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ADJUVANT TAMOXIFEN IN BREAST CANCER: CLINICAL AND PRECLINICAL STUDIES ON THE PREDICTION VALUE OF ESTROGEN RECEPTOR

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Life is really simple, but we insist on making it complicated

Confucius

ABSTRACT

Breast cancer (BC) exhibits great heterogeneity at histophatological, clinical and molecular levels. However, the different clinical outcomes in patients with seemingly similar breast cancer have led scientists to search for subgroups or for factors and characteristics related to the tumor or the patient that could anticipate clinical course (prognosis) of disease and/or response to given therapy (prediction). Estrogen receptor (ER) is the first molecule identified that has had great influence on the management of breast cancer. This thesis focuses on the role of ER and its significance in breast cancer.

In one study, we compared the potential of ER-positive tamoxifen sensitive cells (MCF-7) versus ER- negative cells (MDA-231) to handle DNA repair, transmit signals from DNA damage, initiate apoptosis, control transmitted signals from the cell cycle and synthesize growth factors and receptors. Genes related to these processes were studied by cDNA microarray. We found that the ER-negative cells were characterized by a higher expression of growth factors and cell cycle regulation components, and improved DNA repair.

We explored the long-term pattern of disease recurrence among pre-and post-menopausal patients with primary BC according to ER status. The patients were randomly given tamoxifen versus no systemic therapy. The results showed a reduction of locoregional, distant metastases and breast cancer death in ER-positive patients who received tamoxifen. The pattern of metastases was not different in these two groups. The conclusion was that the differences in term of gene expression appeared mainly to be related to endocrine sensitivity and not metastatic potential. Some more events in the first 5 years in ER-negative patients suggested that ER negativity in some cases is correlated with an increased tumour growth rate.

ER had been measured by cytosol assays prior to around 1990 when these assays substituted of immunohistochemical (IHC) assay. However, ER predictive ability of response to tamoxifen has been assessed based on ER measurement by cytosol assays. We compared these two assays in a clinical trial and found a high concordance between the assays and concluded that IHC is as accurate as cytosol assays to predict long term response to adjuvant tamoxifen.

The introduction of microarray technique a decade ago already has changed our knowledge of BC but it has some pitfalls that question its potential. In two methodical studies we showed the importance of tissue handling, the effect of heterogeneity of BC and standardization on the result from cDNA microarray.

This thesis confirms the importance of ER in BC but also indicates a more complex phenotypic beyond that which can be explained purely by ER content or endocrine sensitivity. Microarray technique can provide useful information besides the traditional one but requires standardization of sample collection, storage, processing, normalization, interpretation of data and requires validation by large studies.

LIST OF PUBLICATIONS

1 Genes related to growth regulation, DNA repair and apoptosis in an estrogen receptor-negative (MDA-231) versus an estrogen positive (MCF-7) breast tumor cell line
Sven Skog, Qimin He, **Reza Khoshnoud**, Tommy Fornander; Lars-Erik Rutqvist
Tumor Biology, 25:41-47 2004

II Time-dependent RNA degradation affecting cDNA array quality in spontaneous canine tumours sampled using standard surgical procedures
Henrik Von Euler, **Reza Khoshnoud**, Qimin He, Aida Khoshnoud, Tommy Fornander, Lars-Erik Rutqvist, Sven Skog
Intern J Mol Med, 16:979-985,2005

III The impact of RNA standardization and heterogeneous gene expression on the results of cDNA array of human breast carcinoma
Reza Khoshnoud, Qimin He, Maria Sylvan, Aida Khoshnoud, Madlen Ivarsson, Tommy Fornander, Jonas Bergh, Jan Frisel, Lars-Erik Rutqvist, Sven Skog
Intern J Mol Med, 25: 735-741,2010

IV Long-term pattern of disease recurrence among patients with early-stage breast cancer according to estrogen receptor status and use of adjuvant tamoxifen
Reza Khoshnoud, Tommy Fornander, Hemming Johansson, Lars-Erik Rutqvist
Breast Cancer Res Treat ,107:71–78,2008

V Immunohistochemistry compared to cytosol assays for determination of Estrogen receptor and prediction of the long term effect of adjuvant tamoxifen
Reza Khoshnoud, Britta Löfdahl, Helena Fohlin, Tommy Fornander, Olle Stål, Lambert Skoog, Jonas Bergh, Bo Nordenskjöld
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LIST OF ABBREVIATIONS

AI	Aromatase inhibitor
BC	Breast cancer
BLBC	Breast-like breast carcinoma
cDNA	Complementary DNA
CKs	Cytokeratins
DCIS	Ductal cancer in situ
DFS	Disease free survival
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
EIA	Enzyme immunoassay
ER	Estrogen receptor
HER-2	Human epidermal growth factor receptor
IDC	Invasive ductal cancer
IHC	Immunohistochemical
LBA	Ligand-binding assay
LCIS	Lobular cancer in situ
LN	Lymph node
mRNA	Messenger RNA
NPI	Nottingham prognostic index
OS	Overall survival
PR	Progestrone
RFS	Recurrence free survival
RNA	Ribonucleic acid
RS	Recurrence score

1 GENERAL INTRODUCTION

Breast cancer (BC) is the most frequent malignancy in women and is a leading cause of death in women in Europe and North America. The incidence rate of BC has been increasing with more than one million cases of invasive breast cancer (IBC) being diagnosed worldwide every year. However, the mortality from BC has remained unchanged during last 3 decades[2] mainly due to an early detection and more effective therapies.

BC is a multifaceted disease and shows substantial heterogeneity at histophatological, clinical and molecular levels. With the knowledge of this heterogeneity, intensive efforts have been made to find some relationship between clinical characteristics and underpinning histopathological as well as molecular features. The classification of BC into subtypes and identification of prognostic and predictive factors are results from these efforts. Even if, a great improvement in the management of BC has been achieved, the vision in management of patients with BC is to offer them an individual-based treatment plan to avoid over and under treatments that can cause unnecessary morbidity and mortality. Estrogen receptor (ER) that was discovered in 1962 [3], become the first molecule with great influence on the treatment of IBC. Since the discovery of ER and later progesterone (PR), intensive research has been concluded on the role and significance of hormone receptors, particularly ER in tumorogensis, progression, metastasis, prevention and the treatment of BC.

About two third of post-menopausal and approximately half of pre-menopausal women with invasive breast cancer have an ER-positive and/or PR-positive breast cancer. Surgery remains as the primary treatment for the majority of non-metastatic BC, whilst, radiotherapy, endocrine therapy; chemotherapy and biological therapy have become an essential part of breast cancer management both as adjuvant and in metastatic diseases. Ovarian ablation is the first form of systemic therapy for the endocrine treatment of BC, originally described at the end of the nineteenth century for the treatment of inoperable disease in Premenopausal women [4], long time before the identification of estrogen and ER. In 1936, Antoine Lacassagne discovered estrogen as the agent in ovaries that caused BC[5]. 1962, ER was identified by Jensen and Jacobson [3] who subsequently correlated the presence of ER with the hormone responsiveness of tumor in BC [6]. Tamoxifen was discovered in the mid 1960s and was initially used in advanced BC[7]. Tamoxifen was approved for endocrine therapy of advanced BC in the United Kingdom in 1973and in the United States in 1977[8]. Since then a large body of evidence has shown the effect of tamoxifen in endocrine sensitive breast tumours. Development of aromatase inhibitors has further improved effectiveness of endocrine therapy. However, in patients with estrogen receptor (ER)-positive disease, only 50-60% of the patients receive clinical benefit from these agents. Furthermore, the vast majority of patients develop resistance to all form of endocrine therapy within few years.

There are a number of important questions about endocrine therapy, which concern researchers today. These include:

1. Why do not all hormone positive breast cancer patients respond to endocrine therapy?
2. Among those with an initial good response, why does this response decrease or become totally irresponsive?
3. What is the molecular mechanism for the development of this resistance?
4. What is the exact mechanism of ER in the development of BC?
5. What is the role of ER in the prevention of BC?

Several different mechanisms for resistance development have been suggested, such as a decreased level of, or lack of ER, a reduced intracellular level of tamoxifen and upregulation of growth factors. Changes in the balance of co-activators/co-repressors leading to the regulation of the antagonist/agonist action of anti-estrogens are now the focus for investigations.

ER is a transcription factor, which stimulates proliferation and differentiation of normal breast epithelial cells and cancer cells. The human ER mRNA is transcribed from a complex gene. The exact molecular mechanisms regulating ER expression in BC are unclear. The microarray implement makes it possible to study the mechanism of action and resistance of ER on the genes levels.

1.1 EPIDEMIOLOGY OF BREAST CANCER

1.1.1 Breast cancer incidence

Cancer of the breast in females is the most frequent malignancy among women worldwide. It accounted for 23% of all cancers cases diagnosed in 2002 and took overall second place when both sexes were considered together[2]. Hence, BC is responsible for more than one million cases of the estimated 10 million malignancies diagnosed each year in both sexes [9]. Furthermore, BC is the primary cause of cancer death among women globally, responsible for about 375000 deaths in the year 2000 [9]. The incidence of BC varies extensively in different parts of the world, because of dissimilarity in life style and occurrences of many known risk factors [10] (Figure 1).The highest incidence occurs in developed areas such North America, Northern and Western Europe, Australia and New Zealand, whereas the incidence is low in Africa and Asia [11]. In developing countries the available cancer data indicates that the incidence of BC increases most likely as a consequence of following Western lifestyles [2, 12]. In China, the incidence of BC started to increase in socially and economically well-developed regions , becoming the number one type of malignancy in women[13]. The geographical and temporal variation in BC incidence rate can be explained by changes in the risk factors. The high rate of BC in western countries is due to a higher prevalence of the well-known risk factors for BC, such as early age at menarche, null or low parity, late age at any birth and late menopause, i.e estrogen related factors [14]. On the other hand, the higher parity and early age at first pregnancy in many developing countries might account for much of the lower incidence. The other explanation is long-standing breast feeding in these countries that shows a protective role[15]. Furthermore, exposure to exogenous hormones such as oral contraceptives[16] and hormone replacement therapy[15] results in an increase in the risk of BC.

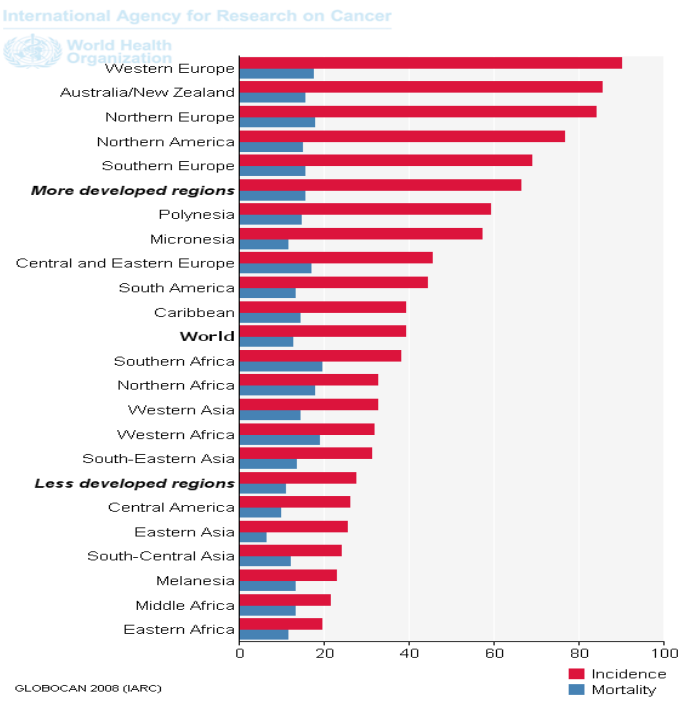


Figure 1.Incidence and mortality in different regions[1].Estimated ASR (world) per 100,000

In Sweden, approximately 7,300 cases of BC in females were diagnosed in 2008. Breast cancer is the most common cancer in women and encompasses nearly 30% of all malignancy in women (The National Board of Health and Welfare, 2008). The incidence rate of BC in Sweden was 84/100,000 women in 1974, increasing to

157/100,000 women in 2008 (figure 2). In other world, the incidence has increased by 1.2% per annum during the last 20 years, although the rate of increase has been slower during the latter 10-years with an average annual change of 0.8%t (The National Board of Health and Welfare, 2008).

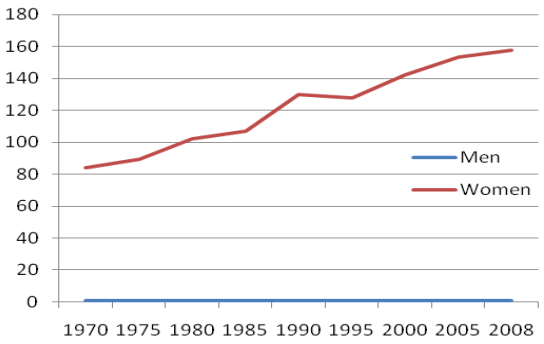


Figure 2.Breast cancer incidence, number cases per 100.000.

1.1.2 Breast cancer mortality

Breast cancer mortality in most European countries increased from the 1950s until the 1980s when it became stable and declined since [17, 18] (Figure 3). The same trend has been observed in North America[19], however, no decrease has been shown for Black American women[20]. Mammographic screening has resulted in early detection of BC, which together with more individual awareness and more aggressive and effective treatment in recent years, accounts for the reduction in mortality in these countries. The mortality from BC in developing countries is higher than the mortality in developed countries due in part to lack of widespread mammographic screening and lesser aggressive treatment. Byers at al, reported that low socioeconomic status was associated with more advanced disease stage and with less aggressive treatment for breast, prostate and colorectal cancer in USA [21].

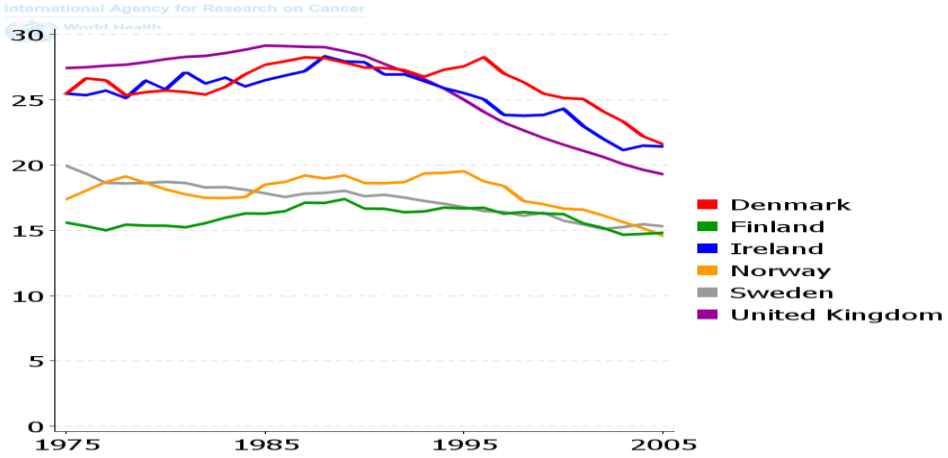


Figure 3.Mortality in North Europe[1]. Age-standardized rate (W) per 100,000.

In Sweden, about 1500 women have died from BC each year in previous decades. However, breast cancer mortality- in contrast to incidence- has been stable since 1960s and started decreasing since the 1980s. The estimated annual reduction in the last ten years has been approximately 1.5% per year.

1.2 HISTOLOGICAL AND MOLECULAR TYPES

Breast cancer is a complex disease, consisting of several subgroups that show different clinical activity and biological features [22-24]. The histopathological heterogeneity of BC has long been illustrated by histopathologists who have attempted to classify BC into meaningful distinct subgroups [25-27]. During last decade microarray-based studies have identified multiple molecular subtypes [28-31]that broaden the idea of heterogeneity of breast carcinoma.

1.2.1 Histological types

Histopathological examinations have revealed specific architectural and cytological patterns that are almost always associated with typical clinical manifestation as well as prognosis in breast cancer. These " histological special types" account for up to 25% of all BC[32]. The latest edition of the WHO classification of BCs discriminates the existence of at least 17 different histological special types[32]. However, the vast majority (50-80%) of BCs are called invasive ductal carcinomas not otherwise specified (IDC-NOS) or of no special type (IDC-NST). This means that majority of BC have not sufficient architectural and cytological characteristics to be classified into one of the special types and show variations in clinical features and outcomes. Even if, the special subtypes of BC have distinct morphological and clinical features and prognostic implications [32], the use of information on histological types have been limited in clinical practice management of patients with BC. This is because of the lack of standardized criteria and low interobserver reproductibility for diagnosis of special type[33]. Furthermore, special histological types of BC have been mainly neglected in studies of microarray-based molecular classification class discovery[28-31] and class prediction[34-38].The 2003 WHO classification gives an accurate definition of IDC-NST, pure and mixed types of breast cancer[32].

Conventionally, invasive human breast carcinomas have been classified morphologically into ductal and lobular carcinoma, tubular carcinoma, mucinous carcinoma, medullary carcinoma, invasive papillary carcinoma, metaplastic carcinoma and some uncommon types. For a long time, it was supposed that special histological types of BC arise from distinct microanatomical structures of the normal breast, hence the terminology of ductal and lobular carcinoma. The influential work by Wellings et al however, showed the vast majority of invasive breast cancer and their in situ precursors, initiate from the terminal duct lobular unit regardless of histological type [39, 40]. Thus, the terms ductal or lobular carcinoma do not imply site of origin or histogenesis, rather these entities are defined on the basis of their architectural patterns, cytological features and immunohistochemical profiles[33].

1.2.1.1 Invasive ductal carcinoma

Invasive ductal cancer is the most frequent BC and accounts for two thirds of all BC. Microscopically, it is characterized by variably thick strands of more than one cell layer, often with tubule formation (Usually with grade II/III nuclei). It sometimes forms solid tumor nodules with central sclerosis, necrosis and with DCIS. With palpation it is stony hard [41]. Tumors with stellate arrangement and focal necrosis have a particularly poor prognosis. This cancer typically metastasizes to bone, lung and liver.

1.2.1.2 Invasive lobular carcinoma

This type includes only 5-10% of BC and is characterized by ill-defined thickening or induration in the breast. Microscopically, it is composed of small cells in a linear arrangement (Indian File) with a tendency to grow around ducts and lobules. Compared to IDC, it has a greater proportion of multicentric tumors. This type is cell-poor and more often spreads to meninges, serosal surfaces, ovaries and retroperitoneum.

1.2.1.3 Tubular carcinoma

Tubular carcinoma is a variant of infiltrating ductal cancer that is usually detected by mammography. This type comprises about 5 % of all BC. Microscopically, more than 75% of tumors are composed of simple, well-formed tubules lined by single layers of cells. It shows low nuclear grade and has better prognosis than IDC. It is often is ER and PR- positive.

1.2.1.4 Medullary carcinoma

About 5-7% of all BC are of this type, which is more common in younger women (<50 years old). Patients may have enlargement of axillary lymph nodes, even in the absence of nodal metastases. This type is characterized microscopically by sheets of tumor cells, poorly differentiated nuclei, severe infiltration of small lymphocytes and plasma cells. There is usually no associated DCIS or just a little DCIS. Typical medullary BC has better prognosis than IDC but atypical medullary has the same prognosis.
Medullary cancer is typically ER-negative, PR-negative, HER-2 negative and usually p53 positive, indicating p53 mutation.

1.2.1.5 Papillary carcinoma

This type of BC is uncommon and accounts for about 1-2% of all BC. Papillary BC usually occurs in older women and is multifocal. It is normally ER-positive and shows good prognosis.

1.2.1.6 Mucinous carcinoma

Mucinous BC is characterized by rich accumulation of extracellular mucin around groups of tumor cells. This tumor type grows slowly and can become large and bulky. About 3% of all BC is of this type and when the tumor is mainly mucinous the prognosis tends to be good. However, mucinous BC with lymph nodes involvement has worse prognosis; about 76% 5 year Disease Free Survival (DFS)[42] . Cerebral infarction due to mucin embolism is an unusual complication in patients with mucinous breast cancer.

1.2.2 Molecular types

Normal mammary glands consist of two layers of cells: a well-differentiated inner (luminal) epithelial layer and an outer layer along the basement membrane. These cell types can be distinguished by the expression of certain cellular markers. Gene expression profiling by cDNA microarray and hierarchal clustering analysis has identified several molecular subgroups within BC. By analyzing gene expression of 115 breast tumor samples, Sorlie et al showed 5 subtypes: two ER-positive subtypes

(luminal A and luminal B) and three ER-negative subtypes (HER-2 enriched, basal-like, and breast-like)[29, 30, 43]. The molecular subtypes can prognosticate clinical outcomes like recurrence free survival and overall survival [30]. Furthermore, these subtypes are constantly present despite systemic therapy and appear to remain concordant during the metastatic process [31, 44, 45].

1.2.2.1 Luminal A

Luminal type A comprises the most cases of BC (56-61%) , is characterized by high levels of ER expression and is associated with relatively good prognosis[46]. The typical immunohistochemical profile of luminal type A is ER-positive and/or PR positive, and HER 2 negative. Based on the molecular profile, all cases with pure lobular carcinoma in situ (LCIS) are luminal type A tumors [47]. Consequently, the large majority of invasive lobular carcinoma have a profile characteristic for luminal A type[48]. Luminal A tumors show conflicting gene expression profile and have very variable prognostic signatures[43].

1.2.2.2 Luminal B

Luminal type B (9-16% of cases) tumours might present a more aggressive phenotype than luminal A and include tumors with high histological grade [29]. In contrast to luminal A, this tumor type is more frequent. They show HER2, EGF1, cyclin E1, ER and/or PR [30, 49].

1.2.2.3 Basal-like breast cancer (BLBC)

This subtype is characterized by the presence of myoepithelial cells that express CK 5/6, CK 14, CK17, vimentin, EGFR, and have a high proliferation index[50]. This group of BC typically lacks the CKs seen in the luminal groups and is often ER and PR negative. Morphology of basal-like BC is not typical and overlaps with many other subtypes. However, basal-like BC is mostly infiltrating ductal carcinoma with solid growth pattern and high nuclear and histological grade. Furthermore, other morphological types like atypical medullary carcinoma can be of basal-like type [51, 52]. They were named basal-like, before the era of gene microarray profiling, because of their expression of basal CKs, such as CK14 and CK17 [53, 54]. Basal-like BC is negative for ER, PR, and HER-2 [53, 55] . HER-1 is positive between 45 to 75 % of basal-like BC [53-57]. P53 gene mutations have been observed in about half of cases [53, 57, 58] and high Ki67 index in 67% of cases [58]of basal-like BC. There is no general consensus on the immunophenotypic criteria of basal-like BC. Therefore a standardized criterion that may facilitate further study on this group of tumor is necessary. The correlation between basal-like BC and clinical outcome has been studied; most studies provided evidence that basal-like BC is associated with a worse clinical outcome than other subtypes of BC [30, 44, 56]. Basal-like BC is more frequent in premenopausal Black women, where the incidence rate is 39% compared to 14 % in postmenopausal Black women and 16 % in non-Black women of all ages [59]. Additional studies have confirmed that basal-like tumors are more frequent in young women [47, 60]. These tumors tended to have aggressive features including high nuclear grade, high mitotic index, and unfavorable histology. Strangely enough, this subtype was not associated with higher regional lymph node involvement.

Disconnection between tumor size and positive lymph nodes have been shown in this subtype of BC by several studies [61, 62].

1.2.2.4 HER-2 enriched

This subtype (8-16%) is characterized by high expression of HER-2-related and proliferation genes and low expression of hormone receptor-related genes [30, 31, 44]. The HER-2 type BC based on the expression of ER, splits in two distinct subtypes: an ER-negative subtype which is closer to the basal-like subtype and an ER positive subtype that is closer to the luminal B tumors[48]. Immunohistochemical profile of HER-2 enriched tumors is ER negative, PR negative, HER-2 positive, EGFR focal positive, CK5 negative and CK 8/18 heterogeneous and moderate positive[48]. HER-2 type is often associated with ductal cancer in situ (DCIS). Many of these cases are less differentiated and have poor prognosis [63]

1.2.2.5 Breast like

About 6-10% of BCs are of this subtype. This type is a triple negative tumor and is close to basal-like tumors in terms of molecular profile. These tumors have a slightly better prognosis than basal-like tumors. The immunohistochemical profile of these tumors was shown negative for ER, PR, HER-2, CK5 and EGFR.

1.3 PROGNOSTIC AND PREDICTIVE FACTORS

1.3.1 Prognostic factors

A prognostic factor is any factor with ability to provide information on the clinical outcome in untreated patients at the time of diagnosis. Thus, prognostic factors help decision making to separate patients with breast cancer who need adjuvant treatment from those who do not need therapy. Several tumor specific and patient specific prognostic factors have been identified. Unfortunately, even using a combination of these factors can not anticipate the outcome of disease in an individual patient. It should be mention that these factors play an informative role in a group of patients. The microarray based gene expression profile has been used to identify a prognostic signature with potential to give information on the clinical outcome in an individual patient. The current trend is to combine the conventionally prognostic factors and genes array based signatures to make decision on treatment plan. In our institution we have used age, tumor size, lymph node status, ER status, tumour grade, ki 67 and HER2 overexpression/amplification to make therapy recommendations.

1.3.1.1 Age

Several studies have indicated that age at the time of diagnosis has prognostic value with a worse prognosis in young patients [64-67]. Although, this less favorable prognosis in younger women can at least to some extent, be a reflection of higher risk for lymph nodes metastasis, ER-negativity and larger tumor in young patients [68, 69]. On the other hand, some studies have shown a negative effect of age even after adjusting for the confounding factors [70-72]. Furthermore, some investigators have shown that BLBC which has poorer prognosis is more frequent in young women [47, 60]. Our local therapy guidelines for treatment of BC at Karolinska University Hospital

make a distinction between ages under and over 40 because of the high risk of unfavorable outcome in the younger ages.

1.3.1.2 Tumor size

The size of primary tumor together with the number of lymph nodes involved has been considered as a powerful prognostic factor and has had major impact on treatment decision making. Tumor size has positive correlation with odds of nodal involvement[73]. In a study of node negative BC, patients without adjuvant treatment and tumor size smaller than 20 mm had a 20 year DFS of 79% whereas patients with tumors larger than 20 mm had a 20 year DFS of 64%[74]. In 767 breast cancer patients with staging T_1/T_2, LN-negative at the time of surgery who received neither radiation nor adjuvant therapy and more than two decades follow up having the tumor of 10 mm or less in diameter, had a 88% RFS at 20 years[75].

1.3.1.3 Lymph nodes status

Axillary lymph node (ALN) metastasis is a strong prognostic factor in breast cancer with poorer prognosis as the number of ALN metastases increases[76]. Patients with no ALN metastasis have about 20% risk of recurrence at 5 years and their 10 year survival is 65-80%, while, patients with more than 4 ALN metastases have 54-82% relapse risk at 5 years and their 10 year survival is 13-24% [76, 77]. About 10-20% of cases with LN-negative BC on histopathological examination may be diagnosed LN-positive by use of monoclonal antibodies. The prognostic value of micro (0.2-2mm) and especially submicrometastasis (<0.2 mm) is being discussed. However, using sentinel node procedure and an intensive examination of these nodes have resulted in an increased detection of ALN metastasis even if the majority of these metastases are small including isolated cells and micrometastases [78]. De Boer at al in a meta-analysis showed that the present of metastases of 2 mm or less in size in ALN was associated with poorer DFS and OS[79]. The American Joint Committee on Cancer (AJCC) and WHO classifications according to size, LN involvement and distant metastasis (TNM staging system)(table 1)have become the most important prognostic tools in breast cancer[80]. However, some aspect of this classification has been questioned [81].

1.3.1.4 Histological grade

Histologic grade is determined as part of the diagnostic microscopic examination. The histologic grading according to Elston and Ellis which is a modification of histologic grading that was originally presented by Bloom and Richardson, is the most frequently used system [82, 83]. This grading involves semiquantitative evaluation of three morphological features, percentage of tumor area with tubule formation, nuclear pleomorphism, and number of mitotic counts per defined microscopic field area. The prognostic ability of histologic grading has been discussed. Some investigators reported a strong connection to prognosis[83] but others were unable to show that the grade is of prognostic significance[84]. Furthermore, its reproducibility between laboratories has been questioned[85].

Table 1.TNM staging system and corresponding survival according AJCC WHO.

Stage	TNM grouping			Overall survival*	
	T	N	M	5 year%	10 year%
0	Tis	N0	M0		
1	T1	N0	M0	87	78
II A	T0	N1	M0	68	52
	T1	N1	Mo		
	T2	N0	M0		
II B	T2	N1	M0		
	T3	N0	M0		
III A	T0	N2	M0	41	28
	T1	N2	M0		
	T2	N2	M0		
	T3	N1-2	M0		
III B	T4	N0-2	M0		
III C	Any	N3	M0		
IV	Any	Any	M1	10	0
T=tumor size, N=nodal status, M=distant metastasis. *American Joint Committee on Cancer (AJCC) Cancer Staging Manual, ID Fleming (ED) Lippincott-Raven,1997					

1.3.1.5 Estrogen and progesterone receptor

Two human ER have been determined, ERα and ERβ. ERα was named ER up to the discovery of ERβ, consequently all data on prognostic and predictive capacity of ER is relevant for ERα. In addition, in breast cancer ERα is in the majority [86], so ERα is more relevant to previous finding. There are conflicting reports regarding the prognostic significance of ERα status in early stage BC. Many early studies reported a more favorable prognosis for patients with ERα-positive tumors suggesting that ERα status was an independent prognosticator [87-90]. However, later studies indicated that the early advantages for ERα-positive BC were not sustained at longer follow-up [91-93]. The conflicting results are probably due to small studies, lack of standard cutoff point for hormone receptors, short follow-up and finally lack of control of relevant other prognostic factors including use of adjuvant tamoxifen. Our results (paper 4) indicated that ER has no or frail prognostic ability but strong predictive ability for respond to endocrine therapy. The overview of all available trials of adjuvant tamoxifen showed a significant improvement of both RFS and OS with tamoxifen in ERα-positive patients. In contrast, no clinically worthwhile treatment benefit was observed in patients whose tumors were classified as ER-negative[94]. The progesterone receptor consists of two isoforms, PR-A and PR-B. progesterone receptor is ER regulated and mediates the effect of progesterone in both normal mammary gland and BC[95]. It reported that a ratio of PR-A/PR-B has confirmed significant for normal development of the mammary glands in rodents [96] and an increased PR-A/PR-B ratio has been described in BC [97]and may be associated with resistance to tamoxifen[98].

1.3.1.6 HER2-neu (ERBB2)

HER 2 gene is a member of the epidermal growth factor receptor (EGFR) family that is located on chromosome 17q21. The protein encoded by this gene is a transmembrane tyrosine kinase growth factor receptor. All epithelial cells express 20,000-50,000 HER2 receptors on their cell surface. However, cells that over express HER2, express receptors numbering in the millions. There is no known ligand, but they form heterodimers with the other family members and; cause kinase-mediated activation of downstream signaling pathways. Over-expression is associated with poor prognosis and occurs most frequently by amplification.[99-101]. It is rarely expressed in lobular carcinoma but always overexpressed in inflammatory BC[101]. HER2 is both a prognostic and predictive factor. Amplification determined by FISH and recently by CISH or IHC determination of protein by IHC is assessed in clinical routine to choose patients suitable for trastuzumab treatment [102]. Furthermore, it has been suggested to have treatment predictive capacity for anthracycline, aromatase inhibitors and tamoxifen.

1.3.1.7 Proliferation rate

The proliferation rate of breast cancer cells has been recognized as a marker for both prognosis and tumor response [103-105]. The cell-proliferation rate can be assessed by synthesis phase fraction (SPF) using flow cytometry, mitotic index (MI) and Ki67 (MIB1) using immunostaining. There is no consensus on which assessment is more precise due to inconsistent results. Differences in methodology should account, at least in part, for these discrepancies. MIB1 has been analyzed in several breast cancer studies and found to provide significant prognostic information [106-108], whereas a better discriminative value was reported by others for MI [104, 109, 110] or SPF[111]. In our institution, the current assessment of proliferation rate uses Ki67 (MIB1) by IHC assay and has substituted SPF which was commonly used up to the late nineties. For instance, based on reports, the median value of MIB1 in breast carcinoma was reported to be less than 10%,[112, 113] between 10-20% [114, 115]and over 20%[107, 108, 116]. These differences in reporting the median value of MIB1 indicate the importance of standardization of methodology to be used for MIB1 assessment. Thymidine Kinase 1 (TK1), an enzyme closely related to DNA-synthesis and thus a marker for proliferation, has recently been compared to Ki67 in breast cancer studies and found to give higher positive rate than Ki67[117]. Cytosolic Thymidine kinase is a specific histopathologic tumor marker for breast carcinomas [117]. TK1 expression in atypical ductal hyperplasia significantly differs from ductal hyperplasia and DCIS; considering to be a useful tool in tumor therapy management[118].

1.3.1.8 Other prognostic factors

Several other factors, such as P53, angiogenesis, bone marrow micrometastases (BMM), cathepsin D and many more, have been suggested and discussed as prognosticator but their used in daily clinic practice very limited if any. It should mention that each individual factor has limited clinical value but considering these factors in combination are of greater value. However, current trends in oncology is analyzing tumor samples with a panel of genes using gene expression profile or the

genes surrogate protein using IHC technique to make diagnosis or determine prognosis and predictive ability to a candidate treatment.

1.3.2 Combined prognosis and predictive approach

1.3.2.1 St Gallen criteria

According the St Gallen criteria[119] and including age, tumor size, lymph node status, histological grade,HER2-neu and peritumoral vascular invasion in risk calculation, patients with operable breast cancer are divided in three risk groups (table 2). Notably, for the first time, the hormone status is not included in risk category.

Table 2.St Gallen risk criteria; Adapted from Goldhirsch et al. Ann Oncol 2005; 16: 1569-158.

Low risk	Intermediate risk	High risk
LN- negative + all of the following •pT <2cm •grade 1 •No peritumoral vascular invasion •HER2/neu negative •Age≥35 years	LN-negative+ at least one of •the following •pT>20mm •grade 2-3 •peritumoral vascular invasion •HER2/neu positive •Age<35 years	LN-positive 1-3 nodes plus •HER2/neu positive
	LN-positive 1-3 plus •HER2/neu negative	LN>4 nodes

1.3.2.2 Nottingham Prognostic Index.

The Nottingham Prognostic Index (NPI) combines three prognostic factors: nodal status, tumor size and histological grade. NPI is not applied in patients with metastatic disease. For NPI, three categories of LN status are used: stage 1 no lymph node metastasis, stage 2, up to 3 low axillary LN involvements or internal mammary node (assessed in medially located tumors) and stage 3, more than 3 low axillary LN metastases and/or the apical axillary node or of both low axillary and internal mammary nodes. All tumor deposits of 0.2 mm and above are regarded as LN metastasis. Tumor size is based on measurement of the invasive component in histological section. The third factor, histological grade is based on Nottingham modification of the Scarff-Bloom-Richardson method of assessing histological grade. The index is calculated by this formula: NPI=lymph node stage (1-3)+histological grade (1-3) + tumor size (cm) x o.2[120]. The NPI has been validated by further studies in Nottingham and by studies from several other countries [121, 122]. In NPI several important factors, such as HER2/neu, age and; peritumoral vascular invasion are not included.

1.3.2.3 Adjuvant Online (www.adjuvantonline.com)

Adjuvant! Online is an evidence based computer program which has designed to make information in the San Antonio Data Base more relevant to clinical practice. The program is based on information from the SEER data base, the overviews of clinical

trials, individual clinical trial results, and the literature in general. The basic format of an early version of Adjuvant! was described by Ravdin et al [123]. There are currently 3 different versions of adjuvant! For BC: adjuvant (standard), adjuvant after 5 years of tamoxifen, and adjuvant genomic version. The factors includes in adjuvant! are: age, performance status, ER status, tumor size, histological grade and nodal status. The results indicate survival chance and suggest treatment recommendation.

1.3.3 Multigene tumor assays

1.3.3.1 Oncotype DX

OncotypeDX is an RT-PCR-based assay from Genomic Health that can be performed on formalin-fixed tissue from paraffin blocks. It is based on analyses of gene expression profiles of 21 genes (16 cancer-related genes including genes related to ER, PR, HER2, proliferation and invasion and 5 control genes) and provides a "recurrence score (RS)" that correlates with outcome, as well as probability of response to endocrine therapy and chemotherapy [124-127]. Oncotype DX recurrence score provides a prognosis for patients with ER-positive BC treated with tamoxifen alone [125]. In one study, the recurrence score predicted benefit from CMF chemotherapy [126]. Although, patients with low RS have not benefited from chemotherapy added to tamoxifen, patients with high RS seemed to benefit from the addition of chemotherapy to tamoxifen. Albain et al reported that the RS is even prognostic for tamoxifen-treated node positive patients and predicts significant benefit of anthracycline based chemotherapy (CAF) in tumors with high RS. A low RS identifies women who might not benefit from chemotherapy, despite positive nodes [128]. Use of RS as a prognostic and predictive tool in ER-positive lymph node negative breast cancer was recommended by the American Society of Clinical Oncology [129]. The usefulness of Oncotype DX will be assessed in an ongoing large prospective trial, TAILORx trial[127].

1.3.3.2 Mammaprint

Mammaprint from "Agendia" uses expression array analysis of 70 genes to identify patients with good and poor prognostic signatures [35, 130, 131]. The prognostic value of this gene signature was confirmed in a study of 295 patients who were classified as having good and poor prognosis. The results showed the gene signature to be a more powerful predictor of disease outcome than conventionally used factors based on clinical and histological criteria [35]. The prognostic value of Mammaprint has been independently confirmed [132].This assay requires fresh frozen tumor tissue. This assay are already being used in patient management, but its ultimate worth will be determined by the results of a prospective clinical trial that currently started, the MINDACT (Microarray In Node-negative Disease may Avoid ChemoTherapy) trial [131].

1.3.3.3 Other multigenic test

Several multigene assays are either in development or on the market but are not approved. These tests and their characteristic shows in table 3.

Table 3.Prognostic and predictive test based on gene array or IHC and their using area.

Test	Gene or protein	Method	Sample conditions	Prognostic established	Guide to therapy
Mammostrat	5	IHC	FFPE	No	Tamoxifen
eXagenBC	3	FISH	FFPE	No	No
Invasivenesssignature	186	Microarray	Fresh7frozen	Yes	No
Molecular portraits	50	Microarray RT-PCR	Fresh/frozen	Yes	Neoadjuvant chmotherapy
Theros Two-gene Ratio	6	RT-PCR	FFPE	No	Tamoxifen
Celera Metastasis Test	14	RT-PCR	FFPE	No	Tamoxifen
Rotterdam Signature	76	Microarray	Fresh/frozen	Yes	Tamoxifen?
NuvoSelect	200	Microarray	Fresh/frozen	No	Neoadjuvant TFAC, tamoxifen
FFPE:formalin-fixed,paraffin-embedded.RT-PCR:reverse transcriptase PCR. TFAC:paclitaxel,fluorouracil,adriamycin,cyclophosphamid					

1.3.4 Predictive factors

Any factor that can predict the effect of certain treatment is a predictive factor. Thus, a predictive factor indicates if the treatment has benefit. Several predictors have been identified in breast cancer and suggest the basis for some of the current systemic therapies.

1.3.4.1 ER and PR

The most known predictive factor is ER status which predicts the effect of endocrine therapy. The predictive value of PR is not clear but its co-expression with ER; raises the probability of endocrine responsiveness in ER-positive BC.

1.3.4.2 HER-2

The overexpression / amplification of HER-2 indicates the usefulness of trastuzumab treatment. In daily clinical practice, the amplification of HER-2 provides the basis for selecting patients who will benefit from trastuzumab treatment.

1.3.4.3 Proliferation rate

Proliferation rate has been correlated to sensitivity to chemotherapy [103, 133] and high proliferation rate may well support chemotherapy. On the other hand, proliferation rate measured by the means Ki67 is related to outcomes. TK1 is another marker for proliferation and its level in serum also predicts relapse within 3 months after surgery [134].

1.4 ESTROGEN RECEPTOR

A majority of human BCs are primarily positive for ERα, and their growth can be stimulated by estrogen and inhibited by antiestrogen [135, 136]. The presence of ER in target tissue or cell is essential to their responsiveness to estrogen action. The cloning of ERβ [137-139] and its high sequence homology to ERα [137, 138] have complicated the mechanisms of breast carcinogenesis of estrogen. ER β can be inhibited by antiesrtrogens and stimulated with estrogen[140] and can form homodimers as well as heterodimers with ER α[140-142]. Thus, the existence of two ER subtypes and their ability to form DNA-binding heterodimerers suggests three potential pathways of estrogen signaling; via ERα and ER β homodimers and via the formation of heterodimers of ER α and ER β in tissue that express both receptor types [140].

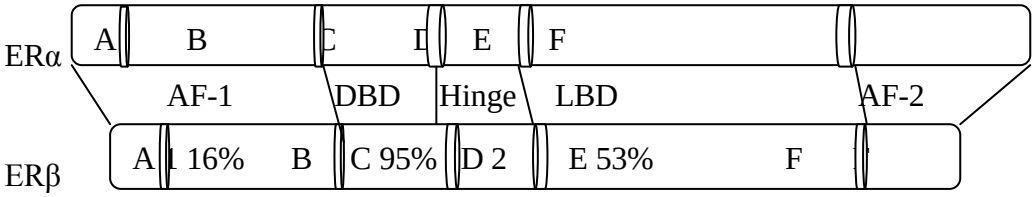
1.4.1 Estrogen receptors structure

Human ERα is a protein consisting of 595 amino acids. It is divided into six separate regions, named A to F and includes at least five functional domains. The domain located in amino-terminal shows a vast variation in both length and sequence and contains a hormone-independent transcription activation function (AF-1). AF-1 can stimulate transcription in the absence of hormone binding and is thought to be responsible for gene and cell specificity [143-146]. AF-1 is also important for the agonist activity of mixed antiestrogens [147]. It has therefore been suggested that the AF-1 domain may play a role in hormone resistant breast cancer [148, 149]. The DNA binding domain is extremely preserved among the nuclear receptors super family (including ER). Hormone binding to receptors induces conformational changes in the ligand-receptor complex that allow the receptor to fasten to the estrogen-responsive element within target genes [150-152] and activate the target genes. The next domain within ER, the hinge domain, allows ER to rotate. Furthermore it may be an important site for binding of accessory proteins [153]. A nuclear localization signal resides in this domain is responsible for the nuclear localization of ER. The ligand-binding domain is where the ligand binds to receptor into a “binding pocket”. Structural studies of the ER ligand-binding domain indicate that the “binding pocket” for the ligand is nearly twice the volume of its estrogen ligand. This difference might help explain the high affinity of synthetic ER ligand to the receptor [154] or the existence of undiscovered endogenous ER modulator[155]. Further crystallography studies with different ligands have showed that the conformational and structural changes induced by various ligands help contribute to their agonist and antagonist effects[156]. The ligand-binding domain has a helix called helix12 with a key function; when the ligand is agonist like estrogen, the helix seals the binding pocket and recruits coactivators to the transcriptional complex on the surface of helix 12. On the other hand, in binding of an antagonist, like raloxifene, helix 12 cannot seal the binding pocket due to the bulky side-chain which causes helix 12 to rotate away from an “agonist” position [155, 156]. The phosphorylation of the ER at tyrosine 537 within the LBD region is implicated in DNA binding, dimerization and in the conformational changes of the ER [157, 158] and its ability to stimulate transcription [159]. The last domain in ER protein, the transactivation function (AF-2) needs an agonist ligand for its activity. There is a third activation domain, termed AF-2a [160, 161] which has either constitutive activity or a stimulatory effect on AF-1. Finally, there is a negatively acting domain which is also involved in binding of the heat-shock protein 90[162].

ERβ is very similar to ERα in its overall structure but to some extent shorter than ERα [142]. ERβ is reported to have 95% homology in the DBD and 53% homology in the LBD. The high degree of homology between the DBD of two ER receptors indicate that they can heterodimerize and bind to EREs. The formation of mixed ER dimmers has been shown both in vitro and vivo [142]. The AF-1 Activity of ERβ is absent or very small. This explains, the differences in transcriptional activation of specific estrogen responsive genes between the two subtypes[163] and the fact that tamoxifen (mixed antiestrogens) shows partial agonist/antagonist activity with ERα but exclusive antagonist activity with ERβ [164]. The homology of ERα and ERβ within LBD, along with their different tissue distribution suggests that the two receptors may exert selective and different responses with different physiological roles. Thus, the balance of ERα and ERβ co-expression in breast cancer might have an effect on progression [165]and their ligand selectivity may become important in the management of BC. A better understanding of the role of ERβ and its significance in BC is fundamental.

Figure 4; A schematic comparison of ERα and ERβ, with the degree of homology shown as a percentage is outlined in figure 4. Both ER proteins consists of six regions (A, B, C, D, E and F) and five domains (AF-1/AF-2 (transactivation domain), DBD; DNA binding domain, LBD; ligand binding domain. and hinge)

Figure 4.Schematic comparison of ERα and ERβ.

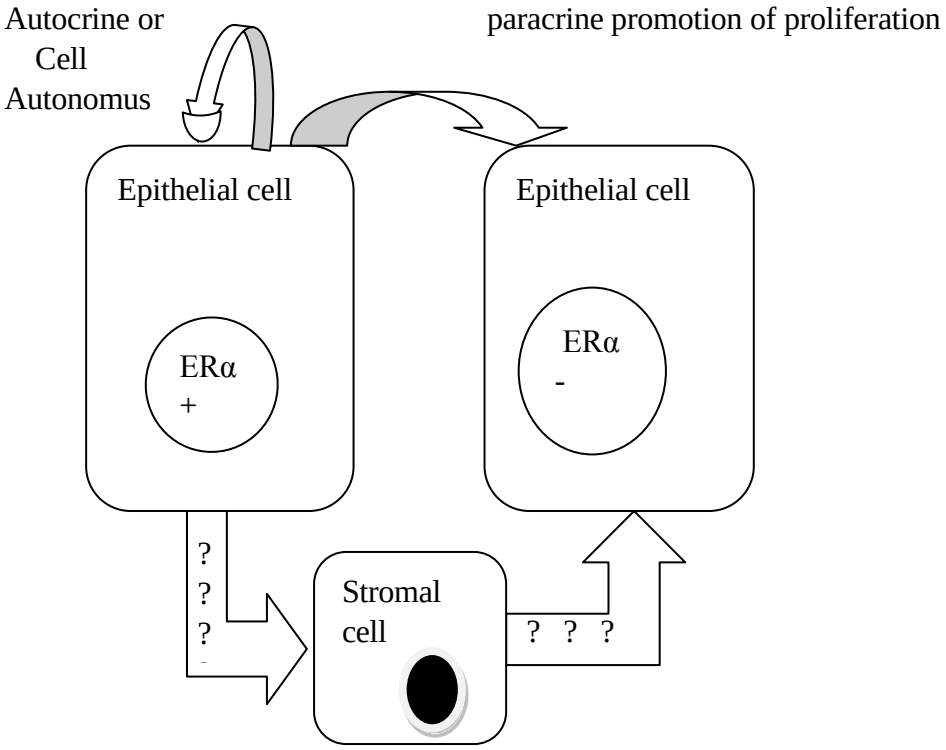


1.4.2 Estrogen receptors expression in normal breast and BC

Epithelial growth and development of normal breast is complex and understanding the factors involving and steering - these event is important as the same factors play a role in the development and progression of malignant breast cancer[166]. The breast gland mainly develops during puberty and afterward throughout pregnancy and lactation. The ovarian function is essential for the development of the breast, it is known that the breast does not develop in the absence of functional ovaries and the premature loss of ovarian function reduces breast cancer risk. Thus, estrogen and progestrone (ovarian hormones) are necessary factors for both normal and abnormal processes in breast glands [167]. The cells that express ERα and PR are found within the luminal epithelial but not the myoepithelial or stromal cells of the human breast[168]. About 10-15% of the premenopausal breast epithelium expresses ERα [168, 169]. In contrast, ERβ is expressed in approximately 85% of both luminal and myoepithelial cells[170]. Furthermore, ERβ is expressed in stromal cells in both fibroblast and endothelial cells. The fact that luminal cells account for more than 90% of the epithelial proliferation that happens in response to cyclical altering of ovarian hormones secretion during the menstrual cycle, shows that they are the major target cells for these hormones. Several investigators have reported that cells in normal breast that proliferate in response to steroid hormones neither express ER nor PR but are usually located next to ERα and PR-positive cells [169, 171]. Dissimilar, ERβ is expressed in many proliferative epithelial cells [172, 173]. These findings have led to the suggestion that ERα-positive cells produce growth factors in response to estrogen and stimulate adjacent cells by a paracrine stimulation leading to their proliferation. The paracrine model was confirmed in steroid receptor knockout mice by Brisken at al [174, 175]. In hormone dependent breast cancer the expression of ERα and PR is increased while the expression of ERβ is decreased. This data fits in with experimental studies indicating that ERβ interacts with ERα and may inhibit estrogenic actions by the means of this interaction [176]. In addition, ERα-positive cells in breast cancer are known to be proliferative, suggesting either the response to estrogen is cell autonomous or that the response to growth factors is in an autocrine way (see schematic figure 1)[177, 178]. Mutation of the ERα gene may elevate its sensitivity to estrogen[179].

Figure 5; demonstrates the; growth in normal and carcinoma cells. In normal cells, estrogen stimulates ERα-positive cells to produce growth factors. These growth factors stimulate proliferation of ERα-negative cells by paracrine way. In cancer epithelium a shift from paracrine to autocrine or cell autonomous growth happens. It is possible that stromal cells so produce local growth factors.

Figure 5.Growth in normal and carcinoma cells.



1.4.3 Methods for the measurement of ER in breast cancer

The assessment of ER status has been a useful prognostic and predictive factor in BC. Following the identification of ER through the 1960s, Jensen suggested that the measurement of ER levels in breast cancers could help predict the response to endocrine treatment ([6]. Since then a range of assay methods have been used to determine ER content in breast cancer samples. Improvement and development of the new assay methods for assessing hormone receptors have led to simpler, less extensive and less time consuming measurement of ER in daily clinical use.

1.4.3.1 Biochemical methods

The biochemical ligand-binding assay (LBA) was the first method that became standard for ER detection and measurement. Dextran-coated charcoal radioactive LBA was most commonly used. This assay was carried out on cytosol from fresh tumor tissue. The tumor tissue had to be frozen immediately after surgery and removed from the patients and stored under special conditions. The main advantage of this method is that it gives an objective and reproducible quantitation of ER under conditions of good

quality control [180]. However, the assay has some disadvantages; it needs a relatively large amount of fresh tissue, it measures ER content of the whole tissue consisting epithelia cells, stroma and saturation of the receptor sites by endogenous or exogenous ligand may lead to low or false-negative results. The assay involves the use of radioactive material and thus requires centralization for accurate performance.

1.4.3.2 Immunohistochemistry

The advance of monoclonal antibodies to the receptor makes the development of new assay methods possible, in order to overcome the difficulties associated with the LBA assays. Enzyme immunoassay (EIA) was developed for tumor cytosol which was somewhat more sensitive than biochemical methods. Finally, immunohistochemical assays (IHC) developed that measure ER only in cancer cells. The IHC assay has many advantages [181]; requires a small amount of tissue and can be performed on the material from fine needle biopsy and core biopsy, making it possible to examine receptor status during therapy in metastatic breast cancer. This assay does not require fresh tissue and works on routine fixed histological sections as well as archival material. The IHC assay can detect ER regardless of its functionality or occupancy. Another advantage is that the IHC assay only measures the ER content in cancer cells. Simplicity, low cost, and no need for specialized equipment, has meant IHC has been the method of choice for determination of ER in clinical daily work since 1990. It should be mentioned that IHC has several drawbacks. Result can vary substantially due to tissue fixation, procedural conditions, and type of antibody [182] or antigen retrieval method [183] used. The semiquantitative and subjective nature of IHC assessment with limited standardization, quality control, and commonly accepted cutoff point and scoring complicates the easy use of IHC analysis in determination of ER in the clinic.

1.4.3.3 Cut-off point

To put an appropriate cutoff point which separates ER-negative from ER-positive tumors is a major concern with any ER assay. It is even more important when cutoff point is used to predict response endocrine therapy. Early studies correlating assay results with clinical response to endocrine therapies indicated that tumors with even a small amount of detectable ER protein had a significantly higher response rate than those with undetectable ER levels[184]. For the DCC LBA, these levels were about 3 fmol/mg protein, which were at the limit to the assay's sensitivity (ref). However, arbitrary cutoff points as high as 20 fmol/mg cytosol protein have been used by some laboratories, perhaps because tumors with higher ER levels were known to be most likely to benefit from hormonal therapy[185]. It is most possible that some patients were misclassified as ER-negative and consequently went without endocrine therapy from which they had a good chance of benefiting. Moreover, such misclassification could have led to the faulty impression that hormone therapy has some effect in patients with ER-negative tumors.

It is even more difficult to adapt an optimal cut-off point for IHC assays. Several studies have assessed the ability of ER by IHC to predict a response to hormonal therapy. However, many of these studies were small, and were performed with antibodies most suitable for fresh-frozen tumor samples [186], a procedure that is not very relevant at present, when practically all IHC determination of ER is performed on formalin-fixed and paraffin-embedded samples. In addition, the definition of ER-

positive and negative varied because of lack of validation and standardization regarding both technical and scoring aspects of this assays. However, recent reports using a validated protype protocol and scoring system, in large studies, are suggesting a stringently low cut-off point. A score value>2, specimens with >1% of cells staining, was considered positive, and was the optimal cut-off point for predicting improved outcome [181, 186]. Moreover, the 11th St Gallen conference defined endocrine responsiveness as the presence of any detectable ER [187].

1.4.4 Microarray

DNA contains all genetic information and gene expression is demonstrated by the transcription of the information limited within the DNA into messenger RNA (mRNA). Every somatic cell has a complete set of chromosomes and identical genes settings but depending on the type and function of the cell, most of these genes are inactivate with only a small portion of genes expressing to give the each cell its characteristic features. Moreover, a cell type responds to different stimuli by means of activating and deactivating a gene or a group of genes, resulting in expression of particular genes as necessary. Recently, investigators have used the microarray technology for analyzing gene expression profile in diseases by studying the steady level of mRNA.

Despite the relatively short life of microarray techniques, increasing numbers of microarray have been performed and the results of these studies have already impacted our knowledge about various diseases, including cancers. Gene expression analysis by the means of microarray in breast cancer has disclosed signatures leading to molecular classification of BC, and has provided gene expression profiles with potential to predict prognosis or/and predict response to a given therapy. However, there are some difficulties associated with the microarray method that can challenge its potential. These pitfalls account for conflicting results and lack of reproducibility-shown in microarray studies. Variations in microarray analysis are caused by: 1) Array manufacturing processes, 2) Preparation of samples, 3) Hybridization of the sample to the assay and 4) Quantification of the spot intensities.

1.4.4.1 Array production

There are several platforms for performing microarray but the most used platforms is cDNA using probes constructed with PCR products of up to a few thousands base pairs and oligonuclotide arrays. In short, the microarray method consists of probe (molecules being immobilized), target (molecules in the sample; mRNA) and a detection device. There are three ways to fabricate a DNA microarray: (1) contact spotted, (2) non-contact printing and (3) in situ synthesis. Probe is a “spot” of known DNA, or oligonuclotide printed on a support of glass, silicon or nylon in defined arrangement. There are some factors during fabricating DNA microarray that affects DNA performance; such as spotter type (pin, inkjet), robotics, humidity, temperature at spotting, probe concentration, spotting buffer, immobilization chemistry, blocking technique, hybridization conditions, probe sequence and target preparation[188]. To avoid or reduce variability associated with DNA microarray, it is possible to use commercially available DNA microarray with associated kits or devices.

1.4.4.2 Samples

Using high quality RNA is recommended for performing microarray analysis. The biological samples can be provided by several means; surgical excision of tumors/ tissues, biopsies (core biopsy, fine needle aspiration), cell culture. Moreover, considerable quantities of human cancer tissues have been conserved as fresh frozen tissue or cells in biobank or are obtainable in formalin-fixed, paraffin-embedded archival materials. However, the tissue processing has not been optimal in most histopathological situations. Furthermore, it has been reported that mRNA degradation depends on type of tissue, type of cells and size of mRNA [189-191]. In addition, fixation of tissue in formaldehyde results in degradation of RNA[192] and paraffin-embedding results in fragmentation of RNA[193]. For these reasons, the amount of RNA extracted from formalin-fixed, paraffin-embedded material is low and modified with poor quality. On the other hand, for microarray proposes, fresh tissues/cells contain high quality mRNA. After surgical removal, samples should as soon as possible be immersed into liquid nitrogen and conserved at -80º to avoid mRNA degradation. An alternative, which has also been shown to prevent mRNA degradation, is putting samples in a RNase buffer before being snap frozen in liquid nitrogen [194, 195] . Another problem regarding tissue sample is the heterogeneity of samples with different amount of cells. Also, several factors, including the specimen type, preservation treatment of the tissue, extraction method, type and length of storage, and freeze and thaw affect the molecular quality of the tissue [196, 197]. To avoid variability related to sample handling it is important to have a standard protocol for sample processing that allows both traditional histopathological diagnostic assessment and molecular investigation. Interlaboratory variability can be avoided by performing microarray analysis in one central laboratory. The MINDACT trial (Microarray In Node-negative Disease may Avoid ChemoTherapy; EORTC) which is a prospective study evaluate the Mammaprint as a risk assessment tool, has adopted standard operating procedures for collection of samples with all microarray being performed in one laboratory in the Netherlands [198].

1.4.4.3 Hybridization

Many instants during hybridization can cause variation in gene expression analysis. The preparation of cDNA, changes in temperature, the agent qualities and labeling are some examples. Following standard protocol, using standard high quality agents and performing the microarray in a central laboratory can reduce variation.

1.4.4.4 Quantification of intensities

Depending on the choice of radioactive label or dye, single or dabble dye, the sort of detective device and the microarray platform some false variation in probe intensities can occur which result in false level of gene expression. After subtraction of background noises, variations in intensity from probe to probe or chips to chips for samples need to be normalized to obtain a trustworthy level of gene expression. The normalization process has been performed in different way. One commonly used method is normalization to a proper internal control. The internal control could be a gene or group of genes with no or minimal variation in their gene expression. Traditionally, the expression of “housekeeping” genes such as; actinβ, GAPDH

(glyceraldehydes-3-phosphate dehydrogenase) and 18s ribosomal RNA is used to normalize gene expression. However, several studies have indicated that the gene expression of "housekeeping" gene may considerably vary between different tissues types, disease states and experimental conditions[199] and have therefore excluded these genes as internal control genes. Another approach is to normalize the expression of each gene to total RNA or the mean expression of all genes included in the experiment (global normalization). Some authors suggested specific internal control (gene/s) for different tissue and pathological condition. We normalized the gene expression level to mg of tissue corresponding approximately to the number of cells.

1.5 TREATMENT OF BREAST CANCER

The treatment of breast cancer in Sweden is based on evidence–based medicine and follows the international and national guidelines. In the Stockholm area, a local therapy synopsis corresponds the recent recommendations on treatment of BC and directs the decision making to suggest adequate treatment to patients. This protocol has been revising regularly to adjust for new recommendations. The other characteristic of breast cancer treatment in Sweden is a multidisciplinary approach in management of BC as well the other cancers. The team includes the oncologist, surgeon, pathologist and radiologist and other disciplines such as the psycho-social team, dieticians and physiotherapists who discuss each case before and after surgery. Multidisciplinary management of BC and guidelines contribute to an improvement in BC overall survival rates [200]. In Stockholm and Gotland County the majority (98%) of patients with breast cancer diagnosis have been discussed in a multidisciplinary team before surgery[200].

1.5.1 Surgery

Surgery is the first therapeutic action for majority of primary BC with removal of the macroscopically tumor. The surgical techniques include breast-conserving surgery, mastectomy and axillary lymph node sampling or dissection. For most patients with stage 1 and 2 breast cancer and for some patient with T3 N0-1 when the ratio of tumor/ breast is allowed or after down-staging by neoadjuvant therapy, breast-conserving surgery in combination with radiation against remaining breast tissue is as safe as mastectomy[201, 202]. In Sweden about 93% of patients with primary BC underwent surgery in 2008, of which about 55 % were operated by means of breast- conserving surgery[200] and the remaining with mastectomy. The removal should be radical, i.e. on microscopic examination; also the excised tumor should be surrounded by a margin of normal tissue.

Information on the lymph node involvement is the most important data and has great impact on decision on postoperative adjuvant therapy. During last 20 years, the use of "sentinel lymph node biopsy" which indicates if the first lymph node/s into which a tumor initially drain, has reduced the more extensive technique of axillary surgery. Also, if the sentinel node is free from cancer cells, there is no need for axillary dissection. However, if the sentinel node is involved, an axillary dissection has to be performed. Axillary surgery (- dissection) is associated with the risk of morbidity in the arm, especially the risk of lymph edema[203] and now is less frequent. In Sweden about 71% of all patients that were operated for primary BC, underwent sentinel node biopsy and about 75% of them had negative lymph node results[200].

1.5.2 Radiotherapy

Radiotherapy is a sort of local treatment that has been given to patients with BC both as adjuvant and palliative. Breast-conserving surgery in patients with invasive breast cancer should be followed by radiotherapy to reduce loco-regional recurrences [204, 205]. Clarke et al reported [206]that radiotherapy after surgery reduced the risk of loco-regional recurrence by two-thirds compared with surgery alone and improved 15-year breast cancer survival by 5.4% in the radiotherapy group including both breast – conserving surgery and mastectomy. In the case of mastectomy, radiation to chest wall is indicated with poor tumor marginal, with multiple tumors in breast, and with large tumor size. With more than three lymph nodes involvement, loco-regional radiation should be given.

1.5.3 Chemotherapy

The purpose of chemotherapy like other systemic therapy is to destroy potential micrometastases when given postoperative (adjuvant) and reduces the risk of recurrences, leading to prolongation of disease free survival and overall survival. Chemotherapy as well has been used in metastatic breast cancer with good results. Recently about 5 to 10 % of patients with typical locally advanced BC have been given systemic therapy with mostly cytotoxic agents before operation (Neo-adjuvant). The main aim for the neo-adjuvant approach is to reduce tumor size, to evaluate the effect of given treatment and to avoid mastectomy. There is no apparent survival advantage to neo-adjuvant chemotherapy compared with adjuvant chemotherapy [207]. Benefit of adjuvant cytotoxic has been shown for several decades [208-210]. In an overview of randomized clinical trials, anthracycline-based polychemotherapy reduced the annual breast cancer mortality by 38% in patients younger than 50 at time of diagnosis and 20% for patients which were 50-69 years old[211]. Furthermore, chemotherapy with docetaxel and paclitaxel agents in comparison with anthracycline has further improved the outcome for patients with invasive BC [212-214].

1.5.4 Endocrine therapy

Endocrine therapy of breast cancer started over 100 years ago, also, long before any knowledge on estrogen, by ovarian ablation of patients with metastatic breast cancer[4] Endocrine therapy of BC with tamoxifen started four decades ago. Since then large bodies of evidence indicate the usefulness of tamoxifen both as adjuvant and in metastatic breast cancer. Early Breast Cancer trialist has with interval overviewed all randomized trial of tamoxifen, with analysis of about 8000 women with ER-negative BC showing no effect of tamoxifen in ER-negative patients. By contrast, the effect of tamoxifen in 18000 ER-positive and 12000 women with unknown status were obvious after 10 years of follow-up: one year of tamoxifen reduced recurrences with 21%, 2 years with 29% and 5 years with 47%, whereas proportional reduction in mortality was 12,17% and 26% respectively[215]. A recent review of randomized studies reported 31% reduction of the annual death rate in ER-positive tumors, as a consequence of tamoxifen treatment for 5 years[211]. In contrast, tamoxifen has no benefit in ER-negative breast cancers [216]. Aromatase inhibitors inhibit conversion of androgens to estrogens. Aromatase inhibitors in comparison to tamoxifen have only marginal effect

on survival [217, 218]. The same result seems to be the case in sequential treatment, tamoxifen for 2-3 years, followed by aromatase inhibitor [219, 220]. However, the new recommendation is first AI followed by tamoxifen because most recurrence happened during first 1-3 years. In premenopausal women ovarian ablation which can be achieved by surgery, radiotherapy and more commonly by gonadotropin-releasing hormone (GNRH) agonist, which is associated with reduction in breast cancer recurrence and mortality [211].

1.5.5 Biological therapy

Biological treatment means treatment with substances that are made in the body or that can block the growth of cancer cells. The first biological treatment for breast cancer that became available in the late 1990s was trastuzumab, which is an antibody against HER2/neu receptor. Initially, was approved in metastatic breast cancer. HER2/neu is amplified in about 15-20% of breast cancer tumors. The mechanism of action is through suppression of HER2 stimulated growth and potential also activation of the immune system to more effectively eliminates the cancer cells. There are several reports that one year of adjuvant trastuzumab in patients with amplified HER2/neu breast cancer tumor reduced risk of recurrence with 50% [221, 222]. A similar result was shown by FinHer trial despite only 9 weeks of adjuvant trastuzumab. However, a recent update of the FinHer trial still showed the benefit of 9 weeks trastuzumab but called for further research about the duration of adjuvant trastuzumab therapy [223].
Bevacizumab (Avastin) is another biological molecule; a humanized monoclonal antibody against vascular endothelial growth factor (VEGF), a pro-angiogenesis factor has shown to be effective in metastatic BC in terms of an improvement in progression free survival. It is indicated for the first line treatment of patients with MBC who have HER2-neu negative tumor in combination with paclitaxel. Several others biological agents, such as lapatinib (Tyverb), Sunitinib (Sutent) have already been used in metastatic patients and others are currently in different phases of clinical investigation. Ongoing studies will evaluate the effectiveness of these agents in adjuvant settings.

2 AIMS

♦ To investigate if the gene expression differences between ER-positive and ER-negative breast carcinoma in vitro is only correlated to phenotypic trait defined by ER status or endocrine sensitivity or if there is further diversity (paper I).

♦ To investigate in clinical material, if the potential of breast cancer for progression and metastasis is only related to endocrine sensitivity and ER status (paper IV).

♦ To investigate if the current use of IHC for determination of ER status is as proper as cytosol assays and has at least the same ability to predict response to endocrine treatment as biochemical methods (paper V).

♦ To investigate how breast cancer heterogeneity as well as sample collection, storage, processing, and normalization of RNA, influence the result of gene expression arrays and the gene expression array's potential to be applied in clinical management of breast cancer (paper II and III).

3 PATIENTS, MATERIALS & METHODS

3.1 PATIENTS

3.1.1 Patients (paper IV and V)

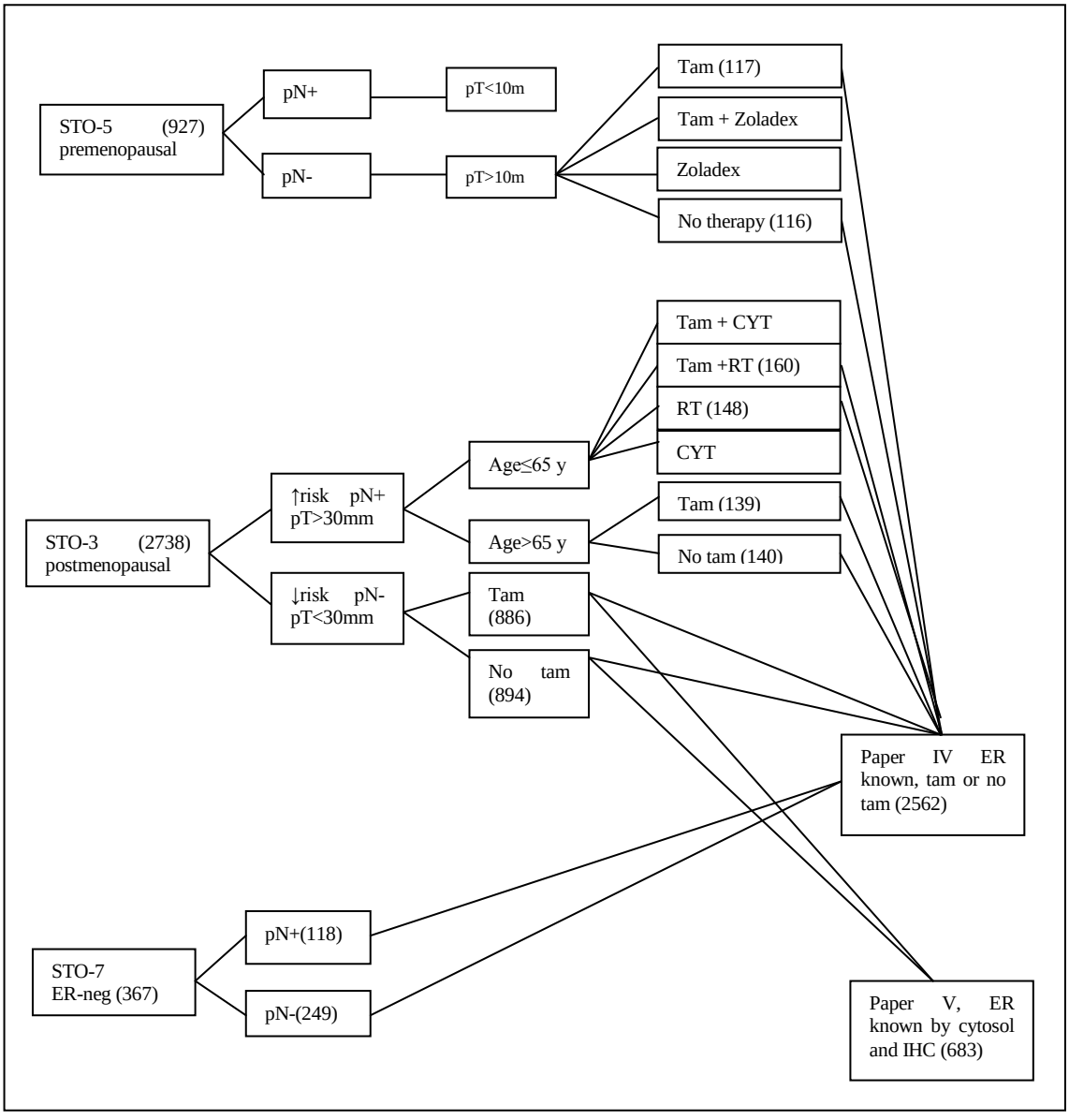
This thesis is partly based on the material of about 2600 patients who underwent surgery for primary breast cancer during the period November 1976 and to December 1996. These patients were included in one of three well-defined, controlled clinical studies of adjuvant endocrine therapy conducted by the Stockholm Breast Cancer Study Group, the Stockholm Adjuvant Tamoxifen Trial 3 (STO-3), the Stockholm part of the Zoladex in premenopausal patients (ZIPP-trial, STO-5), and a continuation of the STO-3 trial which only included patients whose tumors were classified as ER-negative (STO-7) (Figure 6).

STO 3:
From November 1976 through June 1990, a total of 2,738 patients, irrespective of hormone receptor content, entered the trial. Before randomization the patients were stratified according to stage of disease and primary therapy. There were 1,780 patients with a pathological tumor diameter of 30 mm or less and no lymph node metastases (low risk) and 958 patients with "high risk" tumor with either lymph node metastases or tumor size exceeding 30 mm on pathological examination or both. In the "low-risk" group 432 patients were treated with breast conserving surgery including axillary dissection plus radiation to the breast (50 GY/5 weeks), the remaining 1,348 patients had a modified radical mastectomy. The "low-risk "patients were randomized between adjuvant tamoxifen and no adjuvant systemic therapy.

Zipp-trial (STO-5):
This trial included premenopausal patients (< 6 months since last menstruation) with invasive breast cancer treated with a modified radical mastectomy or breast conserving surgery and an axillary dissection or biopsy. The patients with breast conserving surgery also received adjuvant radiotherapy to the breast (50Gy/5weeks). The patients were included in study irrespective of ER status. All patients with node positive disease electively received adjuvant cytotoxic chemotherapy and those with four or more LN metastases as well received radiotherapy.

STO-7:
This study was a continuation of the STO-3 trials and was designed to evaluate the effect of adjuvant endocrine treatment in operable, ER-negative postmenopausal women aged below 75years. The main inclusion criteria were: invasive breast cancer, postmenopausal (> 6 months since last menstruation), operable tumor, ER-negative (ER< 0.05 fmol/µg DNA) and no metastases. Patients who were operated with breast conserving surgery received 50Gy/5weeks radiation to the breast. The node-positive patients electively received post operative locoregional radiotherapy (46Gy/4.5 weeks). Randomization was between tamoxifen 40 mg daily for 2 years versus no adjuvant systemic therapy.

Figure 6.Flow chart for the trials IV and V.STO=Stockholm; pN= pathologic lymph-node; pT= pathologic tumor size; Tam= tamoxifen; CYT=cytotoxic; number of cases in brackets.



For the purposes of paper IV, we selected 2,562 patients from all aforementioned three trials with known ER status randomized to tamoxifen or control. These patients did not receive chemotherapy. The patient characteristics by trial are shown in table 4.

For the purpose of paper V, we chose the 1,780 "low risk" patients from the STO-3 trial because they received no adjuvant chemotherapy known to affect outcomes. These patients were randomized between no systemic therapies versus tamoxifen in contrast to the"high risk" group who also was given chemotherapy.

Table 4.Patient characteristics by trial.

Characteristic	STO3	STO5	STO7	Total
Period of recruitment	1976-1990	1990-1996	1990-1996	1976-1996
Age, median (range)	62 (45-77)	46 (30-55)	62 (44-75)	61 (30-77)
Allocated treatment:				
Tamoxifen	1,007 (50)	101 (50)	173 (50)	1,281 (50)
Control	1,005 (50)	101 (50)	175 (50)	1,281 (50)
Histopathological tumor size:				
<20 mm	1,176 (58)	113 (56)	170 (49)	1,459 (57)
≥20 mm	803 (40)	89 (44)	175 (50)	1,067 (42)
Missing data	33 (2)	-	3 (1)	36 (1)
Histopathological nodal status:				
N0	1,536 (76)	202 (100)	233 (67)	1,971 (77)
N1-3	313 (16)	-	82 (24)	395 (16)
N4+	155 (8)	-	33 (9)	188 (7)
Missing data	3 (0)	-	-	3 (0)
ER status:				
Negative	404 (20)	44 (22)	341 (98)	789 (31)
Positive	1,608 (80)	158 (78)	7 (2)	1,773 (69)
Median follow-up(y)	15.5	6.4	6.7	13.8
Total number of patients	2,012	202	348	2,562

3.1.2 Patients (paper III)

Human breast cancer tissues and normal breast tissues from women who were referred to Karolinska University Hospital, Stockholm were collected. Routine breast surgery procedures were used, and to avoid RNA degradation, the tissue samples were put into RNAse free tubes containing RNA later buffer (Qiagen GmbH, Germany) within 10-15 min after operation. The samples consisted of invasive ductal breast cancer, ductal cancer in situ as well as normal breast tissue. Laser capture microscope (Arcturus Ltd, UK) was used to collect normal (non-malignant) and malignant cells from the tissue section (one patient). The cells were picked by a skilled pathologist and were collected into RNAse-free tubes and immediately prepared for RNA extraction and amplification according the manufacturer's protocol (Arcturus Ltd).

3.1.3 Material (paper II)

Canine breast cancer and lymphoma tumors were taken from dogs referred for routine surgery at the University Small Animal Hospital, Uppsala. Samples consisted of four tubulopapillary carcinoma, two complex carcinoma, two benign mixed tumors, one solid carcinoma, and one fibroadenoma. The lymphoma was a high-grade, large-cell B-cell lymphoma. Routine surgery and procedures were used.

3.1.4 Cells (paper I)

In paper I, human breast cancer cell lines were used, ER-positive cells (MCF-7, ATCC no. HTB-22), ER-negative cells (MDA-MB-231, ATCC No. HTB-23), normal breast cells (H2F), and lymphoblastoid cells (CEM). MCF-7 and H2F were grown in minimum essential medium. H2F were supplemented with sodium pyruvate. MDA-MB-231was cultured in Dulbecco's modified Eagle medium. CEM was kept in RPMI.

3.2 FOLLOW-UP STRATEGIES IN PAPER IV AND V

Clinical visits took place every 3 months during the first 2 years, every 6 months during the next 2-5 years and yearly thereafter. These visits routinely included a physical examination and an annual mammogram. With a possible relapse blood sample, chest x-ray, bone scans, etc were performed. Disease relapse was confirmed when possible by biopsy. However, visceral and bone metastases were sometimes established on obvious radiological evidence. Recurrence was dated from the first evidence of relapse. Death preceded by breast cancer relapse was classified as breast cancer deaths and otherwise considered to be intercurrent death. The proportion of patients lost to follow-up was <1%. In paper IV results were based on follow-up until December 31, 1999 and in paper V until December 30, 2005.

3.3 DETERMINATION OF ER

3.3.1 Cytosol (paper IV and V)

The ER cytosol assays were performed in one laboratory in Stockholm, a participant in the Swedish national quality control program for hormone receptor laboratories. Tumor samples were collected and processed according to study protocols. ER was determined by isoelectric focusing before 1988 when an enzyme immunoassay was used. The

receptor values were normalized to DNA and tumor with a receptor content of≥ 0.05 fmol/µg DNA considered to be ER-positive.

3.3.2 Immunohistochemistry (paper V)

The IHC was performed using the Ventana automatic BenchMark machine (Ventana Medical Systems). A rabbit monoclonal ER antibody (Spl 250) was used and all procedures were performed following Ventana instructions.

3.4 CDNA MICROARRAY

3.4.1 RNA extraction

Total RNA was isolated from cultured cells (paper I) using RNA Quick Kit as described by Qiagen Kit (RNeasy Mini Handbook, 1997). $1x10^7$ cells in 600µl RNeasy lysis buffer were homogenized to which 600 µl of 70% ethanol was added. After mixing, 700µl of solution including any precipitate was placed in an RNeasy mini spin with a membrane in the bottom and centrifuged for 15s at 8,000g. The first washing was performed by adding RW1 buffer and centrifuging for 15s and the second washing by adding a RPE buffer. To dry the RNeasy membrane, RPE buffer was added and centrifuged for 2 min at 14,000g. Finally, the mini spin column with membrane was transferred into a new collection tube and 30µl of RNase-free water was added and centrifuged for 1 min to elute. The total RNA was measured at 260 and 280 nm in a UV spectrophotometer.
Total RNA from canine tissue (paper II) and human breast cancer (paper III) was isolated using an RNA-Bee isolation Kit (BioSite, Sweden). Samples of 50 mg tissue were homogenized in 1 ml RNA-Bee, and chloroform was added, after putting on ice for 5 min and centrifuging for 15 min at 12,000 g. The colorless aqueous phase was collected and 0.5 ml of isopropanol was added and after 10 min in room temperature, it was centrifuged for 5 min at 12,000 [224]g and the RNA pellet was washed twice. The total amount of RNA was measured at 260 and 280 nm in an UV spectrophotometer. The integrity of the total RNA was analyzed on a denatured 1% agarose/formaldehyde gel according to the manufacture's standard protocol (Qiagen GmbH).

3.4.2 cDNA array in paper II and III

Total RNA (5µg) from samples was used for each array. Biotin probe preparation and hybridization were conducted according to the manual provided by Super Array (Super Array Ltd.). The only modification to the protocol was changing the washing temperature and time from 60º C for 15 min to 68º C for 20 min for washing solution no. 1 and 2. Quantitative data was obtained using AGFA Curix 60 photographic film (AGFA, Sweden).
For samples from cell culture (paper 1), the total RNA (5µg) was used as a template for biotinylated probe synthesis using the protocol of Nonrad-GEArray as described by SuperArray (Nonrad-GEArray, 2001). The relative gene expression was detected by chemiluminescence signals using the alkaline phosphatase substrate. The amount of cDNA on the film was scanned and measured by ImagerMaster Total Lab v1.11 (Amersham Pharmacia Biotech, Sweden).

3.4.3 Gene setting

A panel of 18 genes, corresponding apoptosis, DNA repair, cell cycle, DNA damage signal pathway and growth factors and receptors, was used in study I.
Human Cancer Pathway Gene Array with 87 genes (GEArray Q Series, HS-006; SuperArray Ltd.) was used for study II.
Human Signal Transduction Pathway Finder Gene of 87 genes, corresponding to 18 signal transduction pathway (Super Array Inc; Maryland, Bethesda, MD, USA) was used for studies II and III.

3.5 STATISTICAL METHODS

Time to relapse was calculated from the date of randomization to first event and in the absent of an event, to the last day of follow-up. Survival time was calculated from the date of randomization to the date of death or follow-up. Crude cumulative incidence of disease recurrence was estimated using the Kaplan-Meier technique. Proportional hazards regression models were used to relate treatment and ER status to time to recurrence and time to death after controlling for age, tumor size and nodal status. Results are presented as crude cumulative incidence rates at 10 years and hazard rate ratios with 95% confidence intervals. Reported p-values refer to Wald tests. The median follow-up time was estimated with the reverse Kaplan-Meier method. The software used for the statistical analysis was Stata/SE 10.0.

4 RESULTS AND DISCUSSION

4.1 GENOTYPE OF ER-POSITIVE AND ER-NEGATIVE BREAST CANCER IN VITRO

The estrogen receptor content in invasive breast cancer separates these tumors into ER-positive and an ER-negative subtypes. The differences between these subtypes have been investigated in numerous studies both in vitro and in vivo. The endocrine treatment of BC has increased relapse free and overall survival of patients with ER-positive BC. Tamoxifen is the most used endocrine therapy. It has been known that about 30% of ER-positive tumors do not respond to tamoxifen (de novo resistance) and among those who initially respond to tamoxifen the majority develop resistance (acquired). The mechanism(s) of endocrine resistance in BC patients has been studied extensively over the last two decades.

In an in vitro study (cell culture, paper I), we ask how cells with ER-positive and ER-negative genotypes differ in controlling events linked to growth factors, such as cell cycle control and apoptosis. We also included DNA damage signal pathway and DNA repair, because these events are important for responding to antitumor treatment. Using cDNA microarray, we studied gene expression of 18 genes related to above named events and pathways. The results revealed differences in gene expression of 10 genes of 18 investigated between ER-positive CMF-7 cells and ER-negative MDA-231 cells. Genes related to cell cycle regulation, cyclin B1, cyclin E, cyclin D1, and cdc2p34 were overexpressed in both cancer cells compared to normal breast cells, however, the expressions were higher in the ER-negative cells (MDA-231) when compared to the ER-positive cells (MCF-7). The proliferation of cells is controlled at specific checkpoints in the cell cycle. Cyclin D1is involved in the control of events in the GI. Cyclin E is involved in late G1/S phase and cyclin B1has a key role in the G2 to M phase transition. Higher expression of cyclin D1 was found in tamoxifen-resistant MCF-7 these cells also; somehow bypass the inactivated ER, promoting cell growth. The ER-negative MDA-231 cells also grow without functional ER and the event/s behind the resistance may cause an elevation in the level of cyclin D1 and improved proliferation. The expressions of growth factors TGF-α and TGFβ2 and growth factor receptors EGFR, IGFR-1, IGFR-2 and TGFβR2 were elevated in both cancer cell lines with higher expressing of growth factors TGF-α and TGFβ2 in ER-negative cells .It is reported that ER activation stimulates synthesis of growth factors and receptors. In normal breast cells, these growth factors act in a paracrine way but in cancer cells they act in an autocrine way. Thus, despite a lack of ER, growth factors TGF-α and TGF-β2 are synthesized indicating that these genes can be regulated even by a pathway other than ER. Whether or not this is the mechanism of endocrine resistance in breast cancer patients lacking ER is still open to debate. Insulin-like growth factor binding protein-3 (IGFBP-3) was higher in ER-negative cells. However, the expression of some growth factors and growth factor receptors were elevated, which indicates that these factors are related to growth stimulation in BC cells and possibly to the bypasses of the ER pathway in the ER-negative tumors. Furthermore, the higher expression of the cell cycle components in the ER-negative cells may support the observation of the higher expression of growth factors. Genes related to DNA repair were tested by expression of

BRCA1 and BRCA2. BRCA1 showed the highest expression in MDA-231 cells and BRCA2 was expressed in MDA-231 but was undetectable in MCF-7 and normal breast cells (H2F). BRCA1 and BRCA2 are involved in the repair of DNA double strand breaks; in addition, BRCA1 seems to be involved in events regulating cell cycle checkpoints in a P53-independent way [225]. If this finding indicates that a higher DNA repair capacity is present in ER-negative primary breast tumors, these will have a growth advantage over ER-positive tumors when receiving antitumor therapy leading to DNA damages. The genes related to DNA damage signal pathways and apoptosis showed no differences in expression between ER-positive and ER-negative cell lines. Different gene expression between ER-positive and negative breast tumors was reported by other researchers [224]. Whether these profound gene differences between ER-positive and ER-negative tumors are only associated to endocrine responsiveness or indicate two distinct traits requires further investigation.

4.2 CLINICAL OUTCOMES AND ER STATUS

Studies on the pattern of gene expression in ER-positive and ER-negative breast cancer tumors have revealed profound differences according to ER status. However, it remains unclear if these differences reflect phenotypic traits in addition to sensitivity to endocrine therapy. We investigated the long-term pattern of disease recurrence among about 2,600 pre- and postmenopausal women with primary breast cancer according to ER-status. These patients were initially enrolled in three adjuvant trials and received tamoxifen or no systemic adjuvant treatment. A total of 2,048 patients were reported with locoregional, distant metastases and death. 1,041 patients died during the follow-up of whom 543 were classified as breast cancer deaths. The hazard rate for any event was significantly lower among those who had been allocated tamoxifen and the benefit of tamoxifen was more pronounced when the analysis was restricted to the 1,773 patients with ER positive disease. There was no significant heterogeneity between the hazard rate ratios for the different distant metastatic sites. There was no evidence that tamoxifen was more effective in reducing, for instance, bony metastases than visceral metastases. Among the patients allocated to tamoxifen, patients with ER-positive tumors exhibited significantly lower hazard rates than ER-negative patients. In contrast, among those not allocated tamoxifen, there was no significant difference, according to ER status, of neither locoregional nor distant metastases. The results indicating the effect of tamoxifen only in ER-positive breast cancer tumors have been reported by several others groups and also are supported by Oxford overview. This study has several advantages, such as its large size, the long-term and almost complete follow-up, known ER status for all patients with all ER-measurement performed in the same laboratory with well validated methods. Furthermore, the design of the original studies permitted an unbiased analysis of the effect of tamoxifen in relation to receptor status. The results showed that ER-status have little or no prognostic value, that ER-status has strong predictive value for responding to tamoxifen and that there is no significant differences between ER-positive and ER-negative tumors in terms of metastases pattern. There are conflicting reports about prognostic value of ER-status in early stage BC. Many early studies reported a more favorable prognosis for ER-positive patients [87-90, 226]. However, later studies indicated that the early advantages disappeared at longer follow-up [91-93]. The conflicting results are probably due to small studies, short follow-up, mixed study population, different cut-off point and lack of control of

other relevant prognostic factors, including use of tamoxifen. The gene expression differences between ER-positive and ER-negative tumors that have been shown in some studies [224] seem mainly to be related to endocrine sensitivity and not to metastatic potential. However, the minor benefit during the first five years for the ER-positive versus ER-negative tumors in terms of cumulative incidence of events suggests that ER negativity in some cases may also be correlated with an increased tumor growth rate.

4.3 PREDICTION ABLITY OF ER STATUS DETERMINED BY TWO ASSAYS

Estrogen receptor content is a potent predictor of response to endocrine therapy in both primary and metastatic breast cancer. Consequently, the adequate method for determination of ER content is important in daily clinical practice as a false result can lead either withholding patients an effective treatment or receiving a useless treatment with many potential side effects. Also, two factors influence ER determination: firstly, the sensitivity and specificity of the methods and secondly, the establishment of an appropriate cut-off point. ER measurement was performed by cytosol assays up to approximately 1990 when it was replaced by immunohistochemical assays. However, the predictive ability of ER status regarding response to endocrine treatment was based on ER determination by cytosol assays. Furthermore, a high concordance between the two assays has been reported in several studies [227-230]. Several studies have as well shown the similar predictive and prognostic information with both assays [231-233]. The majority of these studies had a short follow-up, and many patients received a range of therapies, and only few studies compared these methods in an adjuvant randomized setting. In one study (paper V) we compared the ER determination by cytosol assays and IHC using material from a randomized trial conducted by the Stockholm Breast Cancer Study Group. The postmenopausal women with primary invasive breast cancer who received tamoxifen versus no adjuvant systemic therapy, whose tumor samples were left in the biobank, were included in the study. A total of 683 patients had tumors with known ER status by both methods (Table 5). 39 had ER-positive tumors by cytosol assays but negative by IHC; whereas the opposite pattern was found in 42 cases. ER status by cytosol assays indicated that 147 of 683tumors were ER-negative and 42 of these tumors were ER-positive by IHC. The concordance between the two assays was high (88%). Patients with ER-negative tumors had no benefit from treatment with tamoxifen regardless by which assay the ER status was determined. Patients with ER-positive BC allocated to tamoxifen showed statistically significant better RFS but a reduction of OS by tamoxifen that was not statistically significant. We divided the patients in three groups by each assay: ER-negative, ER-positive and ER-strongly positive. The results showed that the benefit of tamoxifen was unrelated to the percentage of stained cells, but in the group with ER-positivity measured by cytosol the benefit from tamoxifen appeared to be related to the concentration of ER and an increased benefit with ER≥1.0fmol/µg DNA. We found that RFS and OS were reduced mainly during the first 5 years, among the tamoxifen treated patients. Our data confirms that ER-status measured by IHC is similar to that determined by the cytosol assays.

There is no consensus regarding the ER cut-off level. In the STO-3 study a cut-off level of 0.05 fmol/µg DNA was used because an analysis of treatment benefit versus ER levels did not show any benefit for ER below this cut-off level[234]. For the IHC we

used 10% stained cells as cutoff, however the 11th St Gallen conference, defined endocrine responsiveness as the presence of any detectable ER. Our study could neither support nor reject this statement as the number of tumors stained between zero and 25% were small. It seems the issue of cut-off level needs more investigation.

Table 5.Determination ER by cytosol and IHC.

	ER (IHC)		Total
ER (Cytosol)	ER < 10%	ER≥ 10%	
ER < 0.05	105	42	147
ER ≥ 0.05	39	497	536
Total	144	539	683

4.4 THE USE OF CDNA MICROARRAY: POTENTIAL AND LIMITATION

Gene expression profile by cDNA microarray and other techniques have been extensively used in cancer research, including for breast cancer. However, a number of factors and conditions during microarray can affect the final results of gene expression, with the following being some examples: the way tumor tissues are collected and preserved (which influence the mRNA integrity), the isolation technique, the choice of reference gene(s) or normalization, and tumor heterogeneity. In two method development studies, we check some of these parameters.

The first study (paper 2) investigates alteration in RNA integrity by the means of RNA gel electrophoresis at different post-operative time- intervals on canine mammary tumors and malign lymphoma. We also performed gene expression profile of 87 genes representing human cancer pathway and 87 genes representing human signal transduction pathway using abovementioned tissues. The preparation of tissues was performed according to routine procedures, routine surgery, and handling of samples in the theatre. Thereafter, equal pieces were cut and put in tubes prior to fixation in liquid nitrogen at different time intervals after operation. After extraction of RNA, the quality of RNA was examined by mean of gel electrophoresis. The gel electrophoresis result showed that in lymphoma that has a homogeneous context, all RNA was degraded after 30 min but in mammary cancer tissue degradation started between 15 and 30 minute and aggravated up to 4 hours. Furthermore, the result from cDNA showed that in mammary tissue some mRNA was not degraded, some was partially and some was completely degraded. By contrast, in lymphoma mRNA was not degraded at 15 min but all mRNA was completely degraded after 30 min. These results indicate the importance of tissue handling after surgery to fixation, heterogeneous expression of genes in the same tissue and heterogeneity of tumor tissue.

In the second study (paper3) we analyzed the cDNA microarray results in normal and cancerous human breast tissue concerning standardization of gene expression, gene expression in various part of the tumour, and gene expression in cancer cells compared with cancer tissue. Normalization of gene expression can be done in different ways, such as to total RNA (most common) or mg tissue to DNA or number of cells. It is not easy to count the number of cells but mg tissue corresponds approximately to the number of cells in the tissue. Different results were shown from normalization to total RNA and then to mg tissue malign and normal tissue from the same patient. The

concentration of total RNA was 12 times higher in malign tissue compared with normal tissue but the gene expression was 9 times higher in malign tissue when normalizing to total RNA and 40 times higher when normalizing to mg tissue. A higher gene expression in malign tissue compared with normal tissue is expected because of higher proliferation rate in tumor cells. Gene expression in different parts of the tumor differed between 2-4 times. Furthermore, the expression of housekeeping genes that commonly are used as reference genes deviated markedly. A comparison between gene expression of individual cancer cells which were collected using microdissection by Laser Capture Microscope and gene expression of whole cancer tissue revealed an extensive difference in gene expression of detected genes.

Gene expression analysis by cDNA microarray has the potential of addressing many issues concerning diseases; including breast cancer, and provides investigators a powerful tool. However, three set of drawbacks should be taken into account when interpreting microarray results: 1) the problems related to procedures such as time to fixation, fixation, preservation, isolation and extraction of RNA, probe making and hybridization 2) heterogeneity of gene expression in normal tissue, cancer tissue, between different tumor types and in the same tumor at different stages of development; and 3) the interpretation of data and the validation process. These differences explain at least partially the variations between findings for the same tumor type, which can often be more striking than similar. The results of our studies explain some of these contradictions and that some of these problems could be resolved by implementation of well accepted standard protocols. For these reasons the result from cDNA microarray should be interpreted with caution.

5 GENERAL CONCLUSIONS

5.1 PAPER I

- Certain differences can be demonstrated in gene expression between ER- positive, endocrine sensitive and ER-negative BC cells in vitro.
- The expressions of some growth factors, growth factors receptors and cell cycle regulators and genes related to DNA repair pathway were different in ER negative and ER positive cells.
- Further exploration is required before assigning these differences only to endocrine sensitivity, or ER positive and ER negative BC.

5.2 PAPER IV

- The gene expression differences between ER-positive and ER-negative BC seems mainly to be related to endocrine sensitivity and not metastatic potential.
- ER negativity may also be correlated with an increased tumor growth rate.
- The prognostic value of ER content is apparent only with endocrine treatment; therefore, it is mainly a predictive factor.

5.3 PAPER V

- Current clinical use of IHC for determination of ER status is at least as good as the validated cytosol methods.
- The concordance between cytosol assays and IHC assay for determination of ER is high.

5.4 PAPER II AND III

- Traditional tumor collection, storage and processing are inappropriate and affect mRNA quality and integrity.
- Gene expression shows heterogeneity both in normal and breast cancer tissue.
- Housekeeping genes express differently and are not proper as reference for normalization of cDNA microarray.
- It is possible that normalization to the number of cells (mg tissue) or amount DNA is more appropriate than normalization to total RNA.
- Results from microarray studies should be interpreted with caution and the potential of such studies as complementary to conventional factors needs further validation.

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