Endocrine resistance in breast cancer: gene expression profiling and modifications of the estrogen receptor
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Chapter 1
General introduction and outline
Breast cancer
In 2006, 12,416 women were diagnosed with invasive breast cancer and 3,335 women died of breast cancer in the Netherlands [1]. The development of breast cancer is a multistep process and these steps reflect genetic alterations that drive the transformation of normal breast cells into malignant cancer cells [2]. Cancer is associated with somatic genetic mutations acquired during life. Mutations are predominantly caused by inherited factors (genetic susceptibility), environmental and lifestyle factors. Elderly women have a higher chance to develop breast cancer, as they have more time to accumulate these genetic changes [3]. Specifically, endogenous endocrine factors such as early menarche, late menopause and age at birth of first child influence breast cancer risk [3].

In breast cancer patients, it is not the primary tumor in the breast, but its metastases to distant sites that are the main cause of death. Recently, as a result of widespread mammographic screening, accurate diagnoses and increased number of patients receiving effective adjuvant systemic treatment, a decline in breast cancer mortality rates has been observed [3]. Adjuvant systemic treatment is used to help destroy breast cancer cells that may have already spread to other organs and are not eradicated by surgery or radiation. Breast cancer patients who are at high risk to develop metastases receive cytotoxic chemotherapy, endocrine therapy, the recently introduced targeted drugs in combination with chemotherapy or a sequence of treatment modalities.

Estrogens and endocrine treatment
Hormones have been associated with breast cancer since Beatson showed in 1896 that oophorectomy resulted in tumor regression [4]. Estrogens play a predominant role in the growth of breast cancer. Estrogens bind to the estrogen receptor (ER), leading to dimerization, conformational change and binding to estrogen response elements (EREs) upstream of estrogen-responsive genes including those responsible for proliferation of the tumor cells (Figure 1.1). Approximately 75% of breast tumors express the ER [5]. Patients with an ER-positive breast tumor and who have a likelihood to develop a relapse of disease will receive adjuvant endocrine treatment. The use of endocrine manipulation covers the spectrum of metastatic disease, adjuvant and neo-adjuvant therapy. Adjuvant endocrine therapy is a major contributor to the substantial decline in breast cancer mortality.

Tamoxifen has been the mainstay of treatment for ER-positive breast cancer for more than 30 years [6, 7]. Tamoxifen is a selective estrogen receptor modulator (SERM) that competes with estrogens for ER binding (Figure 1.1). An alternative strategy includes the inhibition of aromatase using aromatase inhibitors that result in a block in the production
of estrogen (Figure 1.1)[8,9]. In addition, selective estrogen receptor down regulators (SERDs), such as fulvestrant, are used in the treatment of metastatic breast cancer patients [10].

![Image of endocrine signaling pathway]

**Figure 1.1.** Mechanism of action of aromatase inhibitors and tamoxifen. Estradiol binds to the estrogen receptor (ER), leading to dimerization, conformational change and binding to estrogen response elements (EREs) upstream of estrogen-responsive genes including those responsible for proliferation. Tamoxifen competes with estradiol for ER binding whereas aromatase inhibitors reduce the synthesis of oestrogens from their androgenic precursors. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer (Johnston SR and Dowsett M. Aromatase inhibitors for breast cancer: lessons from the laboratory. Vol 3: 821-31, 2003), copyright 2003.

**Endocrine resistance**

In patients with operable ER-positive tumors, tamoxifen reduces the risk of recurrence on average by 41% [11]. With that, tamoxifen has changed the clinical management of breast cancer dramatically. However, approximately 30% of the ER-positive breast cancer patients will develop a recurrence of their disease despite five years of adjuvant tamoxifen treatment [11]. Moreover, in the metastatic disease setting, half of the ER-positive breast cancer patients will not benefit from tamoxifen [12]. Endocrine resistance
is a major problem in the clinical management of breast cancer. Figure 1.2 illustrates the impact of endocrine resistance in the Netherlands.

**Figure 1.2. Breast cancer patients in the Netherlands.** Proportion treated with adjuvant endocrine therapy and proportion of endocrine resistant ER-positive tumors. Numbers reflect estimates of patients per year [5, 11, 46].
Several mechanisms may contribute to tamoxifen resistance [9, 13-16]. First, genetic variations in genes coding for enzymes (cytochrome p450, CYP) that convert tamoxifen to its active metabolites can influence the effectiveness of tamoxifen. Patients with variant CYP2D6 alleles had a higher risk of recurrence after adjuvant tamoxifen [17, 18]. Similar metabolic resistance to tamoxifen can occur via co-administration of drugs that inhibit CYP2D6, such as selective serotonin re-uptake inhibitors (SSRIs) [19].

Secondly, a proportion of ER-positive tumors are intrinsically resistant to tamoxifen for example due to high levels of growth factor receptors (GFRs) that may result in activation of signaling pathways in the tumor cells [20, 21]. Mitogen-activated protein kinases (MAPK), protein kinase A (PKA) and p21-activated kinase 1 (PAK1) are well-characterized components of pathways that may be involved in tamoxifen resistance [22-25]. A cross talk between the GFRs and ER has been described [26]. In addition, epigenetic and post-translational regulation of the ER may result in tamoxifen insensitivity via enhanced transcriptional activity [27].

Thirdly, tumor growth can be stimulated by tamoxifen resulting in acquired resistance. Patients will eventually relapse despite an initial response.

Since aromatase inhibitors are introduced recently, it is largely unknown whether the resistance mechanisms known to be involved in tamoxifen resistance contribute to resistance to an aromatase inhibitor as well [9].

Current clinical practice
For premenopausal patients, tamoxifen is considered the standard adjuvant endocrine treatment. In addition, suppression of the ovarian function by means of oophorectomy or a luteinizing hormone-releasing hormone (LHRH) analog is effective [28].

With regard to postmenopausal patients, recent randomized controlled trials showed that aromatase inhibitors are superior to tamoxifen in terms of disease-free survival (4.8% absolute difference at 9 years), but failed to demonstrate a significant difference in overall survival [29, 30]. Sequential tamoxifen for two or three years followed by an aromatase inhibitor for two to three years resulted in a reduction in the risk of breast cancer recurrence and death [31, 32]. Moreover, five years of tamoxifen followed by five years of an aromatase inhibitor resulted in improved overall survival in lymph-node positive disease [33]. The best sequence and timing for tamoxifen and aromatase inhibitors is still unclear [34,35].

Clinicians decide whether a patient is likely to respond to endocrine treatment based on the presence of the ER and/or the progesterone receptor (PR) expression [36]. Although
the predictive capacity of ER is indisputable, data on the predictive value of PR are conflicting and it could well be that PR is a prognostic as well as a predictive marker, just like the ER (definitions in Box 1.1) [11, 37-39]. Up till now meta-analyses have used cut-offs for ER and PR at 1% or 10% positive tumor cells. So, meta-analyses of endocrine treatment benefit have not provided the unambiguous cut-off for the percentages of ER and PR with regard to the predictive value of these markers, which is likely in a much higher range, between 50-100% positive tumor cells.

Human epidermal growth factor receptor 2 (HER2) is tested to identify patients for whom trastuzumab (anti-HER2 therapy) may be of benefit. Although earlier reports showed that HER2 overexpression was associated with tamoxifen resistance, guidelines currently recommend that HER2 should not be used to withhold endocrine therapy for a patient, nor should it be used to select a specific type of endocrine therapy over another [20, 21, 36, 40].

Despite years of research on endocrine resistance, there are no other molecular markers, besides ER and PR, used in daily clinical practice to predict the likelihood of response to tamoxifen [41]. At present, no markers can be used to predict differential benefit from tamoxifen as opposed to aromatase inhibitors.

Box 1.1. Definition prognosis and prediction

Prognostic marker
Any measurement available at time of diagnosis that is associated with disease-free or overall survival in the absence of adjuvant systemic therapy

Predictive marker
Any measurement associated with response or lack of a response to a particular systemic therapy

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Rationale of this thesis

The cured proportion of patients with early breast cancer treated primarily with surgery is estimated to be 18% to 38% (i.e. 30-years breast cancer specific survival) [42, 43] and since the introduction of screening, now probably lies between 40-55%. Although prognosis in breast cancer has been studied extensively, this cured proportion cannot be identified accurately. Therefore, progress in the field of prognostic markers (who to treat?) is crucial. Another pivotal question is ‘how to treat’: what is the optimal systemic treatment for an individual breast cancer patient? This thesis focuses on predictive markers (how to treat?) (see box 1.1. for definitions) [44, 45].

According to current clinical guidelines, approximately 80% of the ER-positive breast cancer patients have a risk of developing a relapse of their disease [46] and should receive adjuvant endocrine treatment to destroy tumor cells that are not eradicated by surgery or radiation alone. However, as described above, ER is not an infallible predictive marker of endocrine responsiveness. The recurrence rate (15 years of follow-up) for ER-positive breast cancer in patients who received adjuvant tamoxifen is approximately 30% [11]. Currently, no accurate biomarkers are available to i) predict the likelihood of response to tamoxifen and ii) predict whether a patient will have more benefit from an aromatase inhibitor or tamoxifen.

Clinical application of such a predictive biomarker could not only rescue the patient from unnecessary treatment and the accompanying toxicity, but will allow selection of the optimal treatment for an individual patient resulting in a decrease in breast cancer mortality. A biomarker that allows identification of tamoxifen resistant tumors may be crucial to enable personalized medicine: In the Netherlands it comprises at least 2,100 breast cancer patients per year who develop a relapse of disease despite tamoxifen (illustrated in Figure 1.2).

This thesis describes the discovery and validation of biomarkers that predict response to tamoxifen in ER-positive breast cancer patients. Both hypothesis-driven strategies using knowledge from the fields of cell-biology and biophysics as well as data-driven methods are presented. Besides the evaluation of ‘single markers’ using immunohistochemistry or genotyping, genome-wide analysis of breast tumors is performed by means of DNA-microarray technology.

The combined chapters of this thesis provide a means for improved upfront identification of those patients who will benefit from endocrine therapy.
Outline of this thesis
The central research question and the different approaches are depicted in Figure 1.3.

The first part of this thesis comprises studies that analyzed the transcriptome of ER-positive breast tumors using DNA-microarray technology. In chapter 2 gene expression studies either attempting to unravel the functional effect of ER or describing the gene expression profiles driven by ER in breast tumors are reviewed. In addition, the development of molecular signatures predicting response to endocrine treatment is discussed.

Recently, an 81-gene signature, a 21-gene assay (Recurrence Score) and a two-gene-index have been discovered. These genomic tests predict outcome after tamoxifen treatment. In chapter 3 a comparison and validation of these three genomic tests is presented in an independent dataset of patients who had received tamoxifen for metastatic breast cancer.

Although the field of cancer stem cell research has emerged, no information is available on the possible endocrine responsiveness of breast cancer cells with stem-cell properties. In order to get insight into the characteristics of putative breast cancer stem cells of ER-positive breast tumors, we cultured ER-positive breast cancer cell lines as mammospheres in vitro. Using mammospheres, cancer cells can be enriched for highly tumorigenic cells. In chapter 4, the expression of ER and PR in mammospheres is evaluated. Next, we analyzed gene expression profiles of mammospheres and correlated these profiles with those of primary breast tumors and with breast cancer outcome.

Prognostic gene-signatures that determine the likelihood of recurrence are urgently needed in the clinic, but it is unclear how information provided by these molecular tests has to be integrated with the conventional predictive markers that are already part of routine clinical practice. Chapter 5 shows a possible integration of the 70-gene prognosis profile and ER and PR as determined by immunohistochemistry.

In the second part of this thesis, studies regarding modifications of ER, the drug target of tamoxifen are presented. The ER can be modified via phosphorylation by kinases such as PKA and PAK1. Using Fluorescence Resonance Energy Transfer (FRET), a phosphorylation of the ER at serine 305 (ER305-P) has been linked to tamoxifen resistance in vitro. In chapter 6 we describe the detection of ER305-P in human breast carcinoma using immunohistochemistry and correlate ER305-P to tamoxifen resistance in patients. In order to capture PKA and PAK1 activity as well as ER305-P in a potential predictive test, we developed and validated an algorithm including PKA, PAK1 and ER305-P. These findings are presented in chapter 7. Additional functional experiments as well as gene expression analysis revealed further insight in the interaction between PKA-PAK1 and ER305-P. The ER can also be phosphorylated at serine 118 (ER118-P). In contrast to ER305-P, ER118-P
seems to be required for a proper ER function. In chapter 8, we analyzed ER118-P in tumors of patients who were randomized between tamoxifen and no adjuvant systemic treatment, and studied the relation between ER118-P status and response to tamoxifen.

Besides characteristics of the tumor cells, response to tamoxifen can be influenced by genetic variations in the germline DNA of the patient (host). Genetic variants in CYP2D6 and CYP2C19 are correlated with outcome in breast cancer patients who received tamoxifen for metastatic disease (chapter 9).

Finally, the results presented in this thesis are summarized in chapter 10 and put in perspective in chapter 11.

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**Figure 1.3.** Outline of the thesis, displaying the objective, the different approaches and respective chapters
References
