Studies of tamoxifen resistance in breast cancer

Pia Palmëbäck Wegman

Division of Cell Biology
Department of Biomedicin and Surgery
Faculty of Health Sciences, SE-581 85 Linköping, Sweden

Linköping 2007
# Table of contents

Abstract  5  
Abbreviations  6  
List of papers  9  
Introduction  11  
  Oestrogen  12  
  The oestrogen receptor  13  
  Molecular mechanisms of oestrogen action in the breast  14  
  Oestrogen and breast cancer  15  
  Adjuvant treatment  16  
  Tamoxifen  16  
  Tamoxifen resistance  22  
    Prediction of tamoxifen resistance/response  24  
Aims of the study  29  
Patients  31  
Material and methods  33  
  DNA-isolation  33  
  RNA-isolation and cDNA synthesis  33  
  Genotyping  33  
  Sequence analysis  34  
  RNA and protein expression  35  
  Statistical analyses  37
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Results and Discussion</td>
<td>39</td>
</tr>
<tr>
<td>Paper I</td>
<td>39</td>
</tr>
<tr>
<td>Paper II</td>
<td>43</td>
</tr>
<tr>
<td>Paper III</td>
<td>47</td>
</tr>
<tr>
<td>Paper IV</td>
<td>51</td>
</tr>
<tr>
<td>Conclusions</td>
<td>55</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>56</td>
</tr>
<tr>
<td>References</td>
<td>59</td>
</tr>
</tbody>
</table>
Abstract

Oestrogen is one of the most important hormonal regulators and is known to play a key role in the development and growth of breast cancer. The majority of tumours have a hormone dependent growth, and this is indicated by the presence of oestrogen receptors (ERs). About two thirds of breast cancers occur after the menopause when the ovaries have ceased to produce oestrogen and despite the low levels of circulating oestrogen’s the tumour concentrations of oestrone, oestradiol and their sulfates have been shown to be significant. Patients with hormone dependent tumours are candidates for treatment with the anti-oestrogen tamoxifen, which acts by competing with oestrogen for binding to the ER thereby, diminish the transcription of oestrogen regulated genes. The drug is mainly metabolised by cytochrome P450 enzymes in the liver and to a lesser extent locally in the breast, where upon several produced metabolites have higher affinity for the ER than the mother substance. Patients treated with tamoxifen have in general a prolonged disease-free survival. Even if most patients respond well to tamoxifen about 30-50 % either fail to respond or become resistant by incompletely understood mechanisms. Therefore, the aim of this thesis was to investigate possible mechanisms responsible for tamoxifen resistance. In paper I and II we studied genetic variants of enzymes participating in the metabolism of tamoxifen and assessed whether these variants correlated to breast cancer prognosis and/or to the benefit of tamoxifen. The results indicate an influence of CYP2D6, CYP3A5, and SULT1A1 genotypes in tamoxifen response. Further, tamoxifen has shown to compete with oestrogen for the binding to ER. In paper III we measured the expression levels of enzymes involved in the local synthesis of oestrogens in order to see if they correlated to clinical outcome. The protein expression of stromal aromatase was shown to have a prognostic significance, especially in ER-positive patients. Finally, tamoxifen and its ER-active metabolites have shown to induce both cell cycle arrest and apoptosis and one central mediator in these processes is the tumour suppressor protein p53. The proapoptotic activity of p53 is dependent on a proline rich domain containing a common Pro-to-Arg polymorphism. In paper IV we examined the value of this genetic variant as a predictive marker for anti-cancer therapy and found that patients carrying the Pro-allele might be good responders of tamoxifen therapy. The present thesis further indicates the complexity of the mechanisms underlying tamoxifen resistance. In summary, genetic variants of metabolic enzymes, genetic variants in p53, as well as expression levels of enzymes involved in local oestrogen synthesis, may have influence on breast cancer prognosis and may be useful markers in the prediction of tamoxifen response.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF</td>
<td>Activation function</td>
</tr>
<tr>
<td>AIB1</td>
<td>Amplified in breast cancer</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2 associated X protein</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>Cdk</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>c-Myc</td>
<td>Cellular myocytosis gene</td>
</tr>
<tr>
<td>Ct</td>
<td>Threshold cycle</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzididine</td>
</tr>
<tr>
<td>DHPLC</td>
<td>Denaturating high performance liquid chromatography</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>ddNTP</td>
<td>Dideoxynucleotide thriphosphate</td>
</tr>
<tr>
<td>E1</td>
<td>Oestrone</td>
</tr>
<tr>
<td>E2</td>
<td>Oestradiol</td>
</tr>
<tr>
<td>E3</td>
<td>Oestriol</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ER</td>
<td>Oestrogen receptor</td>
</tr>
<tr>
<td>ERE</td>
<td>Oestrogen response element</td>
</tr>
<tr>
<td>ERK</td>
<td>External regulated kinase</td>
</tr>
<tr>
<td>HER-2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HR</td>
<td>Hazard ratio</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>HSD</td>
<td>Hydroxy steroid dehydrogenase</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin growth factor</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>NCoR</td>
<td>Nuclear co-repressor</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PgR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3 kinase</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SERM</td>
<td>Selective oestrogen receptor modulator</td>
</tr>
<tr>
<td>Src</td>
<td>Rous sarcoma virus</td>
</tr>
<tr>
<td>SULT</td>
<td>Sulfotransferase</td>
</tr>
<tr>
<td>TGF</td>
<td>Tumour growth factor</td>
</tr>
<tr>
<td>TMA</td>
<td>Tissue microarray</td>
</tr>
<tr>
<td>UGT</td>
<td>UDP glucuronosyl transferase</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
List of papers

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


Introduction

Ever since the late 1970’s, tamoxifen has been the gold standard in the adjuvant setting for endocrine-responsive early breast cancer. Data from an overview analysis of clinical studies demonstrated that the overall effects of tamoxifen appeared to be small in patients with ER-negative tumours, and that 5 years of adjuvant tamoxifen was superior to shorter duration in both pre- and postmenopausal women with ER-positive tumours, irrespective of dose. With an appropriate period of 5 years treatment, this agent has significantly reduced the recurrence and death rates in ER-positive breast cancer patients [Early Breast Cancer Trialists’ Collaborative Group 1998; Bryant et al. 2001]. Even though most treated patient’s benefit from tamoxifen, many breast tumours either fail to respond or become resistant. Several mechanisms for tamoxifen resistance have been suggested but the picture seems to be complex. In order to fully understand this resistance further investigations are needed.

The scientific papers included in this thesis were undertaken to elucidate possible mechanisms responsible for tamoxifen resistance in postmenopausal breast cancer patients. We investigated three potential areas; i) the metabolism of tamoxifen, ii) local oestrogen synthesis, iii) p53 and tamoxifen response.

Initially, tamoxifen is extensively metabolised in the liver and to a lesser extent locally in the breast. There is convincing evidence that tamoxifen is converted to anti-oestrogenic metabolites that are more potent than the mother substance. One suggested resistance mechanism is that altered metabolism might contribute to inter-individual variability in serum concentrations, which in turn may influence the treatment response. Therefore we analysed whether genetic differences in the enzymes participating in the metabolic route could affect tamoxifen response. Further, local production of oestrogens has been implicated in the growth/progression of oestrogen responsive breast cancer in postmenopausal women. Since tamoxifen act by competitively inhibit oestrogen from binding to the ER, we sought to investigate whether increased expression levels of the enzymes responsible for the local oestrogen synthesis/metabolism had an impact on tamoxifen response. Finally, tamoxifen has been shown to induce both cell cycle arrest and apoptosis through ER-dependent and ER-independent mechanisms and one central mediator is p53. The proapoptotic activity of p53 is dependent on a proline-rich domain containing a common polymorphism, which affects the apoptotic potential. We therefore aimed to validate if the p53 polymorphism might influence the efficacy of adjuvant tamoxifen treatment.
**Oestrogen**

The steroid hormone oestrogen is synthesised from cholesterol through several enzymatic steps (Figure 1). Oestrogen is one of the most important steroid hormones of the adult female, progesterone being another [Norman and Litwack 1997], and regulates many physiological processes and plays essential roles in the development, growth control, and differentiation of the normal mammary gland.

![Oestrogen synthesis diagram](image)

Figure 1 – A schematic view of the biochemical steps in oestrogen synthesis from cholesterol.

Oestrogen occurs in several shapes, oestrone (E1), oestradiol (E2), oestriol (E3), oestrone sulfate (E1S), and oestradiol sulfate (E2S), where E2 is the most biologically active hormone in breast tissue. During the premenopausal period the oestrogen is mainly produced in the ovaries, and act in the classical endocrine manner. In the postmenopausal period when the ovaries have ceased to produce oestrogen, the primary source of oestrogen is from circulating androgens secreted from the adrenal gland and ovaries. These precursors are then biosynthesised into oestrogens via
enzymes in peripheral tissues such as adipose tissue (including the breast), skin and muscle [Reed and Purohit 1997; Sasano and Harada 1998]. Peripheral synthesised estrogens act locally in an intracrine or paracrine manner without being released in the circulation. The biological functions of oestrogens are mediated by transcriptional activation through the oestrogen receptors.

**The oestrogen receptor (ER)**

The oestrogen-receptors (ERs) belong to the family of nuclear hormone receptors that can initiate or enhance the transcription of genes containing specific hormone response elements. The classical ERα has been recognised for a long time and is used both as a prognostic and a predictive marker in breast cancer. ERβ on the other hand was first discovered during the mid 1990’s and has high homology to ERα (Figure 2). Both ERs contain six functional domains designated A to F. The DNA-binding domain mediates the binding to oestrogen response elements (EREs) in the promoter of oestrogen responsive genes [Kumar *et al.* 1987]. Two distinct cell-type specific domains, AF-1 and AF-2, where the AF-1 is ligand-independent and AF-2 ligand-dependent mediate the transactivating functions of the ERs. These domains stimulate transcription in mechanistically different manners [Tora *et al.* 1989; Kraus *et al.* 1995].

<table>
<thead>
<tr>
<th></th>
<th>A/B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>hERα</td>
<td></td>
<td>17%</td>
<td>95%</td>
<td>30%</td>
<td>60%</td>
</tr>
<tr>
<td>hERβ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A/B</td>
<td>C</td>
<td>D</td>
<td>E</td>
<td>F</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A/B = Transcriptional activity
C = DNA Binding Domain
D = Hinge Domain
E = Ligand Binding Domain
F = Agonist/Antagonist Distinctions
AF-1 = Transcriptional activation domain 1, ligand independent
AF-2 = Transcriptional activation domain 2, ligand dependent

**Figure 2** – ER homology [Adapted from Enmark *et al.* 1997; Ogawa *et al.* 1998].
Both receptors exhibit similar affinities for the endogenous oestrogen E2 but ERβ has been shown to transactivate oestrogen regulated genes less efficiently than does ERα [Kuiper et al. 1996; Mosselman et al. 1996; Kuiper et al. 1998; Lazennec et al. 2001]. The importance of ERβ in breast cancer however, remains elusive but several studies have found that there is a relative increase in ERα expression and a decrease in ERβ expression in breast cancer compared to normal breast [Iwao et al. 2000; Roger et al. 2001]. The ERs are widely distributed in human tissues, including the breast [Dotzlaw et al. 1997; Enmark et al. 1997; Kuiper et al. 1997; Taylor and Al-Azzawi 2000; Speirs et al. 2002].

**Molecular mechanisms of oestrogen action in the breast**

The biological functions of oestrogens are mediated by transcriptional activation through the oestrogen receptors (ERs). Generally, oestrogen diffuses through the plasma membrane of cells where it binds to ERs in the nucleus and promotes a conformational change. This conformational change brings the two activation-domains, AF-1 and AF-2, exposed that in turn recruit co-activator/co-repressor proteins. The oestrogen-ER complex then binds to oestrogen-responsive-elements (EREs) presented in the promoter region of oestrogen-responsive genes which activates transcription [Klein-Hitpass et al. 1989; Klinge et al. 1992; McDonnell and Norris 2002] (Figure 3).

**Figure 3** – Model of oestrogen action [Modified from Howell et al. 2000]. Oestrogen enters the cell and bind with high affinity to the ER. This result in dimerisation and a conformational change, which brings the two activation domains exposed, and leads to recruitment of coactivators. The ER binds to oestrogen response elements (EREs) in the promoter of oestrogen responsive genes with a following transcription.
ERα is the predominant mediator of the mitogenic effects of oestrogen on the mammary gland in a paracrine fashion. Activated ER can also regulate transcription indirectly through protein-protein interactions with other transcription factors (i.e. AP1 and Sp1). However, little is known about the process in the normal mammary epithelium in humans since most of the studies of oestrogen effects have been performed on breast cancer cells in culture. Several studies have reported that in breast cancer cells the ER-oestrogen complex primarily mediates its proliferative effects through an increase in c-Myc expression. c-Myc’s effect on cell cycle progression seems to be via the induction of cyclin E/Cdk2 through the loss of the cyclin dependent kinase inhibitor p21 [Musgrove et al. 1994; Prall et al. 1997; Prall et al. 1998]. The effects of oestrogen on cell cycle progression are also linked to the up-regulation of cyclin D1 [Musgrove et al. 1994; Watts et al. 1994; Prall et al. 1997; 1998]. In breast tumours of laboratory mice oestrogen has been shown to promote angiogenesis and increase extracellular vascular endothelial growth factor (VEGF) [Dabrosin et al. 2003a; 2003b]. Recently, Garvin et al. [2006] demonstrated that similar events also occurred in ex-vivo cultured normal human breast tissue.

Emerging data suggest that the action of oestrogen when bound to ERα and ERβ may differ regarding the formation and/or progression of breast cancer. It has been reported that oestrogen stimulated cell proliferation and increased tumour formation in the presence of ERα in breast cancer cell lines (MCF-7 & T47D cells). ERβ on the other hand, was related to inhibition of cell proliferation and prevented tumour formation in a mouse model. Others have found by in vitro studies that ERα was associated with breast cancer cell proliferation whereas ERβ was related to decreased proliferation [Lazennec et al. 2001; Paruthiyil et al. 2004; Strom et al. 2004].

**Oestrogen and Breast Cancer**

It is well known that oestrogen plays an important role in the origin and development of breast cancer. A prolonged exposure to oestrogen caused by early menarche, late menopause and hormone replacement therapy has stimulatory effects on cell proliferation and increases the risk of spontaneous DNA replication errors [Feigelson and Henderson 1996; Hahn and Weinberg 2002]. It has also been implicated that genotoxic compounds formed during oxidative metabolism of oestrogens may causes DNA adducts and mutations leading to an increased risk of breast cancer [Cavalieri et al. 1997; Rogan et al. 2003; Fernandez et al. 2006]. The majorities of breast cancers are hormone-dependent and contain oestrogen-receptors and in these cases oestrogen is an important source for the tumour to grow. About two thirds of breast cancers occur during the postmenopausal period when the ovaries have ceased to produce oestrogens and the circulating concentration of these hormones is low. Despite the low levels of circulating oestrogens the tissue concentration of E1, E2 and their sulfates are much higher than that found in plasma or in the area considered as normal, suggesting a local biosynthesis and accumulation of these hormones [Pasqualini et al. 1996; Chetrite et al. 2000].
Adjuvant treatment

The clinical appearance of breast cancer can range from a local tumour to a metastatic disease. For breast cancer the treatment options that can be considered are a primary surgery followed by either local or systemic adjuvant therapy or a combination. The principle of adjuvant therapy is to kill any remaining cancer cell after surgery. Today several standard markers are used clinically for estimating the prognosis of the individual breast cancer. These include age, tumour size, nodal status, histological grade, nuclear grade, oestrogen and progesterone (ER and PgR) receptor status, cellular proliferation and oncogene activation (c-ErbB2/HER2). The presence of ER, PgR, and c-ErbB2/HER2 are also useful markers for the prediction of treatment outcomes [Svenska Bröstcancergruppen - Swedish Breast Cancer Group 2007]. Patients with tumours expressing ER and/or PgR are candidates for adjuvant treatment with anti-oestrogens, primarily tamoxifen.

Tamoxifen

A brief history
There has been a long history before tamoxifen really came into use as an anti-oestrogenic agent for ER-positive breast cancer. As early as in the late nineteenth century, Beatson found that some premenopausal women with advanced breast tumours were responsive to oophorectomy and about 25 years later Allen and Doisy discovered the substance oestrogen. However, the research field was not revolutionised until Elwood Jensen discovered the ER in breast tumours and showed that patients with high levels of ER expression were more responsive to hormone ablation therapy. This opened the possibility to discover anti-oestrogenic drugs for the treatment of breast cancer. The pharmaceutical industry was though only focused on anti-oestrogenic compounds as contraceptives during this time. One such candidate was tamoxifen (ICI 46,474) which was a synthetic non-steroidal compound described by Harper and Walpole in 1967 at ICI Pharmaceuticals Division (today known as AstraZeneca). Tamoxifen was initially used as a contraceptive but was shown to facilitate ovulation rather than prevent it. It was not until tamoxifen was shown to prevent mammary tumours in mice that the pharmaceutical industry undertook clinical studies that led the way to an approval of tamoxifen in the early 1970’s [Jordan 2003; 2006 and authors therein]. The first clinical study on tamoxifen was reported by Cole et al. [1971] and included 46 postmenopausal breast cancer cases. The patients received 10 mg tamoxifen once or twice a day and the response was mainly measured as reduction in tumour size. Their results revealed a definite response with tamoxifen in 22 % of the patients, which was equivalent to other endocrine therapies during this period but tamoxifen showed fewer side effects [Cole et al. 1971].
**Effects of tamoxifen**

Tamoxifen is classified as a member of the selective oestrogen-receptor modulators (SERMs) and the pharmacological features of the compound are complex. It can act as an anti-oestrogen in breast and mammary tissue, but acts as an oestrogen in bone and lowers circulating cholesterol. Tamoxifen in the uterus, function as a mixed oestrogen/anti-oestrogen [Furr and Jordan 1984] (Figure 4). Despite this complexity of tamoxifen it has been the treatment of choice in ER-positive breast cancers during the past three decades, in particular for postmenopausal women.

![Figure 4](image.png)

**Figure 4** – Tamoxifen’s positive and negative effects

The side effects of tamoxifen are relatively few and mild, but the drug has been observed to increase the risk of endometrial cancer probably due to its partial agonistic effects. During early studies of breast cancer cells high doses of tamoxifen had been shown to be cytotoxic. In a rat mammary cancer model it was shown that short-term therapy with high daily doses delayed but did not completely prevent tumour development. In contrast, a prolonged treatment using a lower dose of tamoxifen remained the majority of animals tumour free [Jordan 2003]. In 1996, the Swedish Breast Cancer Cooperative Group reported results from a randomised trial of two versus five years of adjuvant tamoxifen in postmenopausal breast cancer patients and demonstrated that 5 years of treatment lowered the risk of recurrence or...
death by 18% compared to 2 years [Swedish Breast Cancer Cooperative Group 1996]. Recently Nordenskjöld et al. [2005] reported an additional analysis with long time follow-up on all-cause mortality among these patients. They found that patients in the 5-year group had a significantly reduced death rate from cardiovascular diseases, in particular coronary heart disease, compared to those in the 2-year group [Nordenskjöld et al. 2005].

**Molecular mechanism of Tamoxifen action in breast tumours**

Tamoxifen acts by binding competitively to the ligand-binding domain of the ER and influence it’s folding. However, the anti-oestrogen/ER-complex is still able to undergo dimerisation, although its ability to promote transcription is reduced [Pham et al. 1991; Allan et al. 1992] (Figure 5). Studies on the anti-oestrogenic effect of tamoxifen have suggested that tamoxifen causes cell cycle arrest and apoptosis both in vitro and in vivo by affecting growth factors including down-regulation of TGFα, induction of stromal TGFβ-1 and a decrease in the production of the potent mitogen IGF-1 [Noguchi et al. 1993; Chen et al. 1996]. However, the precise mechanisms signalling to and responsible for anti-oestrogen induced cell death are not fully understood. Thiantanawat et al. [2003] studied both the in vitro and in vivo mechanisms and signalling pathways by which tamoxifen exerts its effect and their results indicated that anti-oestrogens inhibit the proliferation of cells by inducing cell cycle arrest in the G0/G1 phase connected with increased apoptosis.
Figure 5 – Model of tamoxifen action [Modified from Howell et al. 2000]. Binding of tamoxifen to the ER is similar to that of oestrogen but leads to an altered conformational change of the activation domain 2 (AF2). When the complex bind to EREs the AF2 recruits corepressors and contribute to antagonism. The partial agonist activity of tamoxifen is affected by phosphorylation of activation domain 1 (AF1) and recruitment of coactivators.

The apoptotic signalling involved down-regulation of Bcl-2, up-regulation of Bax, and the activation of caspase-9, -6 and 7, whereas the cell cycle arrest was associated with up-regulation of p53 and p21 concomitant with down-regulation of cyclin D1 and c-Myc expression [Thiantanawat et al. 2003]. In normal human breast epithelial cells Somaï et al. [2003] demonstrated that the pro-apoptotic effect of tamoxifen was associated with an increase of p53 and caspase-3 protein expression. Tamoxifen also induces apoptosis in breast cancer cell lines via oxidative stress generated in the cell membrane leading to the induction of JNK1 and caspase pathways [Mandlekar et al. 2000]. Tamoxifen has also been shown to exert anti-angiogenic effects by an efficient decrease of secreted VEGF both in vitro and in vivo [Garvin and Dabrosin 2003].

Other antioestrogens
Tamoxifen was the first drug in general use for breast cancer treatment and has now been followed by several new compounds. All are thought to act by blocking oestrogen action, some of them through the obstruction of the ER (i.e. Fulvestrant), whereas others by inhibition of endogenous oestrogen synthesis (i.e. LHRH agonists for premenopausal women and aromatase inhibitors (AI) for postmenopausal women). Fulvestrant has shown promising responses, particular in tumours that initially responded and then recurred on tamoxifen [Howell and Robertson 1995;
Howell et al. 1996]. This agent targets ERα, hindering the complex from entering the nucleus, and promotes degradation by a following ubiquitination [Dauvois et al. 1993; Seo et al. 1998; Reid et al. 2003]. Another newly developed anti-oestrogen acting on both ERα and ERβ is TAS-108. This SERM shows antagonistic property towards ERα signalling accompanied with a partial agonistic effect for ERβ. TAS-108 is undergoing phase II trials for metastatic breast cancer treatment [Yamamoto et al. 2005].

Tamoxifen metabolism
The human body, as well as other animals, harbours a detoxification system making it possible to eliminate foreign chemicals. This system involves two reactions known as phase I and phase II which mostly (but not always) occur sequentially. The main purpose of this system is to modify foreign chemicals, making them more water-soluble and facilitate excretion via the urine or bile. Phase I reactions often consist of oxidation, reduction or hydrolysis which in this manner generate a more chemically reactive product. The functional group added then serves as a point of attack for the conjugation system, phase II reactions. Phase II reactions includes conjugation of groups like glucuronyl, sulfate, methyl, acetyl and glutathione. The resulting conjugate is almost always pharmacologically inactive and less lipid soluble than the mother substance. Phase I and II reactions are catalysed by enzymes whereof some have broad substrate specificity and others have narrower [Rang et al. 1999].

Tamoxifen is extensively metabolised in the liver and to a minor part locally in the breast with the main excretion via the bile and feces. The pharmacokinetics of tamoxifen is complex since several of its metabolites have been shown to be biologically active. The biotransformation of tamoxifen is mediated by cytochrome-P450 enzymes, mainly through demethylation and hydroxylation to form several primary metabolites, principally 4-OH-tamoxifen, α-OH-tamoxifen, N-desmethyl-tamoxifen, and 4-OH-N-desmethyl-tamoxifen (Figure 6). Tamoxifen was first shown to have very low affinity for the ER in vitro compared to oestrogen, but was later considered to be a pro-drug that was converted to the more potent 4-OH-tamoxifen. It has been suggested that this metabolite may be the compound responsible for the anti-tumour effects of tamoxifen in vivo. However, in 2003, Stearns and colleagues discovered a second high potent metabolite, endoxifen,
Tamoxifen metabolism

Figure 6 – Schematic view of tamoxifen metabolism. Tamoxifen is considered to be a prodrug and is extensively metabolised through biochemical reactions mainly catalysed by the cytochrome P450 family of enzymes.

which has similar capacity to suppress oestrogen mediated cell proliferation as has 4-OH-tamoxifen [Stearns et al. 2003; Johnson et al. 2004].

Nevertheless, experimental studies have shown that cytochrome P450 3A4 and 3A5 (CYP3A4 and CYP3A5) are the major catalysts of N-demethylation, whereas the 4-hydroxylation is predominantly supported by the cytochrome P450 2D6 (CYP2D6) enzyme [Jacolot et al. 1991; Crewe et al. 1997; Dehal and Kupfer 1997; Coller et al. 2002]. A further step in the metabolism of tamoxifen is sulfate conjugation, catalysed by members of the sulfotransferase family (SULT) that generally increase the solubility and facilitates excretion of the drug. SULT1A1 is a major form of phenol SULT in adult human liver and has been shown to be the primary sulfotransferase responsible for the sulfation of 4-OH-tamoxifen [Falany et al. 1994; Seth et al. 2000]. Recently, it was also shown that SULT1E1 is capable of sulfate 4-OH-tamoxifen [Falany and Falany 2006]. The 4-OH-tamoxifen is in equilibrium between a trans- and cis-isoform, resulting in a proposed shift in property from a potent anti-oestrogen towards a significantly less potent anti-oestrogen [Jordan et al. 1981; Katzenellenbogen et al. 1984; Malet et al. 2002]. Nishiyama et al. [2002] suggested a geometrical selectivity in both sulfation of trans-4-OH-tamoxifen and glucuronidation of cis-4-OH-tamoxifen, and that these reactions were catalysed by sulfotranseferase 1A1 (SULT1A1) and UDP-glucuronosyltransferase 2B15 (UGT2B15) respectively [Nishiyama et al. 2002].
**Tamoxifen resistance**

Even if most patients respond to tamoxifen therapy many patients experience resistance to endocrine therapy either at the beginning of the treatment or after prolonged use. The mechanisms for this resistance are not completely elucidated but several suggestions have been reported.

**ER mutations**

Tamoxifen effects are primarily mediated via the ER and one of the suggestions is that loss of ER expression through mutations could confer resistance. Fuqua and colleagues [2000] found a mutation leading to a substitution of amino acid 303 (K303R) in 34 % of premalignant breast lesions. This mutation produced a receptor hypersensitive to oestrogen, with enhanced binding of co-activators in the presence of low oestrogen levels [Fuqua et al. 2000].

**ER co-activators and co-repressors**

An inaccurate expression of steroid receptor co-regulators has also been implicated in tamoxifen resistance. In cultured cells where the levels of co-regulators can be manipulated, increased co-activator levels can enhance the agonist activity of tamoxifen-bound ER, whereas increased co-repressor levels can enhance antagonist activity. However, AIB1 is a co-activator that is overexpressed in more than 50 % of breast tumours. In a study by Osborne and coworkers [2003] they analysed AIB1 expression among patients treated with or without tamoxifen. High levels of AIB1 were associated with worse outcome in those who had received tamoxifen compared to those who did not. The outcome was even worse if the patient had a concomitant high expression of HER-2 [Osborne et al. 2003].

Girault and colleagues [2003] investigated the relationship of NCoR1 and tamoxifen response in a cohort consisting of postmenopausal breast cancer patients that all had received tamoxifen therapy. They found that patients with an intermediate or high tumour mRNA level of NCoR1 had a significant improved relapse free survival compared to those with low mRNA levels [Girault et al. 2003].

**Cross-talk**

The oncogene HER-2 is amplified and overexpressed in 20 – 30 % of breast cancers and has been associated with poor prognosis. Several studies have indicated that patients with tumours positive for HER-2 have a reduced benefit of antioestrogen therapy [Borg et al. 1994; Carломagno et al. 1996; Stal et al. 2000]. Others have though failed to observe such correlation [Elledge et al. 1998]. There are however emerging experimental data suggesting that tamoxifen resistance may be due to cross-talk between the ER, EGFR/HER-2 and IGF-1R network [Nicholson et al. 2004 and authors therein]. In tamoxifen resistant MCF-7 cells Knowlden et al. [2003] demonstrated that there was increased signalling activity by enhanced EGFR/HER-2 heterodimerisation and receptor phosphorylation, as well as increased activation of the downstream kinases ERK1/2 and AKT [Knowlden et al. 2003]. Additionally, Hutcheson et al. [2003]
suggested that the ER appears to play a role in maintaining the efficiency of EGFR signalling. They also suggested that ER was involved in modulating the autocrine growth regulatory loop in tamoxifen resistant cells, possibly by regulating the availability of TGFα. Although the exact mechanisms by which ER regulates TGFα expression remains unclear [Hutcheson et al. 2003].

**Non-classical mechanisms**
A growing body of evidence suggests that ER can regulate cellular function through non-classical mechanisms. Studies have indicated that ER not only resides in the nucleus, but also in the cytoplasm or near the plasma membrane [Chambliss et al. 2000; Russell et al. 2000; Razandi et al. 2003]. Membrane ER can directly or indirectly activate membrane tyrosin kinase receptors in many cells including breast cancer cells. ER has been shown to interact directly with HER-2 and this interaction protects HER-2 overexpressing breast tumour cells from tamoxifen induced apoptosis [Chung et al. 2002]. The membrane ER can also directly interact with and/or activate IGF-1R, PI3K/Akt, Src and EGFR [Chung et al. 2002; Shou et al. 2004].

**ERβ**
When the second ER, ERβ, was discovered it was found to be expressed in both normal and malignant breast tissue [Dotzlaw et al. 1997; Fuqua et al. 1999; Roger et al. 2001]. It was also observed that ERβ expression was decreased in pre-invasive mammary tumours compared with normal benign lesions and the authors suggested that ERβ might have a protective effect against the mitotic effect of oestrogen in pre-malignant lesions [Roger et al. 2001]. It has also been suggested by in vitro studies that tamoxifen resistance may be due to increased expression of ERβ [Speirs et al. 1999]. However, Hopp et al. [2004] analysed the expression of ERβ in tumour specimen from breast cancer patients and reported that those with high levels of ERβ had an improved relapse free survival when treated with tamoxifen. Whether changes in the expression of ERβ are an important factor of tamoxifen resistance requires further investigations.

**Influence on tamoxifen bioavailability**
Since tamoxifen is extensively metabolised in the liver, primary by the polymorphic cytochrome P450 family of enzymes, one possible resistance mechanism may be the influence of genetic polymorphisms in these enzymes. This could generate individual variations of antagonistic/agonistic metabolites that will affect patient outcome. Osborne and colleagues [1992] suggested that the ratio of the less antioestrogenic cis-4-OH-tamoxifen and the more potent trans- 4-OH-tamoxifen was altered in breast cancer patients responding versus not responding to tamoxifen [Osborne et al. 1992]. In a study by Nowell et al. [2002] patients with the genotype responsible for a low activity phenotype, sulphotransferase 1A1*2 (SULT1A1*2) was shown to have a significantly increased risk of disease recurrence compared to those with the wild-type alleles [Nowell et al. 2002]. Others have suggested that CYP2D6 is an
independent predictor of therapeutic outcome in postmenopausal breast cancer patients receiving tamoxifen [Goetz et al. 2007].

**Prediction of tamoxifen resistance/response**

The complete mechanisms of the development of resistance to adjuvant tamoxifen and other anti-oestrogens are still unclear. Today the only predictive markers in use for endocrine therapy are the presence and the level of ER and PgR and to a lesser extent HER-2 status. In order to offer the best survival outcome selection for the most optimal therapeutic regime from the time at diagnosis is essential. It is important to consider that some resistance pathways are important in some patients but are irrelevant in others depending on the tumour character, genetic background and other yet undefined factors. Therefore, further research is necessary in order to discover additional biological markers with high selectivity and sensitivity for the choice of endocrine therapy in breast cancer patients.

**Major areas investigated in this thesis**

1. Tamoxifen metabolism  
2. Local oestrogen synthesis  
3. p53 and tamoxifen response

1. **Tamoxifen metabolism**

Tamoxifen is considered as a pro-drug which needs to be bio-transformed to the more potent anti-oestrogens 4-OH-tamoxifen and endoxifen. The precursor of endoxifen is N-desmethyльтamoxifen and the reaction from tamoxifen to N-desmethyльтamoxifen is catalysed by cytochrome P450 3A5 (CYP3A5). The subsequent 4-hydroxylation is predominantly catalysed by the CYP2D6 enzyme. CYP2D6 is highly polymorphic and at present there are more than 80 polymorphisms identified in this particular gene (http://www.imm.ki.se/cypalleles). For CYP3A5 there are approximately 20 polymorphisms identified today. The most frequent and functionally important polymorphism in the CYP3A5 gene consists of an A6986G transition within intron 3 (CYP3A5*3). This polymorphism creates an alternative splice site resulting in a frame shift and truncation of the protein [Hustert et al. 2001; Kuehl et al. 2001]. In CYP2D6 the most common non-functional allele is CYP2D6*4. This polymorphism generates a G to A transition at the first nucleotide of exon 4 in the CYP2D6 gene leading to a truncated non-functional gene product [Hanioka et al. 1990]. Further steps in the metabolism of tamoxifen comprise sulfation and glucuronidation to make the metabolites more inactive and these reactions are catalysed by SULT1A1 and UGT2B15 [Nishiyama et al. 2002]. A frequent polymorphism in the SULT1A1 gene is a G to A transition at nucleotide 638, defining the SULT1A1*2 allele, which is correlated with a diminished capacity to sulfate SULT1A1 substrates [Raftoganis et al. 1997]. Several polymorphisms have also been found within the UGT2B15 gene although only two in the coding region [Iida et al.
One causes an amino acid shift from aspartate (UGT2B15*1) to tyrosine (UGT2B15*2) at position 85 of the UGT2B15 protein, a shift which has been associated with an increase in enzyme activity [Levesque et al. 1997].

2. Local oestrogen synthesis

It is now well established that breast tumours possess enzymatic systems necessary for the local formation of oestrogens such as aromatase, steroid sulfatase, 17ß-HSD1/2 and sulfotransferase. A change in the expression levels of these enzymes is speculated to affect intratumoural levels of oestradiol, which in turn may affect the responsiveness to endocrine treatment. Aromatase is a key enzyme in the synthesis of oestrogens and participate in the aromatisation of androstenedione to oestrone but also testosterone to oestradiol (Figure 7). Data concerning the expression levels of aromatase and the impact on prognosis/endocrine therapy is relatively sparse although in a recent study by Yoshimura et al. [2004] they demonstrated that high mRNA expression of aromatase correlated to improved prognosis. Similar results have also been reported by Zhang et al. [2003]. Steroid sulfatase is a single enzyme that hydrolyses oestrone sulfate to oestrone. The sulfatase pathway has been postulated to form more oestrogen than the aromatase pathway in vivo since the activity of sulfatase has been shown to be 30 – 150 times greater than that of aromatase [Pasqualini et al. 1996; Chetrite et al. 1998]. High levels of this enzyme have been correlated with a shorter disease-free survival and worse prognosis [Utsumi et al. 1999; Miyoshi et al. 2003; Yoshimura et al. 2004]. SULT1E1/SULT1A1 is the counterparts of sulfatase and is responsible for the conjugation of a sulfate group to make the oestrogen molecule biologically inactive. These enzymes have mainly been studied in cell systems and its role in vivo is less known [Falany and Falany 1996; Chetrite et al. 1998; Falany and Falany 2006]. However, Suzuki et al. (2003) studied both the activity and expression of SULT1E1 in breast cancer patients and reported that high protein expression of SULT1E1 was correlated with a decreased risk of recurrence. The importance of SULT1A1 in this manner has been less studied.

17ß-HSD1 is the enzyme that catalyses the final conversion of oestrone into oestradiol whereas the opposite reaction is catalysed by 17ß-HSD2. Several reports suggest that differences in the levels of these enzymes could be of importance for oestradiol levels and thereby affect the prognosis of the disease [Suzuki et al. 2000; Gunnarsson et al. 2001; Miyoshi et al. 2001]. However, the 17ß-HSD enzymes have not been investigated in this thesis.
Figure 7 –Schematic view of local oestrogen synthesis in the breast.
3. p53 codon 72 polymorphism

The apoptotic process is fundamental in the response to the cytotoxic stress generated by anticancer treatment, and the p53 protein is one key determinant in transducing this apoptotic signal. The concentration of p53 in the cell is normally low but is upregulated by stimuli such as DNA-damage or other types of cellular stress. p53 has been shown to be mutated in more that 50 % of human cancers which is indicative for the important role of p53s normal function. The principle of most anticancer therapies is to generate signals that activate apoptosis either through DNA-damage, the reduction of growth factors or by blocking hormone-receptor interactions.

The structural organisation of p53 includes a transactivation domain, a sequence specific DNA-binding domain (DBD) and a tetramerisation domain (Figure 8). Between the transactivation domain and the DBD is a region rich in proline, which has been shown to be a key determinant in the apoptotic function of p53. This proline-rich domain contains a common Pro-Arg polymorphism at codon 72. Data indicate that the two polymorphic variants are functionally distinct leading to different apoptotic potential where the Arg allele induces apoptosis more efficiently than does the Pro allele [Walker and Levine 1996; Sakamuro et al. 1997; Dumont et al. 2003]. Differences in the ability to induce apoptosis may influence the cancer risk or cancer therapy.

![Figure 8](image_url) – Structural organisation of p53 [Weinberg 2007].
Aims of the study

The general aim was to elucidate resistance mechanisms of tamoxifen in order to create new knowledge that might be a step forward to a more personalised therapy.

The specific aims were:

- To examine if genetic polymorphisms in enzymes participating in the metabolism of tamoxifen correlated to clinicopathological factors. We also aimed to validate if genetic variations in these enzymes could predict tamoxifen response in ER-positive postmenopausal breast cancer patients.

- To examine if the mRNA and protein expression levels of aromatase, sulfatase, sulfotransferase 1A1 were associated with clinical/pathological factors and/or had a prognostic importance in tamoxifen treated postmenopausal breast cancer patients.

- To investigated the polymorphism in codon 72 of the p53 gene and its impact on efficacy of adjuvant treatment in postmenopausal breast cancer patients.
Patients

**Paper I and IV**

In 1976, the Stockholm Breast Cancer Group started a trial to compare postoperative radiotherapy with adjuvant chemotherapy and tamoxifen with no tamoxifen [Rutqvist et al. 1989; Rutqvist and Johansson 2006]. Both pre- and postmenopausal patients (age ≤ 70) with a unilateral, operable breast cancer were included. The patients were required to have either histological verified lymph node metastases or a tumour diameter exceeding 30 mm. All patients were treated with a modified radical mastectomy. Using a 2 x 2 factorial study design, the postmenopausal patients were then randomised to four treatment groups: adjuvant chemotherapy, adjuvant chemotherapy plus tamoxifen, radiotherapy, radiotherapy plus tamoxifen (Figure 9).

![Figure 9](https://example.com/figure9.png)

*Figure 9 – A schematic view of the randomisation*

Oestrogen-receptor (ER) content was measured in clinical routine practice by isoelectric focusing and samples with concentrations of ≥ 0.1fmol/μg were classified as positive. Tamoxifen was given postoperatively at a dose of 40 mg daily for 2 years and initiated within 4 – 6 weeks of surgery. For patients randomised to chemotherapy, treatment consisted of 12 courses of CMF (Cyclophosphamide, Methotrexate and 5-Fluorouracil). Radiotherapy was given with high voltage technique. The dose was 46 Gy with 2 Gy per fraction. The mean follow-up time was 10.7 years (range: 0.24 – 18.6 years). Of the 696 postmenopausal breast cancer patients included in the trial, fresh frozen tumour tissues from 226 patients, whereof 112 had received tamoxifen therapy, were available for the current investigations. The percentage of lymph-node positive, ER-positive and large tumours (> 20 mm) were 88 / 89, 71 / 70, and 57 / 61 respectively, in the initial and the current study.

**Paper II**

The 677 postmenopausal patients included in paper II were diagnosed in the South East Health Care Region of Sweden between 1986 and 1997. They had a breast cancer in stage II or III and were between 50 and 96 years old with a mean age of 69 at the time of diagnosis. All patients were oestrogen receptor (ER) positive and had
received adjuvant postoperative tamoxifen therapy. ER and progesterone-receptor (PgR) content was measured in clinical routine practice by isoelectric focusing before 1988, and later on with enzyme immunoassay (EIA). Samples with concentrations of $\geq 0.1$ fmol/μg (or $\geq 0.3$ fmol/μg with EIA) were classified as positive. In this patient cohort, a subgroup of 238 patients was randomised to either 2 or 5 years of tamoxifen. Patients diagnosed before 1994 received a daily dose of 40 mg tamoxifen for 2 or 5 years. After the 1st of January 1994 all postmenopausal breast cancer patients received 20 mg tamoxifen for 5 years. The mean follow-up time in the total population was 7.3 years (range: 0.04 – 17.9 years). For the randomised group, the mean follow-up time in the arm of 2 year was 9.8 years (range: 2.3 – 17.7 years) and in the 5-year arm 10.7 years (range: 2.1 – 17.9 years).

**Paper III**

The study in paper III included 161 postmenopausal patients less than 75 years of age with a breast cancer in stage II or III, diagnosed between 1985 and 1994 in the South East Health Care Region of Sweden. They were participants in a randomised multicentric trial where two versus five years of postoperative tamoxifen treatment was evaluated [Swedish Breast Cancer Cooperative Group 1996]. The daily dose of tamoxifen was 40 mg. ER and PgR content was measured in clinical routine practice by isoelectric focusing before 1988, and later on with enzyme immunoassay (EIA). Samples with concentrations of $\geq 0.1$ fmol/μg (or $\geq 0.3$ fmol/μg with EIA) were classified as positive. In the present study tumour material from a subset of the patients included in the trial was available. The mean follow-up time was 9.5 years (range: 0.08 – 16.9 years, median: 11 years).
Material and Methods

This section explains the principle of the methods used in this thesis. A more detailed description is presented in the respective paper.

**DNA-isolation**
The DNA used in paper I and IV was isolated from fresh frozen tumour tissues using phenol, phenol/chloroform (1:1), and chloroform, disintegrated by proteinase K, precipitated with ethanol and re-dissolved in sterile water.
For paper II, fresh frozen tumour tissues (~30 mg) were homogenised using a microdismembrator (B Braun, Melsungen, Germany) and genomic DNA was isolated with the SV Genomic DNA Purification System (Promega Corporation, Madison, WI, USA). The DNA content was determined by spectrophotometry and purified DNA was stored at -20°C before use.

**RNA-isolation and cDNA synthesis**
In paper III, fresh frozen tumour tissues (~30 mg) were homogenised using a microdismembrator (B Braun, Melsungen, Germany) and total RNA was isolated using SV Total RNA Isolation System (Promega Corporation, Madison, WI, USA). RNA concentrations and A260/A280 ratios were measured with a NanoDrop®ND 1000 spectrophotometer. The isolated RNA was further converted to cDNA using random primers and Superscript™ II RT (Invitrogen™, Life Technologies, Stockholm, Sweden) for reverse transcriptase PCR following the manufacturer’s recommendations.

**Genotyping**

**PCR-Restriction fragment length polymorphism**
After PCR the DNA is digested with a specific endonuclease that recognises the area containing the single nucleotide polymorphisms (SNPs) (paper I, II, and IV). Endonucleases are natural occurring enzymes of bacteria that destroy foreign DNA of invading viruses. The enzyme recognises certain nucleotide sequences (mostly 4 – 8 nucleotides) and cleaves the DNA-backbone at or near these sites [Tamarin 1996]. This method is a classical way to identify SNPs and can be used to genotype the sample without completely sequencing it. At the final step a gel electrophoresis is performed to separate the DNA-bands, which will generate distinct patterns depending on the genotype.

**Denaturating high performance liquid chromatography**
Another way to identify genetic variants is by the use of denaturating high-performance liquid chromatography (DHPLC or Wave) which is an automated technique that detect single base substitutions and small insertions or deletions [Xiao and Oefner 2001; Frueh and Noyer-Weidner 2003] (paper II). DHPLC uses a
combination of partial heat denaturation and reverse-phase chromatography to detect sequence polymorphisms within DNA fragments. The principle of this method is based on that heteroduplexed DNA can be distinguished from homoduplexed DNA by different elution pattern (retention time) when it is separated through a linear buffer gradient at a given optimised temperature (Figure 10).

For example; in an individual heterozygous for the SNP the ratio of wild-type (wt) and variant DNA is 1:1 [Xiao and Oefner 2001; Frueh and Noyer-Weidner 2003]. A PCR of the sequence of interest will therefore generate equivalent quantities of wt and variant DNA in this case. The PCR product is then denatured followed by a stepwise renaturation, which will generate a mix of homo- and heteroduplex. During the partial denaturating elution phase, heteroduplex DNA will, because of the mismatch, have shorter retention time compared to homoduplex DNA. Depending on the genotype, different retention patterns will be generated in a chromatogram.

**Sequence analysis**

In order to achieve standards for the specific genotype patterns obtained with RFLP or DHPLC (paper I, II and IV) we performed direct DNA sequencing by using the Sanger’s dideoxy method [Sanger et al. 1977]. This method is based on the incorporation of a labelled (e.g. isotope) 2’, 3’-dideoxy nucleotide triphosphates (ddNTPs) in a PCR additional to the normal deoxynucleotide triphosphates (dNTPs) included. The difference between ddNTP and dNTP is that ddNTP have a hydrogen
atom attached to the 3’ carbon rather than a hydroxyl group. This prevents further addition of nucleotides during PCR elongation because a phosphodiester bond cannot form and thus leads to a DNA chain termination. Briefly, a PCR reaction is performed, one for each of the four bases G, A, T and C, with for example a reduced concentration of dGTP and an addition of ddGTP in the “G-tube”. As the DNA synthesis proceeding nucleotides are added on the growing chain. However, on occasion a ddGTP is added the DNA chain is terminated. The different tubes will contain fragments of different lengths with DNA terminated at all positions where the ddNTP is incorporated. Finally, the contents in each G, A, T, and C tube are then run in separate lanes in a polyacrylamide gel in order to separate the bands of different lengths. The gel is finally exposed on an x-ray film.

**RNA and Protein expression**

**Real-Time PCR**

In order to measure the relative quantity of mRNA expression of certain genes we performed two-step real-time PCR (paper III). Real-time PCR is a technique, which enables simultaneous amplification and quantification of a specific nucleic acid with detection of the PCR product in real time. This method utilises sequence specific primers, a sequence specific labelled hydrolysis probe and DNA polymerase. The probe is labelled with a reporter dye on the 5’ end and a quencher dye on the 3’ end. When bound to the target sequence the probe is quenched but will be degraded by the DNA polymerase 5’ nuclease ability during the extension phase of the PCR. Upon degradation, the reporter dye will be separated from the quencher dye with a subsequent increase in fluorescence emission (Figure 11).

![Figure 11](image-url)

**Figure 11** – Principle of real-time PCR chemistry:
1. The primer and probe anneal to the target sequence
2. Taq DNA polymerase extends the primer
3. When the polymerase reaches the probe its 5’ to 3’ exonuclease activity cleaves the probe
4. The fluorescent signal from the free reporter is then measured
The increase in fluorescence is measured sequentially during the elongation by a sequence detector and the amount of reporter signal increase is proportional to the quantity of product being produced for a given sample. When the reporter signal reaches a detectable level (threshold cycle, Ct-value) it can be captured and displayed as an amplification plot (Figure 12). To compare the results between different runs/plates, a standard curve is created for the target gene and the housekeeping gene in each plate (in paper III; β-actin from Human Breast Cancer Total RNA, Ambion®). A standard curve slope of -3.32 is indicative for a PCR reaction with 100% efficiency, which is the same as good sample/experimental replicate precision. The final quantitation is calculated by dividing the mean Ct-value of the gene of interest with the mean Ct-value of the housekeeping gene.

Figure 12 – Example of a real-time PCR standard curve amplification plot
**Tissue Microarray**

Tissue microarray (TMA) technology used in paper III is a high throughput technique used to examine DNA, RNA or protein targets in a large number of tissues on a single slide in parallel [Kononen et al. 1998]. Briefly, three 0.8 mm cylindrical cores from each breast cancer specimen were placed in a recipient paraffin block with a maximal of 243 cores, including liver as control. The tissue microarray blocks were then cut with a microtome into 4μm thick sections and mounted onto glass slides and the proteins of interest were examined by utilising immunohistochemistry.

**Immunohistochemistry**

Immunohistochemistry (IHC) is an immunological method based on the use of monoclonal or polyclonal antibodies to detect specific proteins in a tissue section. Monoclonal antibodies are produced by one type of immune cell and are all clones from a single parent cell, which recognises a specific epitope. These antibodies are generally considered to exhibit greater specificity than polyclonal antibodies, which are derived from different B-cell lines and are a mixture of immunoglobulin molecules, each recognising different epitopes on the antigen that they were raised against. There are several systems available for visualising the antibody-antigen interaction. In this thesis (paper III) the DakoCytomation EnVision™System-HRP was used, which is a two-step immunohistochemical staining technique. This system is based on a peroxidase (HRP) labelled polymer that is conjugated to secondary antibodies. After blocking endogenous peroxidase the tissue is incubated with an optimally diluted primary antibody against the protein of interest. Then a secondary antibody labelled with multiple HRP is added. To be able to visualise the antigen-antibody complex a substrate-chromogen solution is used, which consists of hydrogen peroxide and diaminobenzidine (DAB). The peroxidases conjugated to the secondary antibody then use the hydrogen peroxide as substrate to oxidise DAB into a brown coloured deposit. The brown colour demonstrates the presence and the location of the protein of interest. Finally, the tissue is counterstained and the staining intensity is examined by light microscopy.

**Statistical analyses**

The Statistica 6.0 software program was used for the statistical calculations in paper I and IV. In paper II and III the statistics were performed by Statistical package for social sciences (SPSS) advanced model 12.0. In order to show differences in the distribution of genotypes (paper I, II and IV) and to examine the relationship between mRNA/protein expressions (paper III) according to tumour characteristics we utilised the Chi-square test. The survival curves of recurrences were produced according to the life-table method described by Kaplan and Meier and the P-values presented in the survival plots were obtained by the log-rank test. A univariate Cox proportional hazard model was used for the estimation of the hazard ratio (HR) when comparing genotypes or mRNA/protein expression for each treatment group. Further, a multivariate Cox-model was used in order to adjust for the different
tumour characteristics. We also tested whether the calculated HRs were significantly different between treatment groups by using an interaction test according to the Cox-model (treatment (0/1), genotype or mRNA/protein expression (0/1), treatment x genotype or treatment x expression (0/1)). In the calculations of whether the investigation parameters could predict resistance to tamoxifen therapy only oestrogen receptor positive patients were included. Differences between groups were judged significant at confidence levels greater than 95 % ($P < 0.05$).
Results and Discussion

In this section highlights of the results will be presented and discussed. For a more detailed presentation, please refer to the result and discussion part in each paper.

Paper I

Genotype of metabolic enzymes and the benefit of tamoxifen in postmenopausal breast cancer patients.
Wegman P, Vainikka L, Olle Stål, Bo Nordenskjöld, Lambert Skoog, Lars-Erik Rutqvist, and Sten Wingren

Results

First, an evaluation was performed to see if the genetic variants of CYP2D6 and SULT1A1 were of prognostic importance including both ER-positive and ER-negative patients; however results showed no difference in neither tumour characteristics nor recurrence-free survival between genotypes.

Further, it was assessed if different genotypes could predict the benefit of tamoxifen treatment in ER-positive patients. A calculation of distant recurrence-free survival between the randomisation of tamoxifen vs. no tamoxifen was performed for each genotype. Patients possessing at least one CYP2D6*4 allele had a better survival and demonstrated a significantly decreased risk of recurrence (RR = 0.28, 95 % CI = 0.11-0.74) when randomised to tamoxifen (Figure 13). The outcome among patients homozygous for CYP2D6*1 was approximately equal between tamoxifen treated and untreated cases. We could also observe a tendency for a better recurrence-free survival in patients homozygous for SULT1A1*1 when treated with tamoxifen (RR = 0.48, 95 % CI = 0.21-1.12) (Figure 14). When we combined patients with at least one CYP2D6*4 allele and/or a homozygous SULT1A1*1, tamoxifen therapy significantly improved survival in these cases (RR = 0.38, 95 % CI = 0.19-0.74, P = 0.0041).
Figure 13: Distant recurrence-free survival among postmenopausal women with ER-positive breast cancers, in relation to CYP2D6 genotype and adjuvant tamoxifen treatment. Solid line represents patients receiving tamoxifen (Tam+) whereas dashed line represents patients who did not received tamoxifen (Tam-). A. Patients homozygous for the CYP2D6*1 allele. B. Patients homo- or heterozygous for the CYP2D6*4 allele (null-allele).
Figure 14: Distant recurrence-free survival of postmenopausal, ER-positive breast cancer patients in relation to SULT1A1 genotype and adjuvant tamoxifen therapy. Solid line represents patients receiving tamoxifen (Tam+) whereas dashed line represents patients who did not received tamoxifen (Tam-). A. Patients homozygous for the SULT1A1*1 allele. B. Patients homo- or heterozygous for the SULT1A1*2 allele (low-activity allele).
Discussion
The observation of the significantly improved benefit from tamoxifen in patients carrying at least one CYP2D6*4 and/or patients homozygous for SULT1A1*1 is, to our knowledge, the first report on the influence of the CYP2D6*4 polymorphism on tamoxifen therapy. The SULT1A1 polymorphism has been previously investigated by Nowell et al. [2002] and similar to our results, Nowell and colleagues showed that the high activity allele SULT1A1*1 contributed significantly to tamoxifen response [Nowell et al. 2002]. They speculated that sulfation might affect bioavailability of 4-OH-tamoxifen by reducing the clearance of the sulfated metabolites, which in turn may serve as an inactive reservoir. This reservoir might subsequently undergo desulfonation by steroid sulfatase expressed in breast tumours and recover to the active 4-OH-tamoxifen, leading to a prolonged anti-oestrogen effect. In experimental studies CYP2D6 has been shown to be a determinant to form 4-OH-tamoxifen [Coller et al. 2002] and endoxifen [Stearns et al. 2003] that is supposed to be the most ER-active metabolites. In the present investigation we hypothesised that those patients homozygous for CYP2D6*1 would generate more of the ER-active metabolites and therefore benefit from tamoxifen therapy. However, the results demonstrated that patients homo- or heterozygous for CYP2D6*4 had a significantly better response of tamoxifen compared to CYP2D6*1 homozygous patients which is in contrast to the main hypotheses and may be a consequence of the limited number of patients. Others have thought by in vitro studies noted that an absent or decreased CYP2D6-dependent 4-hydroxylation is compensated by CYP2C9 and CYP3A4 to the overall formation of 4-OH-tamoxifen, but the reaction proceeded at lower rate [Crewe et al. 1997; Coller et al. 2002]. Nevertheless, the results from the current investigation demonstrating variability in distant recurrences between patients treated with endocrine therapy may not be a coincidence since Nowell et al. (2002) have shown similar trends. This gives rise to speculations that the entirely picture of tamoxifen and its active metabolites may not be fully elucidated and there might be other yet undefined ER active metabolites.
**Paper II**

Genetic variants of CYP3A5, CYP2D6, SULT1A1, UGT2B15 and tamoxifen response in postmenopausal breast cancer patients
Pia Wegman, Sauli Elingarami, John Carstensen, Olle Stål, Bo Nordenskjöld, and Sten Wingren

**Results**

In this study we found that patients homozygous for CYP2D6*4 had a significantly better prognosis compared to patients homo- or heterozygous for CYP2D6*1 (P = 0.05 and P = 0.04, respectively), although in a multivariate Cox-model the result was partly weaker (P = 0.055). For genotypes of CYP3A5, SULT1A1 and UGT2B15, no differences in prognosis were seen.

Among the 677 ER-positive patients a subgroup were randomised to receive either two or five years of tamoxifen. In this subgroup (n = 238) we examined if the genotypes of CYP3A5, CYP2D6, SULT1A1 and UGT2B15 could predict the benefit of tamoxifen duration by comparing the genotypes for each randomisation. We found that patients homozygous for CYP3A5*3 tended to have an increased risk of recurrence if they were treated with tamoxifen for two years, although this was not statistically significant (Figure 15A).
In the group randomised to five years tamoxifen the survival pattern shifted towards a significantly improved recurrence-free survival among CYP3A5*3 homozygous patients (Figure 15B). The decreased risk of recurrence with five years of tamoxifen in CYP3A5*3 homozygotes was also true in a multivariate Cox-model (HR = 0.13, 95% CI = 0.02-0.86, P = 0.03). No conclusive association could be seen for CYP2D6, SULT1A1, or UGT2B15, even though patients homo- or heterozygous for CYP2D6*4 showed a tendency for an improved benefit of five years tamoxifen compared to patients homozygous for CYP2D6*1.

Discussion
In paper I we found that genetic polymorphism in CYP2D6 and SULT1A1 may predict the benefit of tamoxifen with a significantly improved recurrence-free survival in patients that were carriers of the CYP2D6*4 allele and were homozygous for SULT1A1*1.

In paper II, we could not confirm previous findings. We did however, find that five years of adjuvant tamoxifen seems to be beneficial for patients homozygous for the CYP3A5*3 allele, and we could also see a trend for similar result in patients who were carriers of the CYP2D6*4 allele. The result of CYP2D6 is partly in agreement with Nowell et al. [2005] who noted that the CYP2D6*4 variant seemed to be associated with a decreased risk of death or recurrence. Others have found contrary
results. In a recent study by Goetz et al. [2005], who investigated the CYP2D6*4 genotype in 180 postmenopausal breast cancer patients treated with tamoxifen, they demonstrated that patients homozygous for CYP2D6*4 had significantly worse relapse- and disease-free time but not overall survival. However, their homozygous CYP2D6*4 constituted only thirteen patients and there is a substantial lack of patient information (i.e. ER-status, tumour stage, other therapies etc.), which makes difficulties in interpreting their findings.

Our novel findings of an improved recurrence-free survival with 5 years tamoxifen in patients homozygous for CYP3A5*3 was unexpected since this genotype represents an inactive form and should not be able to form the primary metabolite N-desmethyltamoxifen, which is the precursor of the ER-active metabolite endoxifen. Even though studies have been performed concerning the CYP3A5 enzyme and tamoxifen results are controversial. Recently, Tucker et al. [2005] as well as Goetz et al. [2005] reported no influence on CYP3A5 genotype, metabolite concentration and/or disease-free or overall survival, whereas Jin and colleagues [2005] proposed that subjects who carried at least one CYP3A5*1 allele had higher plasma levels of endoxifen than those lacking functional CYP3A5 alleles.

The current investigation and earlier reports [Nowell et al. 2002; 2005], including paper I, indicate that patients who carry the SULT1A1*1, CYP2D6*4 and CYP3A5*3, which generate less ER active metabolites, might have benefit of tamoxifen. This gives rise to questions about the hypothesis that genotypes contributing to the biosynthesis of ER-active metabolites improve tamoxifen response.
Paper III

In situ levels of oestrogen producing enzymes and its prognostic significance in tamoxifen treated postmenopausal breast cancer patients
Barbara E. Licznerska, Pia P. Wegman, Anna Karlgren, Bo Nordenskjöld, and Sten Wingren

Manuscript

Results
Protein expression of sulfatase and SULT1A1 was detected in the cytoplasm of breast cancer cells and aromatase protein expression was found in the cytoplasm of both carcinoma cells and stromal cells. We also measured the mRNA expression of aromatase and sulfatase but there were no correlation between mRNA and protein expression of these enzymes.

We then evaluated if the protein and/or mRNA expression levels of aromatase, sulfatase and SULT1A1 were associated with tumour characteristics. The mRNA expression of these enzymes did not correlate with any of the tumour characteristics. Protein expression of cytoplasmic aromatase showed a significant correlation with tumour size, lymph nodes and S-phase fraction (Table 1). Next, we assessed whether the different expression parameters were related to the risk of having a relapse. Patients with high protein expression of aromatase in the stroma had a significantly decreased risk of recurrence (RR = 0.50, 95 % CI = 0.33-0.76, P = 0.003). No significant differences could be seen among the other expression parameters, neither for mRNA nor for protein expression.
<table>
<thead>
<tr>
<th>Tumour characteristics and protein expression of sulfatase, aromatase, and sulfotransferase SULT1A1 in breast cancer patients calculated with Pearson Chi-square (unless otherwise specified).</th>
</tr>
</thead>
<tbody>
<tr>
<td>§Weak or high staining was grouped together because of few cases for separate analysis.</td>
</tr>
<tr>
<td>*Pearson's correlation (R).</td>
</tr>
</tbody>
</table>
In the Kaplan-Meier estimates, including ER-positive and ER-negative patients, cases with weak or high protein expression of stroma aromatase had a significantly better prognosis than those with negative expression ($P = 0.0008$) (Figure 16). The improved prognosis was even more obvious when we combined patients with weak and high expression of stromal aromatase and selected only ER-positive patients ($P = 0.0000$) (Figure 17). In a multivariate Cox-model high stromal aromatase was demonstrated to be an independent significant factor, at least in ER-positive patients ($HR = 0.27$, $95\% CI = 0.09-0.83$, $P = 0.022$). The protein expression of cytoplasmic sulfatase, aromatase and SULT1A1, as well as the mRNA expression of aromatase and sulfatase revealed no prognostic significance, even though patients with a high protein expression of sulfatase showed a trend for a better recurrence-free survival ($P = 0.09$).

The predictive value regarding the expression levels of aromatase, sulfatase, and SULT1A1 have to be performed in a larger patient cohort since the subgroup randomised to 2 vs. 5 years of tamoxifen therapy comprised a limited number of patients.

**Figure 16** - Recurrence-free survival in patients with immunohistochemical staining of stromal aromatase. $P$-value between negative and weak staining was $0.0001$, $P$-value between negative and strong staining was $0.08$, and $P$-value between weak and strong staining was $0.33$.

**Figure 17** - Recurrence-free survival in ER-positive patients with immunohistochemical staining of stromal aromatase. Patients with weak and strong staining was combined and compared to those with negative staining.
Discussion

We investigated the protein and/or mRNA expression of aromatase, sulfatase, and SULT1A1. Immunoreactivity of aromatase was found both in stromal cells surrounding the tumour cells but also in tumour epithelial cells, which is in agreement with what others have seen [Esteban et al. 1992; Lu et al. 1996]. Moreover, similar to Yamamoto et al. [2003], sulfatase expression was found in the cytoplasm of breast cancer cells. The immunoreactivity of SULT1A1 was found in the cytoplasm of carcinoma cells and to the best of our knowledge, SULT1A1 protein expression has not previous been studied in breast cancer tumours. However, Richard et al. [2001] examined SULT1A1 protein expression in fetal livers, although by another antibody. Similar to our findings they described cytoplasmic immunoreactivity of SULT1A1. Interestingly, in ER-positive patient’s stromal aromatase was demonstrated to be an independent significant factor showing that patients with high or weak expression had an improved recurrence-free survival. Previous reports on aromatase and clinical outcome in breast cancer have though been controversial [Girault et al. 2002; Salhab et al. 2006], however, Yamamoto et al. [2003] partly support our findings by their conclusion that patients who had tumours expressing aromatase showed a longer time to relapse compared to patients lacking aromatase. Similar results have also been found by Yoshimura et al. [2004].

Finally, the results on aromatase and sulfatase immunoreactivity were contrary to our hypothesis where those with high expression were expected to relapse earlier as they retain the capacity of oestrogen synthesis given the tumour an advantage to grow. In an attempt to explain this, we speculated that since all patients received tamoxifen the increased levels of aromatase and/or sulfatase may reflect the oestrogen dependency of the tumour and the improved prognosis might be the consequence of tamoxifen response.
Paper IV

p53 polymorphic variants at codon 72 and the outcome of therapy in randomized breast cancer patients
Pia Wegman, Olle Stål, Marie Stenmark Askalm, Bo Nordenskjöld, Lars-Erik Rutqvist, and Sten Wingren
Pharmacogenetics and Genomics 2006, 16: 347-351

Results
We first examined the total population, including both ER-positive and ER-negative patients, to see if the codon 72 polymorphism was of prognostic importance. In all statistical analyses patients harbouring at least one Pro allele were grouped together. Results showed that there were no differences between genotypes and clinical outcome. When we calculated the benefit of tamoxifen only ER-positive patients were included and we compared tamoxifen vs. no tamoxifen for each genotype group. We found that patients carrying at least one Pro72 allele had better survival when treated with tamoxifen compared to those not receiving tamoxifen (P = 0.0033) (Table 2, Figure 18). Among individuals homozygous for the Arg72 allele the outcome was almost equal between tamoxifen treated and untreated patient

Table 2 - The hazard ratio (HR) of distant recurrence by Cox analyses according to genotype of codon 72 polymorphism of the p53 gene in oestrogen receptor positive patients randomised to either tamoxifen or no tamoxifen (TAM+/TAM-).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>TAM-</th>
<th>TAM+</th>
<th>P value, CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg72/Arg72</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of patients</td>
<td>33</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>Number of recurrences</td>
<td>15</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>HR = 1.0</td>
<td>HR = 1.2</td>
<td>0.65, CI = 0.61-2.2</td>
<td></td>
</tr>
<tr>
<td>Arg72/Pro72+/Pro72/Pro72</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of patients</td>
<td>40</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Number of recurrences</td>
<td>25</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>HR = 1.0</td>
<td>HR = 0.28</td>
<td>0.0033, CI = 0.12-0.65</td>
<td></td>
</tr>
</tbody>
</table>

The test for interaction demonstrated that the risk reduction of distant recurrence with tamoxifen was significantly higher in patients harbouring the Pro72 allele (P = 0.0088).
To estimate if there were any differences in hazard ratios between the genotypes and tamoxifen randomisation, an interaction test was performed. The interaction demonstrated that the risk reduction of distant recurrences was significantly higher in patients harbouring the Pro allele. These results indicate that tamoxifen therapy seems to be more beneficial for patients homo- or heterozygous for the Pro allele.

**Discussion**

p53-dependent apoptosis is an important mechanism through which DNA-damaging anticancer agents exert their biological effects [Lowe et al. 1993; 1994]. The codon 72 polymorphism of p53 has been known since the mid 1980’s, but it was not until recently the two variants were shown to differ both biochemically and biologically [Thomas et al. 1999; Dumont et al. 2003; Pim and Banks 2004]. Several reports have currently addressed the importance of this polymorphism in cancer predisposition, clinical outcome and therapeutic response, although results have been controversial [Papadakis et al. 2000; Goode et al. 2002; Sullivan et al. 2004; Tommiska et al. 2005; Xu et al. 2005; Santos et al. 2006].
In paper IV, we found that the p53 codon 72 polymorphism significantly influenced anti-oestrogen response in randomised breast cancer patients. The results demonstrated a better relapse-free survival in patients harbouring the Pro-allele if they were treated with tamoxifen. This was not the case in Arg homozygous patients. To the best of our knowledge, this is the first report concerning the p53 codon 72 polymorphism and the prediction of tamoxifen response in breast cancer. There have though been several reports on different tumour types and their response of chemotherapy and most results have pointed out a beneficial effect in homozygous carriers of the Arg-allele [Sullivan et al. 2004; Xu et al. 2005; Santos et al. 2006]. In addition, Bonafè et al. [2003] studied the p53 codon 72 polymorphism in 67 breast cancer patients who underwent therapy with either chemotherapy or tamoxifen. They found that retention of the Arg-allele was associated with reduction of both disease-free and overall survival in Arg/Pro heterozygous patients. Few studies have reported beneficial effects correlated to the Pro-allele, however, Goode et al. [2002] analysed data from a large population based study investigating common polymorphisms in several genes, including the codon 72 polymorphism in p53, in order to determine if these genes were linked to clinical outcome in breast cancer. For the genetic variants in p53 they demonstrated borderline significance for a decreased risk of death in patients homo- or heterozygous for the Pro-allele.

It can be questioned why it should be preferable to carry the Pro-allele since the Arg-allele has been shown to induce apoptosis more efficiently. Since mutations in p53 are thought to be the most common genetic alteration in human tumours and there are reports showing that hot spot mutations in p53 selectively occurs on the Arg-allele compared to the Pro-allele in breast cancer [Marin et al. 2000; Langerod et al. 2002], one explanation could be that Arg homozygous patients not responding well to tamoxifen might have p53 mutations. Among our patients 36 out of 220 had mutations in p53 although data on allele specificity were not available. Furthermore, mutations in the Arg- allele of p53 are more common in heterozygous individuals, and Marin et al. [2000] reported that certain mutations in the Arg- allele within Arg/Pro heterozygous patients enhanced the interaction with, and consequently impaired the function of the p53 family member p73. The p53 homologue p73 has been shown to be able to bind to canonical p53 binding sites and activate the transcription of p53 target genes, at least when overproduced, and inhibit cell growth in a p53-like manner by inducing apoptosis [Jost et al. 1997].
Conclusions

- Genetic variation of enzymes involved in the metabolic route of tamoxifen seems to influence treatment response in ER-positive postmenopausal breast cancer patients

- The current investigation indicate that patients carrying genotypes generating less ER-active metabolites (i.e. CYP2D6*4, CYP3A5*3, SULT1A1*1) have benefit of tamoxifen

- The protein expression of stromal aromatase had a prognostic significance, particularly in ER-positive patients, showing that individuals with weak or high expression had a significantly improved recurrence-free survival

- The p53 codon 72 polymorphism may be a predictive factor of tamoxifen response. ER-positive patients harbouring at least one Pro-allele had a significantly decreased risk of recurrence when treated with tamoxifen. Cases homozygous for the Arg-allele might therefore be candidates for therapy with other antioestrogens.
Acknowledgements

I would like to take the opportunity to express my sincere gratitude to everyone that has contributed to this thesis. Especially I would like to thank;


My supervisor Sten Wingren, for introducing me to the fascinating world of cancer research and for your great scientific knowledge and excellent guidance during these years. For lots of interesting and inspiring discussions and for always taking your time whenever I needed your advice.

Professor Bo Nordenskjöld, and Professor Olle Stål for their great scientific knowledge and generous support.

Professor John Carstensen, and Johan Rosell for their guidance during the statistical calculations.

My co-authors for collaboration and scientific discussions.

Malin Bergman, my previous room-mate for accepting me in the research group and for lots of nice discussions both scientific and non-scientific.

Agneta Jansson, for sharing your scientific and methodological knowledge.

Piiha-Lotta Jerewall, for all the help and for sharing your great computer knowledge, lots of good advice and last but not the less, for your “hawk eyes”.

All the people at the Division of Oncology, Gizeh Perez-Tenorio, Karin Söderlund, Marie Ahnström, Birgit Olsson, Åsa Wallin, Daniella Pfeiffer, Andreas Lewander, Jing Fang Ghao, Xiao Feng Sun, Annica Knutsen, Josefin Bostner, Marie Stenmark Askmalm for good cooperation, discussions, and for nice dinners.

My colleagues and friends at the Division of Cell Biology for a friendly atmosphere, nice discussions and for the weekly happening: “fredagsfika”. Lotta Harnevik, for all interesting discussions (including everything in life), and for your wise and nice personality. I hope (know) that some day you will be a professor. Annelie Karlsson for all our discussions (both scientific and non scientific) and I appreciate our
friendship. I hope you will be back soon. Lena Thunell for your great knowledge about almost everything and for your kindness. I would like to wish you good luck with your position as a post doctoral fellow and with everything else in life. Åsa Schippert and Anette Molbaek, for your outstanding knowledge about laboratory things and for always being so friendly and helpful. Patiyan Andersson, the guy with a great sense of humour! I wish you good luck with your research and everything else that is important in life. Just to remind you, “DNA är inte rakt”!! Cecilia Gunnarsson, for sharing your scientific and clinical knowledge, and for always being friendly and open minded. Nashwan Jalal Marcus, for being my new friendly roommate and a good collaborator. I hope we will have more time for discussions later on, when I’m not that busy. Pernilla Eliasson, Amanda Nordgården, Pia Druid, Jan-Ingvar Jönsson, Jonas Ungerbäck, Deepti Verma, Nils Elander, Ahmad Ahmadi, Linda Vainikka, Karin Fransén, Peter Söderkvist, Sigrun Liedgren, Jon Jonasson, Catarina Trofast, Gunnel Karlsson, Birgitta Persson, Lena Tudegård for good company and friendly chats during lunches and coffee brakes.

Cecilia Trinks for good company during the student laborations, for fruitful discussions and for being my friend.

Birgitta Holmlund and Lilian Ferraud, for all your help and for being so nice and friendly.

My dear family;  
My mother Deris Palmebäck for your endless love and support. My father Curt Palmebäck, I wish you were with us now, but I know that we will see each other in “Nangijala”, and in that time I will tell you… Peter Palmebäck (and his family) for just being my very best big brother. Have you been studying the area of breast cancer properly? I expect you to ask questions you know… My little sister Lisa Palmebäck Karlsson (and her family) for your peptalk, all discussion about nothing and everything, and for just being my sister.

My mother in law Viviann Wegman for being a warm and kind person, and for your endless encourage.

Stefan, the love of my life, for always being there for me and for your endless love and support. But watch out! I’m going to beat you in golf this season…; )

Joachim, Patrik and Emil, my beloved wonderful children for bringing energy and sunshine in life, and for your patience during my “never ending” studies. Hopefully, this was the last big challenge.


Musgrove, E.A., Lee, C.S., Buckley, M.F. and Sutherland, R.L. Cyclin D1 induction in breast cancer cells shortens G1 and is sufficient for cells arrested in G1 to complete the cell cycle. Proc Natl Acad Sci U S A 1994; 91(17):8022-8026.


