lesions and contributes to overtreatment [10,11,14,15]. In Sweden, in 2017, two thirds of the breast cancers in patients aged 40-74 years were detected by screening[16].

1.4   PATHOLOGY

The surgical breast cancer specimen and axillary lymph nodes are assessed and classified histologically according to the World Health Organization (WHO) classification system from 2003[17]. The majority of invasive breast cancers are ductal carcinomas (80-90%). About 10% are classified as lobular carcinoma with no tendency to form ductal structures. Besides defining the tumor as malignant, the pathologist provides prognostic information by performing histological grading as described by Bloom and Richardson in 1957[18] and later revised by Elston and Ellis in 1991[19]. This grading system (BRE) contains assessments of the components; tubular formation, nuclear atypia and number of mitoses, where all the components are given scores from one to three. A total ranking of three-to nine is then determined with nine as the most malignant grade. A ranking 3-5 is classified as grade 1, 6 and 7 as grade 2 and 8 or 9 as grade 3. Currently pathologists also stain breast cancer surgical specimens for the Ki 67 antigen as a measure of cancer cell replication, receptors for estrogen and progesterone indicating hormone dependence and also the HER2 surface antigen for information on sensitivity to anti HER2 therapy. For modern pathology IHC is a very important tool and also used as described below in this thesis using formalin fixed tumors. The IHC procedure allows the qualitative identification by light microscopy of antigens in sections of formalin fixed, paraffin-embedded tissue, via sequential steps with interposed washing steps.

Prior to staining, endogenous peroxidase activity is blocked and sections are subjected to epitope retrieval. The section is subsequently incubated with the primary antibody. A biotin-conjugated secondary antibody formulation that recognizes in our case mouse immunoglobulins is used to detect the primary antibody. A streptavidin-peroxidase conjugate is then applied and binds to the biotin present on the secondary antibody. Sections are further incubated with the substrate/ chromogen, 3,3' - diaminobenzidine (DAB), and DAB Substrate Buffer. Reaction with the peroxidase produces a visible brown precipitate at the antigen site. Sections are counterstained with hematoxylin and coverslipped. Results are interpreted using a light microscope.

Molecular pathology with gene expression profiling is not yet included in Swedish standard care but has in clinical trials allowed subgroups of breast cancers to be correlated to prognosis and treatment response[20-23]. When gene expression profiling is used messenger-RNA is extracted from the tumor and the enzyme reverse transcriptase is used to copy the mRNA into stable double stranded-cDNA. The cDNA is fragmented and fluorescently labelled. The labelled fragments bind to an ordered array of complementary oligonucleotides, and measurement of fluorescent intensity across the array indicates the abundance of a

predetermined set of sequences. These sequences are typically specifically chosen to report on genes of interest within the tumor. Based on gene expression, eventually four groups of breast cancer have emerged: luminal A, luminal B, HER2 and triple negative. The concordance between molecular subtypes measured by gene expression and estimated from the IHC staining described above is relatively high (75-90%)[23]. The use of the following IHC markers to determine molecular subtype has therefore been suggested according to St Gallen International Expert Consensus 2011[24] as described in the table 1 and figure 2 below from the Swedish national guideline.

Table 1 Breast cancer subtypes according to the St Gallen expert consensus guidelines[24]

Type	ER	PR	HER2	Ki67
Luminal A	ERpos	PRpos or neg	HER2neg	Low Ki67
Luminal B	ERpos	and/or PRpos	HER2+ with any Ki67	High Ki67
HER2	ERneg	PRneg	HER2pos	
Triple negative	ERneg	PRneg	HER2neg	

Figure 2 Breast cancer subtypes according to the Swedish national guidelines[25], which follows definition of subtypes by Maisonneuve et al[26]

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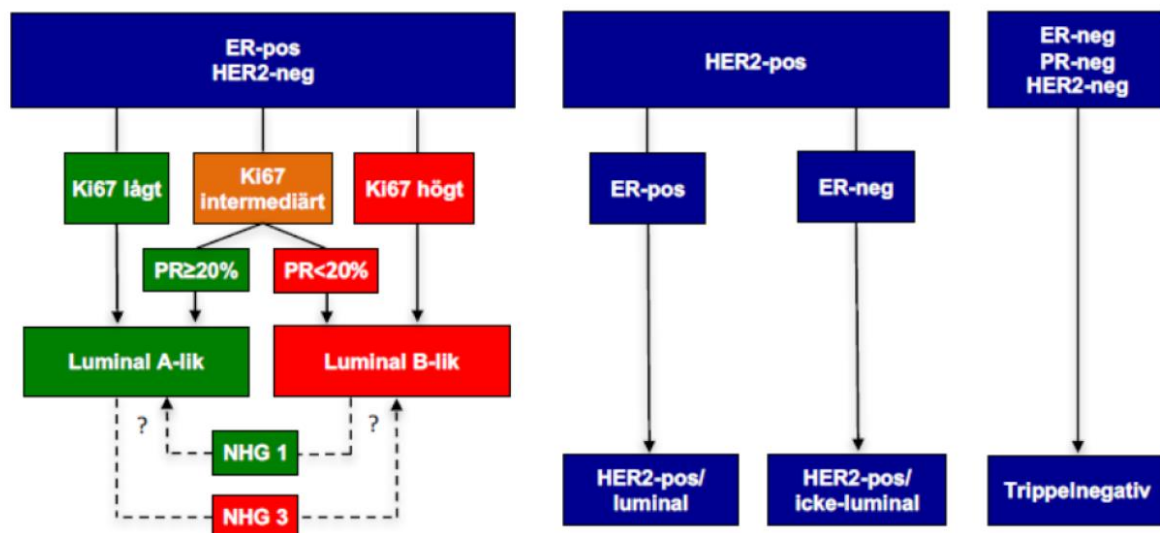
1.5 STAGES OF BREAST CANCER

Breast cancer is commonly further described according to the TNM system (Tumor, Node, Metastasis) where the local and distant extent of the disease is numerically defined and divided to stages I-IV[27]. Accurate definitive staging is based on pathology reports. Such staging is referred to as Pathology TNM or pTNM as illustrated below.

Stage 0 or carcinoma in situ means that the cancer only grows in the cell layers where it started and has not yet penetrated the basal membrane. This is a very early stage that can be considered as a precursor of breast cancer, where 20-30 % may develop invasive breast cancer.

Stage I means that the tumor is up to two centimeters in diameter, and has not spread to the axillary lymph nodes and is referred to as T1N0M0.

Stage II compromises tumors that are two to five centimeters in diameter without nodal involvement (T2N0M0) or tumors five centimeters or less that have spread to less than four axillary lymph nodes (T1N1M0) or (T2N1M0).

Stage III intends tumors larger than five centimeters (T3N0M0), or lymph node involvement of more than three lymph nodes (T1-T3, N2M0).

Stage IV means that the cancer has metastasized locally or to other parts of the body (M1).

Lymph nodes are classified as positive with metastasis if the metastasis is more than two millimeters in size. N1 indicates one to three positive axillary lymph nodes, N2 4-9 positive axillary lymph nodes and N3, ten and more positive axillary lymph nodes or ipsilateral supraclavicular lymph nodes.

Table 2 illustrating breast cancer stages[27]

Stage 0	Tis	N0	M0
Stage 1A	T1	N0	M0
Stage 1B	T0	Nmi	M0
Stage 1B	T1	Nmi	M0
Stage 2A	T0	N1	M0
Stage 2A	T1	N1	M0
Stage 2A	T2	N0	M0
Stage 2B	T2	N1	M0
Stage 2B	T3	N0	M0
Stage 3A	T0	N2	M0
Stage 3A	T1	N2	M0
Stage 3A	T2	N2	M0
Stage 3A	T3	N1	M0
Stage 3A	T3	N2	M0
Stage 3B	T4	N0	M0
Stage 3B	T4	N1	M0
Stage 3B	T4	N2	M0
Stage 3C	Any T	N3	M0
Stage IV	Any T	Any N	M1

1.6   TREATMENT

Nearly all the newly diagnosed breast cancer patients are offered therapy with curative intention but approximately 20 % of the patients will despite this develop metastatic disease. Distant metastatic disease cannot yet be cured but treated with the intention to relieve symptoms and prolong life for months or many times for years.

Surgery is the main curative treatment in most breast cancer cases. In the early 70s *mastectomy* was the standard operation[28]. The tumor size, the localization of the tumor, and the possibility of nodal involvement are taken into consideration in the choice of surgical extent. The options are *complete mastectomy* or *breast-conserving surgery*. Several prospective randomized studies have compared *complete mastectomy* with *partial mastectomy* followed by breast radiotherapy, showing no difference in survival between the two techniques[29-32].Therefore in the early 80s *breast-conserving surgery* became more common[28], and the use of adjuvant postoperative radiation therapy increased. In situations when *mastectomy* is needed, it may lead to long lasting psychosocial problems[33-35]. To lessen these problems immediate reconstruction with oncoplastic surgery or later breast reconstruction have become more common[36]. Aesthetically successful breast conserving surgery was reported to yield high quality of life scores[37].

During the last two decades sentinel node surgery was established as a safe technique to investigate the axilla. No difference in the overall survival, disease-free survival or in regional control was found when sentinel node biopsy was compared with conventional axillary dissection[38]. Since the sentinel node technique is associated with fewer complications[39-41] such as arm edema; it is now considered standard care of treatment.

Radiotherapy of breast cancer was introduced more than 100 years ago when it was demonstrated that breast cancer was relatively sensitive to radiation. It was first introduced for palliation but soon also for postoperative therapy to reduce recurrence rates[42]. Radiotherapy after mastectomy became a common treatment of early breast cancer. This remained until the 1970s[28,43-45]. As described below, breast-conserving surgery with radiotherapy and other adjuvant therapies later became common.

Postoperative radiotherapy is currently recommended for patients with a risk of local recurrence of more than 20 per cent in ten years. Radiotherapy of the preserved breast is offered to patients after breast-conserving surgery [46], and patients operated with mastectomy receive radiotherapy of the chest wall and of axillary lymph nodes if the tumor is more than 50 mm in size, or there is significant lymph node involvement in the surgical specimen[25].

Early side effects of radiotherapy, defined as occurring up to three months after termination of radiotherapy, are erythema and pneumonitis[47,48]. Later the skin may become more fibrotic and stiff[49-51]. Lymphedema of the arm can also be seen as a late side effect of radiotherapy with chronic swelling of the arm. Arm swelling is handicapping and difficult to treat but promising results with liposuction were reported[52]. Other more uncommon late side effects, are late appearing pneumonitis, pulmonary fibrosis, rib fractures, secondary malignancies and brachial plexus neuropathy[53,54].

Late side effect from radiotherapy revised from Dan Lundstedt[55]

Variable	Axillary dissection and RT No. (%) RR (ci 95%)	Axillary dissection no RT No. (%) RR (ci 95%)
Paraesthesia in the hand	38/192 (19.8) 1.47 (1.02–2.11)	68/505 (13,5) 1.00 (Reference)
Oedema of the hand	43/192 (22.4) 1.46 (1.04–2.03)	78/507 (15.4) 1.00 (Reference)
Pain in the arm	38/192 (19.8) 1.02 (0.73-1.42)	98/504 (19.4) 1.00 (Reference)
Decreased strength in hand	26/192 (13.5) 1.47 (0.94-3.47)	47/509 (9.2) 1.00 (Reference)

The majority of the node-positive as well as node- negative patients are now offered hypofractionated radiotherapy of 40 Gy in 15 fractions based on recent data[56]. It has recently been shown that partial breast radiation may be equally safe as whole breast radiation for women more than fifty years old with small luminal cancers[57]. The ACOSOG Z11[58] (an American College of Surgeons Oncology Group study) and the AMAROS[59] study (a multicentric study by European cancer departments collaborating in the European Organisation for Research and Treatment of Cancer, EORTC) both show that patients with a positive sentinel node can safely be spared axillary dissection and receive radiotherapy only, with little risk of lymphedema. The AMAROS study showed a significantly lower rate of lymphedema at five years in the radiotherapy group as compared to the axillary dissection group (11% versus 23% and a p-value of less than 0.0001).

The Oxford overview has shown that at five years postoperative radiotherapy results in a reduction in local recurrences of almost 20% and a 15 year overall mortality reduction of 5%[60]. The conclusion is therefore that for every four local recurrences avoided, one breast cancer death is avoided. The effects on recurrences and death were similar for all patients, irrespectively of age and tumor characteristics. In the Oxford overview published in 2011 on the effect of radiotherapy after breast conserving surgery only, similar effects were found, with recurrences reduced by 50%, and breast cancer death rate by 16%[61]. The absolute reductions in recurrences and deaths were greater for node-positive women. Also, among node-negative women the absolute recurrence reduction varied according to ER-status, tamoxifen use, and extent of surgery, with most benefit found for patients with the largest recurrence risk[61].

Endocrine therapy was introduced in 1896 when it was shown that oophorectomy had a good clinical effect on locally advanced breast cancer in premenopausal women[62].

One risk factor for developing breast cancer is exposure to estrogen. Most invasive breast cancers (70%-80%) express the estrogen receptor (ER), and are dependent on estrogen for their survival. Response to endocrine treatment tends to improve with increasing proportion of ER positive tumor cells and with the homogeneity of the ER staining between cells[63,64]. In clinical practice, a cut-off of 10% positive cells is currently used to classify tumors as ER-positive. However, a cut-off of 1% is sometimes used in i.e. the US[65]. Some previous studies and data presented in this thesis show that combined ER and PR positivity increase the probability of response to endocrine therapy[66,67]. However, these results contrast to a meta-analysis by the EBCTCG that did not find that PR positivity independently predicted tamoxifen response in ER positive tumors[30].

Both estrogen and progesterone receptors are members of the steroid–thyroid hormone–retinoid receptor superfamily of ligand-activated nuclear transcription factors[68,69]. The two most common isoforms of progesterone receptors (PR-A and PR-B) can act as homo-or hetrodimers[70,71]. They are transcribed from two different promoters of the same gene on human chromosome 11 q22–q23[69].

In premenopausal patients the ovaries are the main source of estrogen, while in postmenopausal women estrogen is predominantly produced by aromatization of the adrenal and ovarian androgens in the liver, muscle and fat tissue[72]. The ER pathway can be targeted by inhibiting the receptor with tamoxifen or fulvestrant, or by removing estrogen via oophorectomy or Luteinizing hormone releasing hormone agonists (LHRH) in premenopausal patients or by aromatase inhibitors (AIs) in postmenopausal patients.

In humans, tamoxifen has an antiestrogenic effect in some tissues and an estrogenic effect in other tissues. The antiestrogenic effects are most prominent in breast tissue and the vagina, where tamoxifen reduces glandular as well as epithelial developement[73,74]. The antiestrogenic effect on breast tissue decreases the risk of primary and contralateral breast cancer[75,76]. Among the antiestrogenic effects of the drug are also vasomotor symptoms, such as hot flushes, the most common side effect of tamoxifen. However, in some tissues the estrogenic effects of tamoxifen are dependent on menopausal status.

Among postmenopausal women, estrogenic effects are predominant, in the uterus with an increased risk of endometrial cancer, in the heart with decreased risk of coronary events, in bone with decreased rate of fractures and in coagulation with doubled risk of thrombosis. In premenopausal women tamoxifen is antiestrogenic[77-84] generating hot flushes, cold sweats, genital itching and pain in intercourse.

Side effects revised from the NSABP P-1 Study[85]. A double blinded prevention trial with five years of 20 mg tamoxifen daily for women 35-70 with a quality of life questionary at baseline, at 3 months, and then every 6 months until 36 months. Side effect counts when reported positive at least one time.

Side effect	Tamoxifen	Placebo
Vaginal discharge	55 %	34 %
Cold sweats	21 %	15 %
Genital itching	47%	38 %
Hot flushes	78 % (severe 30 %)	65 %
Pain in intercourse (age 35-49)	32 %	24 %

Side effects revised from the international multicenter ATLAS-study with patients almost always treated with 20 mg per day in five respectively ten years.

Out of 48064 patients in the group treated for ten years and 46959 patients treated for five years from the ATLAS study[86]

Deaths	*Ten years number*	*Five years number*	*event ratio*
Pulmonary embolus	10	8	1.21 (0.48-3.04)
Endometrial cancer	17	11	1.49 (0.71-3.13)

Disease	*Ten years number*	*Five years number*	*event ratio*
Contralateral breast cancer	415	460	0.88 (0.77-1.01)
Endometrial cancer	116	63	1.74 (1.30-2.34)
Pulmonary embolus ever hospitalized or leading to death	41	21	1.87 (1.13-3.07)

Aromatase inhibitors (AI) were widely introduced in clinical practice in the 1990s but are only effective in postmenopausal patients, since aromatase inhibition does not inhibit ovarian estrogen production. In contrast, tamoxifen can be used for all women regardless of menopausal status. Tamoxifen or AI adjuvant treatment of ER positive disease can reduce breast cancer mortality by 30% during 15 years[87] with tamoxifen reducing the event rate from 4-8% yearly to 2-8% yearly.

While tamoxifen, AIs and ovarian suppression are currently used in the adjuvant settings, additional endocrine therapies are used in metastatic disease. Examples are fulvestrant, a pure estrogen antagonist that also down regulates the ER, and synthetic progestins such as megestrol acetate. Palbociclib is a CD4/6 inhibitor used in addition to fulvestrant or AI in the metastatic setting of hormone receptor positive breast cancer[88]. In addition, high doses of estrogen can be effective in patients, that initially responded to endocrine therapy but later became resistant to previous endocrine therapy[89].

Chemotherapy became available after World War 2 and around 1960 it became evident that three of the early agents cyclophosphamide, methotrexate and fluorouracil could induce short lasting tumor shrinkage and palliation. After 60 years of use, cyclophosphamide is still a standard drug widely used in the therapy of breast cancer.

Around 1970 the three agents were combined and polychemotherapy was introduced for palliation and soon also for adjuvant treatment. Later several other cytotoxic agents were found to be active against breast cancer and epirubicin, paclitaxel, docetaxel, vinorelbine and capecitabine are cytotoxic drugs currently used in Sweden.

Targeted therapy aims at specific changes in malignant cells. Genetic changes or their corresponding proteins can be targeted with drugs.

Tamoxifen targeting the estrogen receptor was the first successful targeted therapy in cancer and is listed as an essential medicine by the World Health Organization. Tamoxifen is in a class of selective estrogen receptor modulators (SERMs), having tissue-dependent as well as species-dependent effects. For example, tamoxifen has a pure antiestrogen effect in chicks[90].

Another well-known targeted therapy is trastuzumab (Herceptin). This drug became commonly used around the year 2000. Trastuzumab is a humanized monoclonal antibody targeting HER2[91]. Approximately 10% of breast cancer tumors have an amplified HER2 gene resulting in a disease with high risk of recurrence and most often resistance to endocrine therapy. These patients are candidates for one year of adjuvant trastuzumab therapy[25].

Bisphosphonate treatment targeting osteoclasts is able to reduce skeletal events and reduce mortality for high risk postmenopausal breast cancer patients by approximately two per cent at ten years[92]. This is now standard of care[25].

1.7 ADJUVANT TREATMENT

After the introduction of radical mastectomy more than 100 years ago[93], it became evident that breast cancer patients in spite of radical surgery carried a substantial risk of developing local and distant disease recurrences. Based on this experience, trials of postoperative treatment were introduced.

These are referred to as adjuvant treatment. Based on the marked effect of radiotherapy and ovarian ablation for palliative treatment of breast cancer the first trials of adjuvant treatment included radiotherapy[44,94] or castration[95] and later trials with cytotoxic chemotherapy[96,97] and tamoxifen[98,99], were introduced. Highly significant reductions in the annual rates both of recurrence and death were produced by radiotherapy, by tamoxifen, by ablation below age 50 and by polychemotherapy[99]. Tamoxifen was introduced in the palliative setting during the 1970s[100], and became commonly used in the adjuvant setting in the 1980s[101].

Most current patients with breast cancer are therefore offered adjuvant treatment.

During the nineteen seventies randomized trials demonstrated that postoperative combination chemotherapy with cyclophosphamide, methotrexate and fluorouracil could significantly reduce breast cancer mortality [87,102]. Later introduced regimens containing the anthracyclines doxorubicin or epirubicine[87,103] and the taxanes paclitaxel and docetaxel have resulted in further prolonged survival[104] but also in new types of toxicity such as cardiotoxicity, oncogenicity and allergic reactions. Adjuvant chemotherapy is now individualized and more toxic combinations are reserved for patients with high-risk disease. That is patients with triple negative disease, Her 2 positive tumors larger than 5 mm and luminal B tumors, but also luminal A type tumors with extensive lymph node metastasis. For anthracycline-based therapy, the acute side effects are nausea, alopecia, vomiting, lack of appetite, sensibility to infections, mucositis, acute menopause and fatigue. Anthracycline therapy can also give late side effects such as acute myeloid leukemia and heart failure. Toxicities for taxanes are pain in muscles and bones, sensibility to infections, neurotoxicity, hypersensitivity reactions and fatigue[105]. For premenopausal patients, chemotherapy may reduce breast cancer mortality by about 40% and for postmenopausal patients, this reduction is about 20%[87] during 15 years.

For some patients receiving adjuvant therapy, it is lifesaving. However, for the majority of the patients it adds unnecessary risk, trouble and toxicity. Unfortunately, we are unable to precisely identify the patients that should survive without adjuvant therapy and those who die from cancer in spite of adjuvant therapy. This is an ethically difficult issue that motivated my studies to more precisely identify patients benefitting from radiotherapy and adjuvant tamoxifen. It needs consideration by those treating cancer and it was discussed by the last St. Gallen panel[106] recommending breast cancer therapy. The panel agreed on escalating radiation therapy with nodal irradiation in high-risk patients, but was in favor of omission of boost in low-risk patients. High risk means patients with tumor bigger than 5 centimeters and/or extensive lymph node engagement. The panel recommended Gene expression signatures that permit avoidance of chemotherapy in many patients with ER positive breast cancer. The panel further escalated the recommendations for adjuvant treatment for women with high-risk ER positive tumors to include ovarian suppression in premenopausal women and extended therapy for postmenopausal women. The low-risk patients, however, can avoid these treatments.

2   AIMS OF THE STUDY

Adjuvant treatment of breast cancer after radical resection is based on our understanding of the prognosis and the predictive information we can obtain from the pathology report and staging. Retrospective correlation of overall survival, disease free survival and the pretreatment findings are the bases for patient groupings. Randomized trials then give us information on the effects of treatment. Based on the interpretation of trial results, international and national guidelines are developed, where different subgroups/stages are recommended treatment aiming to achieve a well-judged balance of treatment effects versus side effects.

The overall aim of this thesis is to add knowledge that could improve the selection of patients, that is to fine tune treatment. The particular aim includes:

i)   investigating the prognoses of patients diagnosed with different stages of breast cancer

ii)   analysis of the benefits from postoperative radiotherapy in stage 2 patients with 1-3 metastatic axillary lymph nodes and

iii)   analysis of the influence of the progesterone receptor (PR) level on the benefit from postoperative tamoxifen therapy.

The first study focuses on the effect of adjuvant radiotherapy given to patients with one to three positive lymph nodes. The hypothesis is: loco-regional radiotherapy given to breast cancer patients with 1-3 positive axillary nodes prolongs survival as compared to that of patients receiving less or no locoregional radiotherapy.

The second study compares the benefit from adjuvant tamoxifen in patients with tumors positive for both estrogen receptor (ER)and PR with that for patients with tumors positive for ER only. The hypothesis is: PR positivity as compared to PR negativity is a marker for increased benefit from tamoxifen treatment.

The aim of the third article was to study the development in breast cancer survival after primary diagnosis divided by stage and age. The hypothesis is: the gradual improvement of survival in breast cancer is evenly distributed between stages of disease and age groups rather than unevenly distributed between stages and age groups.

The fourth article extends the study in the second article to include tumor content of PR estimated by immunohistochemistry in tumor sections and expression of the PR gene. The hypothesis is that PR positivity as compared to PR negativity measured with gene expression or whole section IHC is a marker for increased benefit from tamoxifen treatment.

2.1 STUDY 1

2.1.1 BACKGROUND

The early studies comparing postoperative radiotherapy versus control in patients with lymph node positive disease reported unfavorable survival rates in the experimental arms and even worse rates in the control arms Overgaard[61,107]. Overgaard et. al. recruited premenopausal patients from 1982 to 1989. The ten year survival was only 54 % in the group that received both CMF and radiotherapy. There was a high loco-regional recurrence rate (30%) observed in patients with one to three involved nodes. However, only a mean of seven lymph nodes were identified in the surgical specimens indicating that some patients had metastatic nodes left in the axilla partially explaining the high recurrence rate. In the EBCTCG metaanalysis of studies beginning before year 2000 the survival rates are also low[61]. For node negative patients the risk reduction at 15 years for patients receiving radiotherapy was from 25, 5% to 21,1% that is 4,4% reduction in breast cancer deaths. The corresponding risk reduction at 15 years for node positive patients was from 51,3% to 42,8% that is 8,5%. In our study with more modern treatment the relative survival is more than 75% at ten years for the whole cohort. It has therefore been questioned whether the early randomized studies are relevant for patients participating in mammography screening programs and treated with modern surgery and systemic therapy. A survey among European radiation oncologists on the use of post mastectomy radiotherapy in women with 1-3 positive nodes showed wide variations among those advocating radiotherapy from as little as 19% in Italy and up to 74% in Spain and Portugal[108]. We therefore wanted to provide more modern data from a large cohort on the effect of postoperative radiotherapy on survival.

2.1.2 PATIENTS AND METHODS

For our first study we selected patients from two population-based cohorts of all patients diagnosed with breast cancer between January 1 1989 and December 31 2006. 18 697 were diagnosed in the western heath care region (W-region) and 11 032 in the South Eastern region (SE-region). As seen in the figure 4 below, 2750 from the W-region and 1698 from the SE-region were found to have 1–3 positive axillary lymph nodes in the surgical specimen. To these patients postmastectomy radiotherapy was generally given in the southeast region (89% of all cases) and generally not given in the west region (15% of all cases). For patients with 1–3 positive nodes who underwent breast-conserving surgery, patients in the west region had breast radiotherapy only, while patients in the southeast region had both breast and lymph nodes irradiated. The patients were followed up for vital status until August 2010 through record linkage to national population registers.

We retrieved information from the quality-management registers regarding age at diagnosis; type of surgery; tumor size; number of examined nodes; hormone receptor status; grade; and adjuvant therapy regarding radiotherapy (Yes/No), endocrine therapy (Yes/No), and chemotherapy (Yes/No).

Relative survival was computed using the Ederer II method[109]. Mortality data for the general population in Sweden were used to estimate expected survival rates for the study populations. The mortality data comprised the probability of death for single-year age groups in 1-year calendar periods. Relative risk between different groups was estimated by Poisson regression. A *p* value of <0.05 was considered to be statistically significant.
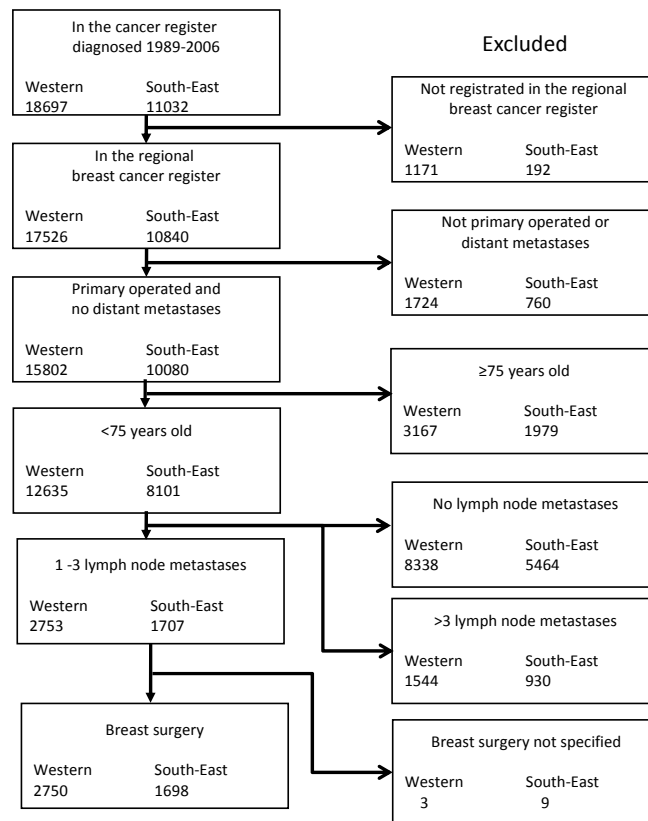
Figure 4. Consort diagram study 1 We selected patients with one to three positive nodes: 2750 from the W region and 1698 from the SE region, both representing 15% of the respective total populations as illustrated. All registered incident breast cancer cases in the Swedish Cancer Register from these two regions from 1989 to 2006 comprised the initial study base. From the total cases, all primary operated cases without initial distant metastases and age <75 years with one to three positive lymph nodes were selected.

2.1.3   RESULTS AND DISCUSSION

The 10-year relative survival for patients with 1–3 positive lymph nodes was 78% in the west region and 77% in the southeast region (*p*=0.12).

Separate analyses depending on type of surgery, as well as number of examined nodes, also revealed no significant differences in relative survival rates.

The survival rate in this study of Swedish cohorts with 1–3 positive nodes is similar to t that of groups without lymph node involvement in the EBCTCG analysis, in which no apparent effect on breast cancer specific survival was seen for postmastectomy radiotherapy[61].

One reason that the effect of radiotherapy was not detected in this cohort study is that the survival rates are high in both cohorts. These data need to be interpreted with caution, the first conclusion that radiotherapy has no effect on the survival of patients with high survival rates is not justified. Indeed, it has been demonstrated that postoperative radiotherapy effectively reduces local recurrence rates and that approximately 25% of the local recurrences result in breast cancer specific mortality. In a nonrandomized study like ours, there certainly are minor differences between the groups that may affect outcome. Differences that we detected were that in the western region there were more lymph nodes removed and more cytotoxic agents given as compared to the south-eastern region, where more endocrine therapy was given.

Our major conclusion is that in a mammography screened population receiving modern surgery and adjuvant therapy, the presence of 1-3 involved node in the surgical specimen does not strongly motivate postoperative radiotherapy. Other indicators must also be considered.

2.2   STUDIES 2 AND 4

2.2.1   BACKGROUND

The independent predictive information from progesterone receptor (PR) positivity for breast cancer treated with tamoxifen has been questioned after an overview by the Early Breast Cancer Trialists' Collaborative Group (EBCTCG). However, most studies in the overview were performed with different cytosol assays before modern PR immunohistochemistry (IHC) was developed[75]. We therefore decided to analyze PR with more modern techniques.

In study 2 we investigated the predictive value of PR determined with IHC in tissue micro arrays from estrogen receptor (ER)-positive tumors from patients participating in the Stockholm trial of adjuvant tamoxifen therapy. A second aim was to investigate if the effect varies over time and/or with increased levels of PR positivity. See figure 5 left flow-chart.

2.2.2   PATIENTS AND METHODS

In study 2 the IHC staining was performed using the Ventana HX automatic system BenchMark (Ventana Medical System, SA IllKirch, Cedex, France). Primary monoclonal anti- bodies were the CONFIRM[TM] mouse anti-ER antibody (clone 6F11) and the CONFIRM[TM] mouse anti-PR anti- body (clone 16) from Ventana Medical Systems. Antigen retrieval and staining procedure were performed according to the instruction by the Ventana manufacture. Positive controls were run with each batch. Only the invasive part of the carcinoma was assessed, and for each

case, all three cores of the TMA were reviewed. The receptor levels presented are based on an average of the three cores of the TMA. The proportion of stained nuclei was recorded as 0, 1–9 %, 10–24 %, 25–49 %, 50–74 %, 75–89 %, and >90 %. The scoring was done by two pathologists Lambert Skoog and Britta Löfdahl.

To compare the association between PR expression and clinical parameters, the Pearson chi-squared test (categorical variables) and the Student's t-test (continuous variables) were applied.

Cumulative recurrence risk, cumulative distant recurrence risk and for cumulative breast cancer specific mortality were estimated by the Kaplan-Meier method. Hazard ratios (HR) and 95% confidence intervals were estimated using the Cox's proportional hazards model. When we investigated the influence of different levels of PR positivity on recurrence free survival Helena Fohlin, expert statistician and shared first author, performed subpopulation treatment effect pattern plots (STEPP)

STEPP is an exploratory technique for graphical testing of interactions between treatment and continuous covariables. It considers sequences of potentially overlapping subpopulations defined with respect to covariate and is especially useful when trying to identify subpopulations of patients for whom the overall results may be less representative. There are several advantages of the method, e.g., that no assumption about the nature of the relationship between the outcome and the covariate in each treatment group must be done and that is avoids dichotomization of co-variables, which may be subjective. Though, the size of the subpopulations is critical to the performance of the method and, hence, to the interpretation of the results[110,111]. In study 2 we used STEPP to investigate the interaction between continuous PR values and the effect of tamoxifen. To avoid results based on one single subgroup division, we performed the STEPP analysis with different numbers of subgroups and ensured that the achieved results were similar independently of the chosen number of subgroups.

In study 4 of 582 patients with ER positive tumors, we included PR IHC of whole tumor sections, PR H-scoring, PR scored by gene expression, and subanalyses of patients with luminal A subtype tumors. See figure 5 right.

In 2014, immunohistochemistry (ER, progesterone receptor [PR], human epidermal growth factor receptor 2 [HER2], and Ki-67) was performed on 727 patient whole-tumor sections in a random order at a single medical CLIA laboratory (University of California Davis Medical Center, UCDMC). The FFPE tumors were sectioned at 5 microns and mounted on plus coated glass slides in the Tissue Profiling Facility at the Science for Life Laboratory in Uppsala University. The slides were stained in the UCDMC IHC laboratory using DAKO Link48 Autostainer. Antibodies used were: ER (SP1; Spring Bioscience M301), PR (PgR 636; DAKO IR068), HER2 (HercepTest; DAKO SK001), and Ki67 (MIB-1; DAKO M7240). EnVision+ detection was used, following standard recommended procedures and with per-run positive controls assessed by quantitative image analysis to ensure consistent run-to-run staining intensity.

Pathologists at the University of California with expertise in breast diagnostics, as a part of the ATHENA Breast Health network, scored the percentage of positive breast cancer cells for each ER and PR intensity level (0, +1, +2 or +3) compared to established standards. In addition, the

percentage of positive cancer cells for HER2 and Ki-67 were also scored. We computed the total percentage of cells stained positive for ER and for PR (at intensity levels +1, +2 and +3) and the ER and PR H-score defined as the sum of the percent of positive tumor cells at each intensity level multiplied by an ordinal value corresponding to the intensity level (0=none, 1=weak, 2=moderate, and 3=strong).

Gene expression data was generated using custom designed Agilent arrays containing approximately 32.1K probes, representing approximately 21.5K unique genes from FFPE breast cancer tumor tissue. Gene expressions from each chip were log2-scaled and upper quartile normalized. In total 652 breast cancer tumors passed the quality check.

Survival curves were constructed by Kaplan-Meier analyses and differences between groups were assessed using the Log-rank test.

Multivariate analyses of tamoxifen benefit in groups based on PR status were performed using Cox proportional hazard modeling adjusting for classical patient and tumor characteristics such as age and year of breast cancer diagnosis, HER2 status, Ki-67 status (15% used as positive cut-off), tumor grade and tumor size.
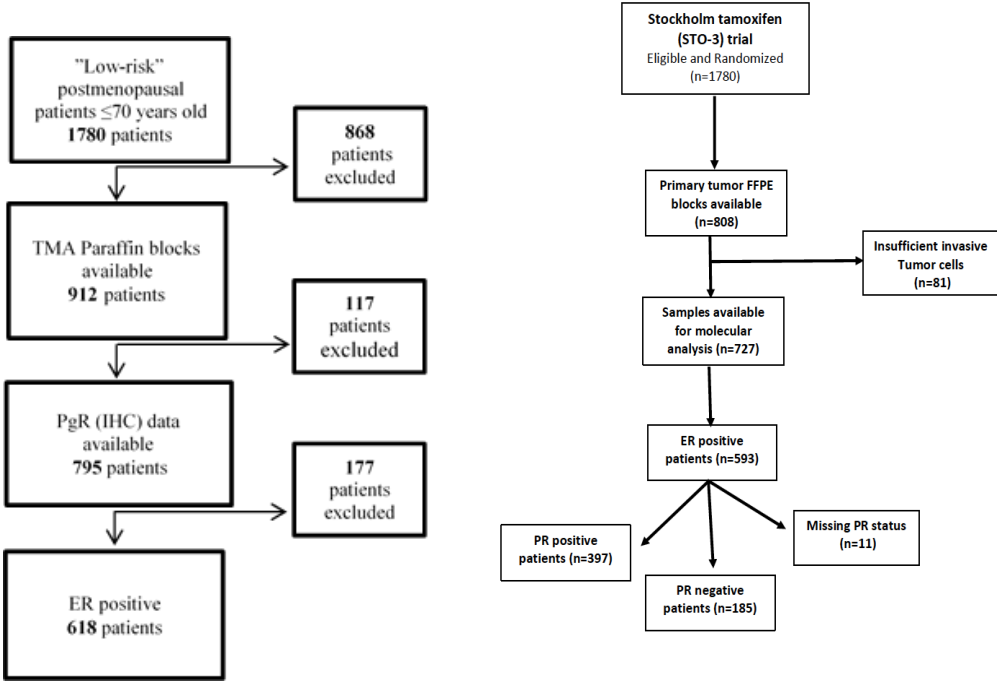
Figure 5 Consort diagram from study 2 left and study 4, right

Comments regarding study group selection. For study 2, from November 1976 through June 1990, 2738 patients entered the Stockholm 3 trial. Among them, 1780 patients (65 %) with no lymph node metastases and a tumor diameter of 30 mm or less (established by histological examination) were classified as "low risk" and did not receive cytotoxic chemotherapy. In this group, 432 patients were treated with breast conserving surgery including axillary dissection plus radiation to the breast (50Gy / 5 weeks). The remaining 1348 patients had a modified

radical mastectomy and no radiotherapy. From the low-risk patients, we were able to retrieve paraffin blocks from 912 for construction of microtissue arrays (TMAs). The trial included patients irrespectively of hormone receptor content, but prospectively collected data on ER and PR status were available and archived tumor tissue had sufficiently high quality for IHC analysis in 795 cases. These patients had similar age distribution, tumors of similar size, and proportion of ER-positive tumors as the entire group of 1780 patients with low-risk tumors. The proportion of patients randomized to tamoxifen therapy was 52 % as compared to 50 % in the entire group. Among the tumors analyzed for PR by IHC, 591 were ER-positive as determined by IHC, while 27 tumors with missing data on ER by IHC were ER-positive according to cytosol analysis, resulting in 618 ER-positive tumors (Fig. 5 left).

Study group selection to study 4. The right part of figure 5 illustrates the selection of the subset of formalin-fixed paraffin-embedded tumor material available from the same clinical trial and used in study 2. This subset was also well balanced to the original STO-3 trial cohort with regards to tumor characteristics, such as tumor size (78% vs 81%), ER status (78% vs 80%) and treatment arm assignment (52% vs 50%). All patients included in the STO-3 randomized trial have detailed patient and clinical information.

2.2.3   RESULTS AND DISCUSSION

Study 2 showed that patients with ER positive tumors that were also PR positive by IHC had significantly prolonged recurrence free survival benefit from tamoxifen as compared to those with tumors positive for ER only.

There was no trend indicating more benefit with increasing proportion of stained cells. Results from the trial have previously shown a significantly reduced recurrence rate among patients with ER-positive tumors randomized to tamoxifen therapy versus control (HR = 0.53 (0.37–0.74), p< 0.001)[112].

Patients with ER positive as well as PR positive tumors receiving tamoxifen had a reduced recurrence risk compared with those who were not treated with tamoxifen (HR = 0.43, 95 % CI 0.29–0.62, p< 0.001) For patients with ER positive and PR negative tumors, the effect of the treatment was time-dependent. The first 5 years after diagnosis the tamoxifen treated patients had a reduced recurrence risk (HR = 0.39, 95 % CI 0.15–1.00, p = 0.05), whereas it increased thereafter (HR = 1.34, 95 % CI 0.69–2.60, p = 0.39). Seen over the whole time period, the relative risk ratio for tamoxifen treatment versus the control group when comparing PR positive and PR negative tumors was 0.49 (95 % CI 0.25–0.92, p = 0.03).

Study 4 demonstrated that tamoxifen treated patients with ER-positive tumors that were also PR-positive by IHC or gene expression had a reduced long term risk to develop metastasis as compared to untreated patients with ER and PR positive tumors (PR-positive IHC Hazard ratio [HR] = 0.34, 95% confidence interval [CI] 0.21 to 0.56, PR-positive by gene expression HR = 0.38, 95% CI 0.24 to 0.63. For treated patients with ER-positive but PR-negative tumors by IHC or gene expression there was not a significant risk reduction (PR-negative IHC HR = 0·58, 95% CI 0·32 to 1·06, PR-negative by gene expression HR = 0·61, 95% CI 0·32 to 1·16)) compared to untreated patients with the same receptor profile and the risk reduction tended to

be limited to the first decade after surgery. We used both metastasis-free survival and breast cancer-specific survival as endpoints and they yielded similar results. Two years of tamoxifen therapy had a very marked effect on risk of metastasis for patients with ER positive and PR positive luminal A tumors as compared to patients in the untreated arm with the same receptor pattern (HR = 0.23, 95% CI 0.11 to 0.49).

One reason that the EBCTCG study does not show any extra benefit from tamoxifen for patients with tumors positive for both ER and PR may be that 41% of the patients in the overview was additionally treated with cytotoxic agents.

Another reason may be that the cytosol techniques used in the metaanalysis from the EBCTCG was not good enough[113].It is stated in the overview that as much as 21% of the ER-negative cancers were PR-positive indicating that the PR technique used resulted in many false positive PR classifications. It has been clearly demonstrated with IHC and gene expression assays that PR-positivity and PR gene expression is a rare event only present in 1-4 % of ER-negative tumors [114]. Our data on the increased benefit from tamoxifen therapy for patients with both ER and PR positive tumors may be compared to those obtained by Elebro et al. demonstrating favorable outcome for patients with ER and androgen receptor (AR) positive tumors[115,116]. A study of the effect of endocrine therapy of patients with triple positive (ER+PR+AR) tumors is required.

2.3   STUDY 3

2.3.1   BACKGROUND

Before approximately 1750 when breast surgery was introduced, breast cancer was not considered curable. Around 1850 when general anesthesia was available radical mastectomy was introduced by Halsted at al [93]and local recurrences became less common but long term survival was not reported. However, in the 19th century some untreated breast cancer patients survived long periods[18]. From 1960 after the introduction of cancer registration in Sweden we have information on population based breast cancer survival and Talbäck et al [117] reported that 10 year relative survival increased from 53% 1965 to 74% in 1990. In study 3 we report that after 1990 survival has further increased. The survival rates had clearly increased for the whole group of breast cancer patients but there was limited information on the development of survival for patients with different stages of disease. Therefore, we performed study 3 to investigate stage specific and age specific survival rates

As in study 1, relative survival was computed using the Ederer II method[109]. Age standardization was applied according to International Cancer Survival Standard 1 (ICSS 1) with the weights 0.04, 0.15, 0.37 and 0.44 for the age groups < 40, 40 – 55, 55 – 69 and ≥ 70, respectively. The weights 0.44, 0.22, 0.17, 0.14 and 0.03 were used for the stage standardization for stage I, II N0, II N+, III and IV, respectively. Survival time was calculated from date of diagnosis to 31 December 2014 or to date of death if it occurred before that date. For each stage, excess mortality rate ratio between different calendar periods was estimated

using Poisson regression, including the categorical variables *year of diagnosis* (in groups of five calendar years each) and *age group*.

2.3.2   RESULTS AND DISCUSSION

Using patients diagnosed 1989-1993 as a reference the relative risk of 5 year mortality decreased with 49% for patients diagnosed in the end of the observation period (ci95% 45-58). The mortality tended to decrease for patients with all stages of breast cancer and test for trend resulted in a statistically significant improvement over time in 5 year relative survival in stage III and IV and in 10 year survival in stage I and III.

For each operable stage of disease, patients aged below 40 years or more than 70 years when diagnosed tended to have less favorable survival than patients diagnosed between 40-69 years of age. Test for trend resulted in statistically significant improvements over time for patients diagnosed at ages below 40, 40-54 and 54-69 but less marked improvements for patients older than 70 when diagnosed. Our data on age related to survival are in line with those from Malmö Sweden from a large cohort of single institution patients collected 1961-1991 mainly before the introduction of mammography screening showing decreased 10 year survival for patients below 40 and above 80 as compared to patients 40-49 years of age[118].

Notably, we observed a 40% increase in in breast cancer diagnoses between 1989 and 2013. Part of this might be explained by the aging society, as increasing age is a major risk factor for breast cancer. Gradually increased participation in mammography screening introduction of digital mammography with excellent detection rate and increased use of ultrasound and MR for breast examinations may also have contributed to the increased detection rate. Some of the increased survival may be explained by an increased lead-time bias by this increased detection rate.

During the two decades of patient recruitment, there has been a gradual development of CT-scanning, MR and PET technology facilitating the detection of distant metastasis possibly resulting in an increased rate of patients with stage IV disease. However, this rate remained low around 2.6% during the entire period. Therefore, we think that improved treatment rather than stage migration explains the improved five year outcome.

Moreover, the stage distribution during the time period was relatively stable, which contradicts stage migration as a major explanation for the improved relative survival. However, the proportion of patients with stage II N+ disease markedly increased with time possibly reflecting the improved pathological and radiological techniques available to detect lymph node metastases.

3   CONCLUSIONS

From study 1 where we investigated relative survival of two cohorts of patients with 1-3 axillary node metastasis there are two main findings. The first is that more recent patients with access to mammography screening, dedicated breast surgeons and modern adjuvant therapy have much improved survival rates as compared to the patients participating in the old trials comparing patients treated with regional postoperative radiotherapy with control groups without radiotherapy. Therefore, the old trials have limited relevance for current patients with 1-3 positive nodes. The second conclusion is that with increased survival rates for patients with 1-3 positive nodes there is less room for improvements and a possible improvement was not detectable by comparison of our two cohorts treated with, respectively without regional radiotherapy. Therefore, we conclude that not all patients with 1-3 involved nodes should be recommended locoregional radiotherapy. Other risk factors for locoregional recurrence including lymphovascular invasion, tumor grade and HER2 status should be considered.

In papers 2 and 4 we studied the predictive value of PR for the response to tamoxifen in ER positive breast cancer. The protein synthesis of PR is known to be dependent of a functioning ER[119]. It is therefore reasonable to speculate that truly estrogen dependent cancers should have both receptors to be able to respond to antiestrogen therapy. It was therefore unexpected when the EBCTCG overview found the same benefit from adjuvant tamoxifen therapy in patients with tumors positive to ER only as for those with tumors positive for both ER and PR. Using both immunohistochemistry and gene expression to study PR, we confirm that patients with tumors positive for ER only may benefit from adjuvant tamoxifen therapy, but in contrast to the findings in the overview patients positive for both receptors tended to have more marked and prolonged metastasis free survival rates. Our studies do not have the statistical power to convincingly demonstrate a difference in metastasis free survival between patients with tumors positive for ER only and patients with tumors positive for both receptors but our data strongly indicate more benefit from adjuvant tamoxifen for patients with tumors expressing both receptors. We are strongly motivated to continue the studies with more tumors and patients to be able to study the issue with more statistical power.

The third study investigated the development of relative breast cancer survival divided by age groups and stages. Using patients diagnosed 1989-1993 as a reference the relative risk of 5 year mortality decreased with 49% for patients diagnosed in the end of the observation period. In fact, breast cancer mortality has gradually decreased since the start of the Swedish cancer registry in 1960 and thus continued the years we investigated. The mortality tended to decrease for patients with all stages of breast cancer and test for trend resulted in a statistically significant improvement over time in 5 year relative survival in stage III and IV and in 10 year survival in stage I and III. However, in line with previous observations, patients aged below 40 years or more than 70 years when diagnosed tended to have less favorable survival than patients diagnosed between 40-69 years of age. Also, during the two decades of observation, we observed no statistically significant improvement in survival for patients older than 70 years at the time of diagnosis. We conclude that there is much room for improvement of care for patients below 40 and above 70.

4   FUTURE PERSPECTIVES

I would like to contribute to future studies with the following aims:

1. To investigate the correlation between tumor steroid hormone receptor levels and the benefit from endocrine therapy of breast cancer.
2. To identify gene expression signatures for endocrine therapy benefit

In Sweden alone one in ten women is diagnosed with breast cancer during her lifetime. Over the past decades a gradual increase in survival has been observed due to early detection, improved diagnostics and treatment. However, despite the improvements approximately one out of four women diagnosed with breast cancer will later develop distant metastatic disease and eventually die from breast cancer[75,87].

Breast cancer is a diverse disease with a natural history, occasionally spanning more than 20 years between primary tumor diagnosis and metastatic disease. We need improved procedures to identify patients at high risk for metastatic disease and improved predictors to tailor adjuvant and palliative therapy.

As discussed in this thesis the benefit from adjuvant endocrine therapy depends on the expression of the ER and PR receptors. However approximately 50% of the receptor positive tumors fail to respond to endocrine therapy.

To be able to contribute to future studies of endocrine therapy I need and want to continue fruitful collaboration with my current collaborators including my tutors Per Albertsson and Per Karlsson, the Stockholm breast cancer group including Tommy Fornander, Lambert Skoog, Linda Lindström and Huma Dar, via Linda also extended to Laura Essermans group in San Fransisco, the Linkoping breast cancer research group headed by Olle Stål including Helena Fohlin, Johan Rosell and Gizeh Perez Tenorio and the South Swedish breast cancer group including Mårten Fernö, Lisa Rydén, Pär-Ola Bendahl and Maria Ekholm.

4.1   RANDOMIZED TRIALS FOR CURRENT AND FUTURE ANALYSES

1. *The Swedish breast cancer group comparison of two and five years or adjuvant tamoxifen.*

When the study was published in 1996 it showed that five years of treatment resulted in prolonged recurrence free survival as compared to two years of treatment. The database for this trial is kept at Regional Cancer Center in Linkoping and has been updated by statistician Johan Rosell. We are now, in collaboration with docent Tommy Fornander, professor Mårten Fernö, professor Olle Stål and collaborators using this material to investigate the correlation of ER and PR receptors with long time recurrence free survival. The study, organized by the Swedish breast cancer group, recruited 4610 patients. 2346 were given two years of tamoxifen and 2264 five years of therapy. Out of this group were have selected 1691 from the South

Eastern region, 1320 from the Southern region and 811 from the Stockholm region for analyses.

2. *The Stockholm-3 trial used in our previous work recruited patients between 1976 until the end of 1989, randomized to receive tamoxifen versus not.*

Low-risk patients were defined as patients with lymph node-negative disease and a tumor size of less than or equal to 3 cm in diameter. All patients included in the trial have detailed patient and clinical information along with a complete follow-up of more than 25 years. As described in the thesis the trial information is updated to include the standard breast cancer immunohistochemical markers (727 patients) namely estrogen receptor (ER), progesterone receptor (PR), HER2 and Ki-67 along with information on intra-tumor heterogeneity of these markers. Gene expression data was generated using custom designed Agilent arrays containing approximately 32.1K probes, representing approximately 21.5K unique genes from FFPE breast cancer tumor tissue. Approximately 90% (or 652 breast cancer tumors) passed the RNA quality check (according to the diagnostic quality model). This information is available and currently used.

3. *The Stockholm-2 trial of high risk patients.*

All patients enrolled in this randomized trial, between 1976 until the end of 1989, received chemotherapy or locoregional radiotherapy and patients that were postmenopausal were randomized to tamoxifen versus not. High-risk patients were defined as patients with lymph node-positive breast cancer and /or/a tumor size exceeding 3 cm. All patients included in the trial have detailed patient and clinical information along with a complete follow-up of more than 25 years. Tumors from the trial are currently collected and updated with standard breast cancer markers (ER, PR, HER2 and Ki-67) and intra-tumor heterogeneity of these markers along with gene expression data using custom designed Agilent arrays as described above.

5 ACKNOWLEDGMENTS

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