

# Tamoxifen in the treatment of luminal breast cancer

Implications of active metabolites on gene expression, side effects and clinical outcome

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Thomas Helland

Avhandling for graden philosophiae doctor (ph.d.)  
Universitetet i Bergen  
2019

UNIVERSITETET I BERGEN



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## **Scientific environment**

The present work was conducted in the period January 2016 to December 2018 at the Hormone Laboratory at Haukeland University Hospital and Department of Clinical Science, University of Bergen.

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I want to dedicate this thesis to my mother Eva, who is a breast cancer survivor, and to my father Øyvind, who lost his life to lung cancer in 2002.

## Abbreviations

4OHTam	4-hydroxy-tamoxifen
AF	Activation function
AI	Aromatase inhibitor
BCSS	Breast cancer specific survival
BRCA	Breast cancer gene
CK6	Cytokeratin 6
Cp	Crossing point
CYP	Cytochrome P 450
DBD	DNA-binding domain
E2	Estradiol
Endoxifen	4-hydroxy-N-desmethyl-tamoxifen
ER	Estrogen receptor
ERE	Estrogen response element
FACT-ES	Functional Assessments of Cancer Therapy-Endocrine subscale
FC	Fold change
FDR	False discovery rate
GG	Genetic grading
HER-2	Human EGF receptor-2
HG	Histological grading
IHC	Immunohistochemistry
LBD	Ligand-binding domain
LC-MS/MS	Liquid chromatography tandem mass spectrometry
NDtam	N-desmethyl-tamoxifen
NorPD	Norwegian prescription database
NR	Nuclear receptor
PR	Progesterone receptor
QDP	Quadrupole
QoL	Quality of life
Q-rt-PCR	Quantitative real-time polymerase chain reaction
SAM	Significance analyses of microarray

SERD	Selective estrogen receptor degrader
SERM	Selective estrogen receptor modulator
shRNA	Short hairpin knockdown
SiRNA	Silencing RNA
SNP	Single nucleotide polymorphism
SRC	Steroid receptor coactivator
TamNoX	Tamoxifen-N-oxide
TDLU	Terminal ductal lobular unit
VVA	Vulvovaginal atrophy
Z-4Ohtam	Z-geometric isomer of 4Ohtam
Z-endoxifen	Z-geometric isomer of endoxifen



## Abstract

Estrogen is a driver for the development and progression of luminal breast cancer, and up to 80% of all breast cancers diagnosed belong to the luminal subtype. The estrogenic effect on breast tumors can be reduced by the adjuvant use of the anti-estrogenic drug tamoxifen, which has significantly improved outcomes in luminal breast cancer patients over the last several decades. The 10-year recurrence rate in luminal breast cancer patients is approximately 25%. Thus, some patients do not achieve the desired curative effect of adjuvant tamoxifen. Tamoxifen is regarded as a weak anti-estrogen, and the anti-estrogenic effect is believed to be dependent on its active metabolites endoxifen and 4OHTam, which have up to 100-fold higher affinities to the estrogen receptor (ER) compared to tamoxifen itself. Worryingly, a significant subgroup of patients has a reduced ability to metabolize the drug, which translates into lower concentrations of the important active metabolites. Studies have indicated that these patients derive suboptimal therapeutic effects from tamoxifen. In addition, a large number of patients using tamoxifen discontinue treatment before the pre-determined treatment time, which may leave these patients at higher risk of relapse.

Therefore, increasing knowledge about the function of and determining possible therapeutic thresholds for the active metabolites represent promising potential progress towards individualized tamoxifen treatment and thereby improve patient outcomes. Identifying biomarkers that predict tamoxifen discontinuation may also be used to further tailor individual treatment.

In paper I, we performed microarray global gene expression analyses on NDtam, 4OHTam and endoxifen-treated MCF-7 cells to elucidate the gene regulative roles of these metabolites. Global gene expression analyses revealed a step-wise regulation of genes in which endoxifen and 4OHTam resulted in the strongest and second strongest regulation of both up- and down-regulated genes, respectively. The change in global gene expression after treatment with NDtam was minimal. The two active metabolites regulated genes in similar gene ontology classes, implying a degree of similar function between the two metabolites. We also provided evidence for all three isoforms of

*CytoKeratin6* (CK6) being estrogen regulated, making CK6 a potential anti-proliferative target of tamoxifen that should be researched further.

In paper II, we retrospectively compared the predictive value of CYP2D6 phenotype groups and concentrations of active tamoxifen metabolite on long-term clinical outcome. Blood and serum from 99 operable breast cancer patients treated with 5-year adjuvant tamoxifen were analyzed for *CYP2D6* genotypes and concentration levels of active tamoxifen metabolites, respectively. CYP2D6 phenotypes were found to be correlated to Z-endoxifen and Z-4OHTam concentrations. However, Kaplan-Meier analyses showed that CYP2D6 phenotypes were not associated with breast cancer specific-survival (BCSS). The same analyses were repeated using concentrations of tamoxifen metabolites; patients with concentrations below 9 nM and 3.26 nM for Z-endoxifen and Z-4OHTam, respectively, had significantly worse BCSSs compared to patients with concentrations above these cut-off points. When we included a third cut-off point, representing patients with high concentration levels found in CYP2D6 ultra-rapid metabolizers, we found that these patients had no BCSS endpoints up to 19 years after surgery. The BCSS results from these three groups also translated into overall survival. Our findings indicate that the concentrations of active tamoxifen metabolites may be used to predict the therapeutic effects of tamoxifen.

In paper III, we investigated the association between tamoxifen metabolites and side effects in 220 patients that delivered blood samples and patient reported outcome measures yearly from 2011 to 2017. Analyses of side effects revealed hot flashes, vaginal atrophy symptoms, and joint pain to be most prevalent. Association analyses demonstrated that patients with high levels of tamoxifen, Z-4'OHTam and tamNoX were more likely to report vaginal dryness than patients with lower levels. Our secondary objective was to compare discontinuation rates obtained through the Norwegian prescription database to rates determined by longitudinal drug monitoring of systemic tamoxifen concentrations. Drug monitoring showed that only 6% of patients were not taking tamoxifen during a period that was covered by a tamoxifen prescription. Our results indicate that drug monitoring of tamoxifen metabolites may be used as biological predictor of vaginal dryness and that discontinuation rates obtained through pharmacy refill registries represent valid results.

## List of publications

**Helland T**, Gjerde J, Dankel SN, Fenne IS, Skartveit L, Drangevåg A, Bozickovic O, Fågeng MH, Søiland H, Mellgren G, Lien EA. (2015). The active tamoxifen metabolite endoxifen (4OHNDtam) strongly down-regulates cytokeratin 6 (CK6) in MCF-7 breast cancer cells. PloS one, 10(4), e0122339.

**Helland T**, Henne N, Bifulco E, Naume B, Borgen E, Kristensen VN, Kvaløy JT, Lash TL, Alnæs GIG, van Schaik RH, Janssen EAM, Hustad S, Lien EA, Mellgren G, Søiland H. (2017). Serum concentrations of active tamoxifen metabolites predict long-term survival in adjuvantly treated breast cancer patients. Breast Cancer Research, 19(1), 125.

**Helland T**, Hagen KB, Haugstøyl ME, Kvaløy JT, Lunde S, Lode K, Lind AL, Gripsrud HG, Gjerde J, Bifulco E, Hustad S, Jonassen J, Aas T, Lende TH, Lien EA, Janssen EA, Søiland H, Mellgren G. (2018) Drug monitoring of tamoxifen metabolites predicts vaginal dryness and verifies a low tamoxifen discontinuation rate from the Norwegian Prescription Database (NorPD) in a real-world data set of breast cancer patients. Manuscript.

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# 1 Introduction

## 1.1 Tumorigenesis and the hallmarks of cancer

Cancer is a vastly diverse and heterogeneous disease with over 100 different types arising from a range of tissues and cell types within the body. The various cancer types grow and behave differently, and cancer is therefore often referred to as a “collection of diseases.” Yet, there are common traits that are shared by all cancers, described by Douglas Hanahan and Robert A. Weinberg as the hallmarks of cancer (Fig. 1) (1, 2).



**Figure 1. Hallmarks of cancer.** Edited from Hanahan and Weinberg (1, 2).

## **HALLMARKS OF CANCER**

**Genome instability and mutations**, changes in genes are caused by accumulation of mutations through enhanced sensitivity to mutagenic agents and/or attenuating mutations of genes involved in controlling growth and proliferation.

**Tumor promoting inflammation**, release of growth factors and mutagenic chemicals into the tumor microenvironment by immune cells can fuel mutagenesis and proliferation of the cancer cells.

**Ability to sustain proliferative signaling**, cancer cells can become self-sufficient in growth factors by abnormal autocrine growth factor signaling and/or acquire increased sensitivity to growth factor stimulus. Cancer cells may also stimulate normal cells to produce growth factors and/or become independent of growth factors by consecutive activation of downstream signaling pathways.

**Ability to evade growth suppressors**, cancer cells exhibit inactivating mutations in tumor suppressors that halt the progression of the cell cycle in response to growth inhibitory signals, DNA damage, stress and other abnormalities.

**Ability to resist apoptosis**, cancer cells survive through loss of tumor suppressors, down-regulation of pro-apoptotic factors, up-regulation of anti-apoptotic and survival signals, and/or disruption of extrinsic ligand-induced apoptosis pathways.

**Ability for unlimited replication potential**, up-regulation of telomerase, a DNA polymerase that adds telomere repeats to the telomeric DNA, allows cancer cells to continue dividing without shortening of telomeres. Also, in the absence of p53 some cancer cells may continue dividing with eroded telomeres and commence chromosomal breakage-fusion-bridge cycles resulting in massive chromosomal alterations.

**Inducing angiogenesis**, a normally strictly regulated process can be constantly “turned on” in cancer by deregulation of signaling proteins including vascular endothelial growth factor-A (VEGF-A) and thrombospondin-1 (TSP-1) thereby providing a constant flow of nutrients and draining of waste products to and from the tumor.

**Activating invasion and metastasis**, a multi-step process that frequently starts with the loss of cell-to-cell or cell-to-matrix adhesion molecules such as E-cadherin allows for local invasion. Further steps include the intravasation of cancer cells into blood or lymph vessels facilitating transport to distant sites where the cells can escape the vessels through extravasation and form micro metastases. An important facilitator of this process is the epithelial to mesenchymal transition (EMT) facilitated by the expression of developmental transcription factors like the Snail, Slug and Twist. The last step is colonization in which the micro metastases grow into new tumors distant from the primary tumor.

**Ability to reprogram energy metabolism**, surprisingly, most cancer cells apply the cytoplasmic glycolysis instead of mitochondrial oxidative phosphorylation, a far less efficient method of producing energy (2 vs. 38 ATP). It is believed that glycolysis allows for improved generation of nucleosides and amino acids, important components when producing new cells. Certain areas of the tumor may also be hypoxic, requiring anaerobic energy production through glycolysis.

**Ability to evade destruction by immune cells**, normally, the immune system will destroy incipient cancer cells, however natural selection towards weakly immunogenic cancer cells over time is believed to create cancer cells that avoid detection by immune cells. Other mechanisms for avoiding immune detection include secretion of immunosuppressive factors and recruitment of immune cells that suppress the action of cytotoxic lymphocytes.

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**Table 1. Hallmarks of cancer.** The first two hallmarks are enabling characteristics.

Adapted from (1, 2).

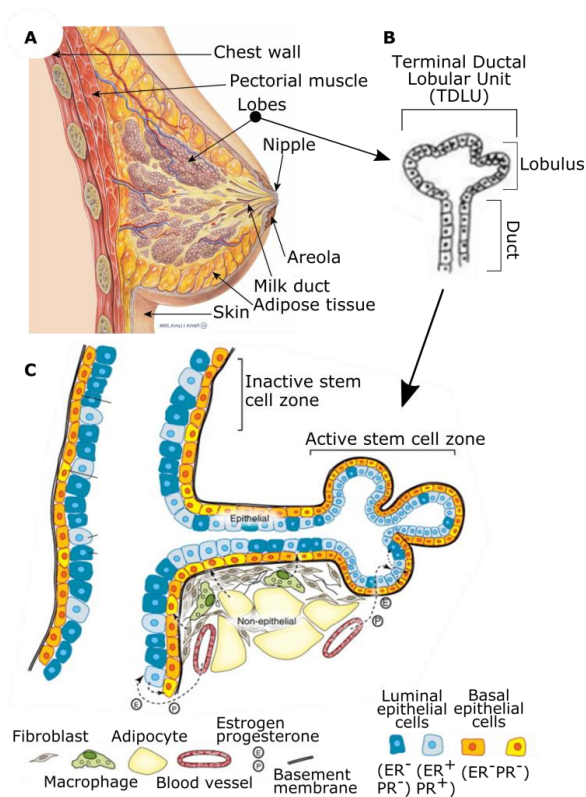
## **1.2 Breast cancer**

### **1.2.1 Breast anatomy**

The female breast is an exocrine modified apocrine sweat gland overlying the pectoral muscles, predominantly consisting of glandular and duct epithelium and adipose and connective tissues (Fig. 2A). From the formation of the epidermal breast bud, commencing in the 7th gestational week (3), to old age, the breast is constantly undergoing changes due to ever-changing estrogen and progesterone levels throughout life (4). Under the influence of increased estrogen and progesterone production in puberty, the stem cells in the terminal ductal lobular unit (TDLU) of the breast bud (Fig. 2B and C) initiate sprouting and growth of parenchymal breast tissue into the adjacent connective and adipose tissues (3). There are two major types of stem cells in the TDLU: luminal stem cells and basal stem cells. Luminal stem cells are orientated towards the lumen of the apocrine glandular alveoli and the lumen of the terminal ducts of the breast. These are cells that predominantly express ERs and progesterone receptors (PRs), or ER+/PR+ cells. Basal stem cells are located between the luminal cells and the basement membrane (Fig. 2C). These stem cells are ER-/PR- and are also the origin of the myoepithelial cells, which comprise smooth muscular fibers that can contract and squeeze excreted milk from the alveoli through a network of lobules, lobes and ducts to the nipple. The lobes and ducts are surrounded by fibroblasts, macrophages, and adipose tissue that make up the microenvironment of the TDLU. The ratio between connective, adipose, and glandular tissues shows substantial individual variation. In addition, this ratio is highly influenced by age. In the postmenopausal breast, an involution of glandular tissue and incremental increase of adipose tissue occur. A high ratio of glandular and connective tissue compared to adipose tissue is described as high breast density, a condition associated with increased risk of developing breast cancer (5). Lymphatic vessels of the breast drain to two major lymph node locations: the intra-pectoral and the axillar locations. The latter route is the most accessible during surgery, and in a normal breast, the average number of lymph nodes in each axilla is 12 - 15. The presence of tumor cells in lymph nodes–



the first recipient lymph node is known as the sentinel node—is an important prognostic marker in breast cancer and is used in the staging of the disease (6).



**Figure 2. Breast anatomy.** A) Gross anatomy of the breast. Artwork by Patrick J. Lynch, medical illustrator. B) Illustration of branching of the ductal system down into the TDLU. Modified from (7). C) Overview of various stem cells, luminal and basal cells in the TDLU and the various cells in the microenvironment of the TDLU. ER, estrogen receptor; PR, progesterone receptor; TDLU, terminal ductal lobular unit. Modified from (8).

### 1.2.2 Breast cancer epidemiology

Breast cancer is the most frequent form of cancer for women throughout the world, with over 1.6 million incidences registered in the latest update from 2012 (9). In the same year, over half a million breast cancer deaths were registered, making breast cancer the second most deadly form of cancer in the western world (9). Estimates of

the world-wide cancer burden for 2018 suggest over 2 million new incidences and 600 000 deaths from breast cancer (10). Breast cancer is also the most frequent form of cancer among Norwegian women, with 3371 new incidences in 2016. Approximately 1 in 12 women in Norway will develop breast cancer by the age of 75, and the median age of developing the disease is 62 (11). In Norway, breast cancer ranks third in mortality, behind lung and colon cancer, with 585 deaths in 2015 (not updated for 2016).

Despite the high number of deaths and increasing incidences, breast cancer mortality has been decreasing in the western world over the last several decades (9). The improvement in survival is attributed to earlier detection through screening programs and improvements in treatment (12). This trend has also been observed in Norway where from 1965 to 2015 there was an increase in incidence, but at the same time, an increase in the 5-year survival rate and a decrease in mortality (11).

### 1.2.3 Breast cancer etiology and risk factors

The lifetime risk of developing breast cancer for a Norwegian woman is about 10 % (11). The etiological and risk factors can be classified into four groups: genetic-, hormonal/reproductive-, lifestyle-, and environmental factors.

#### 1.2.3.1 Genetic factors

The strongest germline genetic factor is the female X sex chromosome. Being female (homozygote XX) is associated with a risk of developing breast cancer 100 times greater than being male (XY) (13), and this increased risk is attributed to the higher estrogen levels in women compared to men. Besides sex chromosomes, approximately 5-10% of all breast cancers are caused by germline mutations in somatic chromosomes. Founder mutations in breast cancer genes 1 and 2 (*BRCA1*, 2) on the 17th and 13th chromosome, respectively, are high-penetrant gene mutations, leaving a woman who has a *BRCA-1* mutation with a 45-75% lifetime risk of developing breast cancer (14). Mutations in *TP53*, *PTEN*, *STK11* and *CDHI* have also been associated with increased risk of breast cancer (14, 15).

### 1.2.3.2 *Endocrine factors*

Three types of sex-hormones, estrogens, progesterone, and androgens all influence the risk of acquiring breast cancer. However, estrogen is the most important factor due to its dual carcinogenetic action: mutagenic effects and activation of ERs. The mutagenic effect is caused by DNA adducts that are formed when DNA is bound by oxidative metabolites of estrogen. These DNA adducts can cause DNA damage through depurination of adenine and guanine, oxidative damage, and alkylating reactions, all of which can lead to mutations (16). Stem cells in TDLUs are under constant influence of estrogens throughout the fertile years and are therefore at risk of accumulating mutations created by endogenous estrogens and exogenous mutagen factors. The second carcinogenic action of estrogens is mediated through the activation of ERs, which leads to alterations in the transcription of over 1000 genes (17). The cellular effects translate into increased proliferation, reduced apoptosis, and increased growth via activation of PR production (16).

Reproductive factors that increase the endogenous estrogen influence on stem cells are early menarche, late menopause, late 1st pregnancy, lack of breastfeeding and nulliparous status (never given birth). Postmenopausal obesity is also associated with higher levels of endogenous estrogens as conversion of adrenal androgens to estrogens occurs in adipose tissue. An exogenous factor is prolonged hormone replacement therapy (estrogens and progestins). Oral contraceptive use is a putative factor, but results from various studies are ambiguous (13).

### 1.2.3.3 *Environmental and lifestyle factors*

Chemical agents with estrogen-like action, such as polychlorinated biphenyls (widely used in the past in cooling liquids for electric apparatuses) and pesticides like DDT, have been shown to cause mutagenic effects through the formation of DNA adducts (18). Similar to estrogen, they are able to bind to ERs and activate transcription. Electromagnetic fields (19) and high exposure to ionizing radiation (20) are additional environmental factors which have been linked to increased risk of developing breast cancer. Lifestyle factors associated with increased breast cancer risk include the usual suspects such as high fat intake (21), high BMI (postmenopausals), and increased

alcohol consumption (18). A high level of physical activity, however, has been shown to have a protective effect (22).

#### 1.2.4 **Breast cancer predictive and prognostic factors**

Breast cancer is a heterogeneous disease and can be divided into four clinical subtypes based on predictive and prognostic markers (Table 1) (23). Prognostic factors are predictive of clinical outcome and prognosis independent of treatment, while predictive factors give information on probable patient response to treatment (24). Importantly, prognostic factors can only be determined in materials comprising treatment naïve patients (25). Nodal status and tumor size are prognostic factors determined by histopathological analyses of primary breast cancer samples. One of the most important microscopic features with substantial prognostic information is the histological grading (HG) system. It consists of an algorithm comprising three cellular features; percentage tubular formation (1-3 points), nuclear pleomorphism (1-3 points) and mitotic index (1-3 points). These add up to a scale from 3 to 9 points. A higher score indicates poorly differentiated cancer. A sum score of 3-5 is categorized as HG 1, 6-7 points as HG 2, and 8-9 as HG 3, and there are clear prognostic differences between these grades, with HG 1 representing the best prognosis and HG 3 the poorest prognosis. Interestingly, gene profiling of these three histological grades reveals that there are only two genuine genetic grading (GG) groups; genetic grade 1 and genetic grade 2. All HG1 cancers cluster in the GG1 group and all HG3 cluster in the GG2 group, while HG2 cancers are a mixture of both GG1 and GG2 cancers (26).

Predictive factors include expression levels of ER, PR, and the human epidermal growth factor-2 (EGFR2) also known as the human EGF receptor-2/neu (HER-2/neu). ER and PR status is assessed by immunohistochemistry (IHC) and HER2 status is determined by fluorescence in situ hybridization. These growth stimulatory receptors can be directly or indirectly targeted by drugs and their expression levels are predictive of response to these treatments. However, outcome studies of untreated patients show that these predictive factors are also prognostic, as HER-2 positive patients have poorer outcomes compared to HER-2 negative patients, and patients with high expression of ER have more favorable outcomes compared to ER-negative patients

(27). An additional factor that has mixed prognostic and predictive significance is the cell cycle protein Ki67. High Ki67 protein levels in cancer cells indicate high proliferation and poorer prognoses. But high Ki67 may also be predictive of better response to chemotherapy. However, Ki67 is somewhat controversial as its IHC measuring method has not been adequately standardized resulting in variations between laboratories, and no consensus on cut-off values has been established (28, 29). All these prognostic and predictive factors are routinely measured in the clinical setting, and their combinations are used to guide clinicians in their decisions on treatment for breast cancer patients (Table 1) (23, 24). In breast cancer, there exist over 100 prognostic factors, but few predictive factors. There is thus a need to establish more and reliable predictive factors (25).

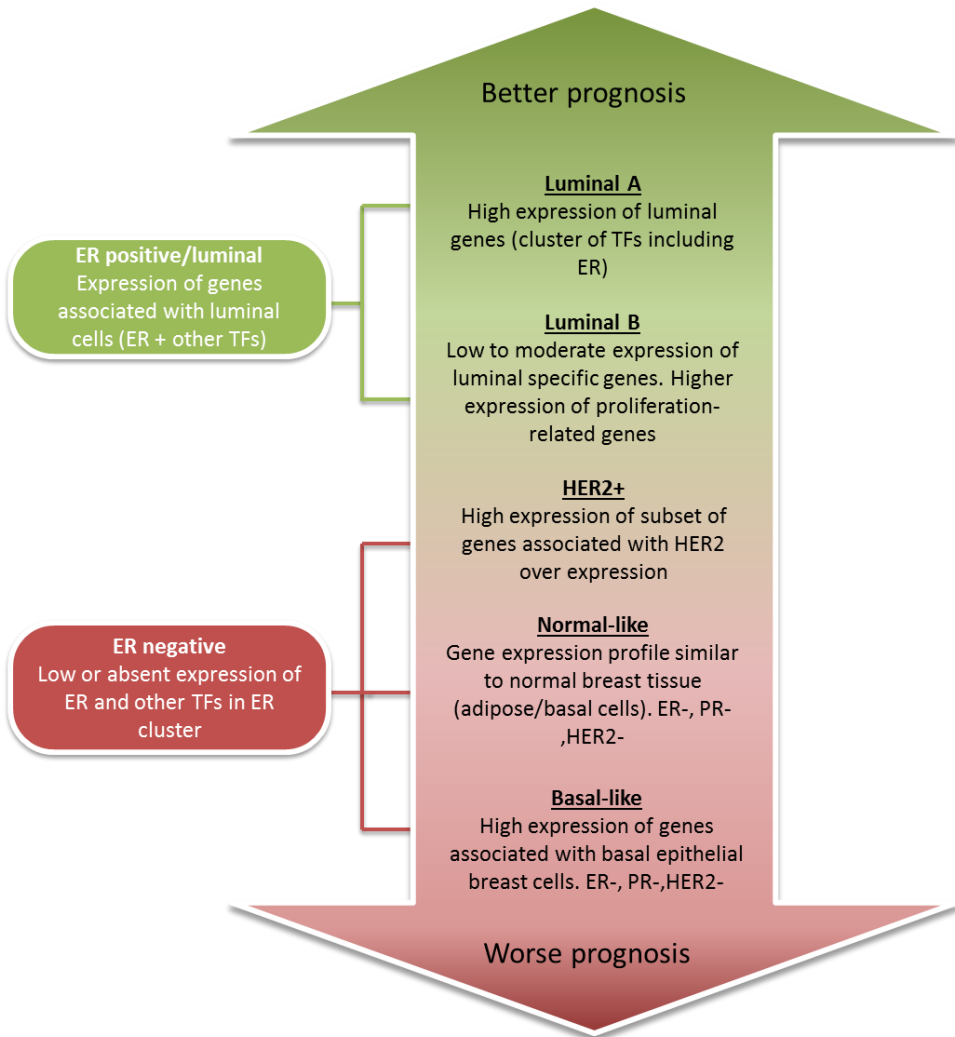
Clinical grouping (subtypes)	Characteristics	Systemic treatment options
Triple negative (ER-, PR-, HER2-)	ER-, PR-, HER2-	CT
HER2 type	HER2+, HR +/-	CT, anti-HER2 antibodies, ET
Luminal A-like	HER2-, HR+, high ER/PR and low Ki-67 or low grade	ET, (CT if stage>2)
Luminal B-like	HER2-, HR+, lower ER/PR, high Ki-67, grade 3	ET, (CT)

**Table 2. The four main subtypes used in the clinical setting.** ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor-2; HR, hormone receptor; CT, chemotherapy (either taxanes or Epirubicin and Cyclophosphamide); ET, endocrine treatment. Adapted from (23).

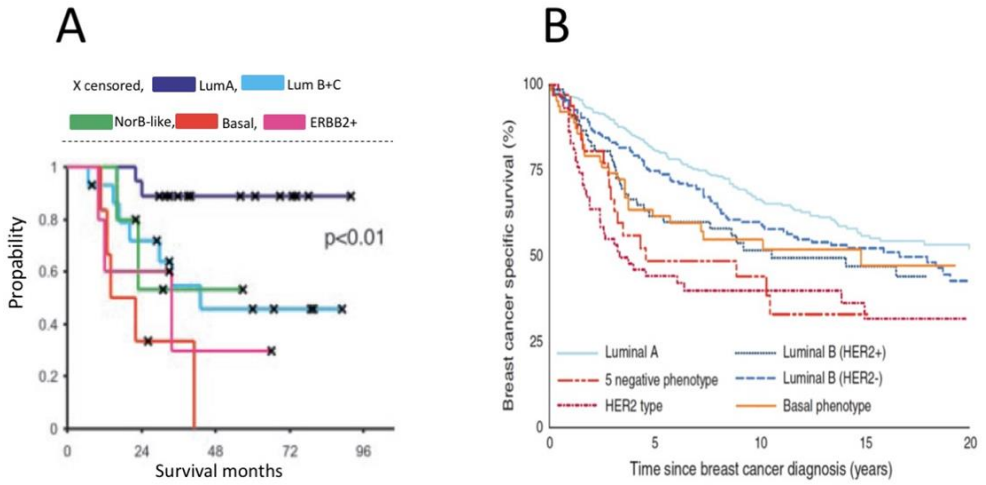
**1.2.5 Molecular subtyping of breast cancer**

The heterogeneity of breast cancer cannot be fully captured by the clinicopathological markers discussed above. In the early 2000s two microarray studies using gene sets that reflect the intrinsic properties of breast cancer tumors showed that breast cancer can be classified into molecular subtypes based on unsupervised hierarchal cluster analyses of the expression of 456 genes (30, 31) (Fig. 3 and 4A). These studies

showed that ER-negative and positive tumors are inherently distinct on a molecular level and comprise subgroups that are separated based on expression of genes associated with specific cell types (Fig. 3). In their pivotal study, Sørli and Perou demonstrated that the ER-negative tumors express genes associated with basal cells and adipose cells, while ER-positive tumors (discussed in more detail in sections below) express genes associated with luminal cells. The subtypes were also shown to be prognostically distinct in which the luminal subtypes exhibit the best prognosis and the basal subtype the worst after 5 years of follow-up (31) (Fig. 4A). However, a Norwegian study with 20 years follow-up using surrogate biomarkers to determine subtypes showed that the luminal subtypes are subject to late breast cancer events which leads to similar survival rates to the basal subtype at 20 years (27) (Fig. 4B). More recently, two additional subtypes have been suggested: the claudin-low (32) and the molecular apocrine (33) subtypes. Molecular portraits of tumors laid the foundation for the commercial gene signature assays, such as the MammaPrint, Oncotype Dx, and PAM50 (34-36), that are now used to clinically identify low-risk patients and avoid over-treatment (23, 37).



**Figure 3. Molecular subtypes of breast cancer.** Prognosis based on short-term follow-up (~5-years). TF=transcription factor, ER=estrogen receptor, PR=progesterone receptor, HER2=human epidermal growth factor-2. Adapted from (31).



**Figure 4.** **A.** Short-term relapse-free survival analysis comparing the six molecular subtypes described in an unsupervised gene expression analysis (31). **B.** Long-term breast cancer specific survival among molecular breast cancer subtypes (27). Notably, the survival curves of the luminal subtypes have a negative slope of all the way down to 20-years follow-up. Thus, they have a good short-term survival (5 years), but a considerable worse long-term survival (20 years). HER2, human epidermal growth factor-2.

### 1.3 The luminal subtype of breast cancer

#### 1.3.1 Molecular characteristics of luminal breast cancer

The luminal subtype of breast cancer is characterized by expression of a cluster of transcription factors including the following: the estrogen receptor  $\alpha$  (*ESR1*), X-box binding protein 1 (*XBPI*), GATA-binding protein 3 (*GATA3*), hepatocyte nuclear factor 3 $\alpha$  (*HNF-3 $\alpha$* ), and increased expression of genes normally expressed in breast luminal epithelial cells (30). The luminal subtype is further divided into luminal A and luminal B based on the level of expression of the above-mentioned transcription factors. Luminal A tumors presents with higher expression of these genes compared to luminal B tumors. The luminal B subtype is further differentiated from luminal A by higher expression of proliferation-related genes such as *MKI67*, *CCNB1* and *MYBL2* (38), which clinically translates into more aggressive tumors with poorer prognoses



(39). In addition, luminal B cancer may or may not be HER2 positive while luminal A is always HER2 negative. In one study, a third subgroup, luminal C, was distinguished from A and B by its expression of a novel gene set with unknown function, which was also observed in ER-negative subtypes, but this could not be reproduced in later studies (39). Importantly, the luminal breast cancer subtypes comprise a special intrinsic biology, which extends into a characteristic survival pattern that comprise breast cancer related events during the whole course of the disease (27) (Fig. 4).

### 1.3.2 Nuclear receptors

Clinically, the most important characteristic of luminal subtypes is the expression of ER, a member of the nuclear hormone receptor (NR) family. This family of 48 evolutionarily related, ligand inducible transcription factors (40) regulates genes involved in development, reproduction, homeostasis, and differentiation (41). Structurally and functionally conserved domains of nuclear receptors include the DNA-binding domain (DBD) and the ligand-binding domain (LBD) (Fig. 5). The latter also incorporates domains responsible for nuclear localization, dimerization, and activating function (AF)-2. A ligand-independent activating function domain, AF-1, is found in the length and sequence variable N-terminal domain. Less conserved hinge regions connect the ligand-binding domain and the DNA-binding domains. Generally, binding of a ligand to the LBD leads to dimerization to other ligand-bound NRs, nuclear localization, and binding of the activated dimer to promoters or enhancers of target genes. Binding of a ligand also leads to change in the conformation of the AF-2, which allows binding of co-regulators. However, other mechanisms for the activation of NRs that differ from the general mechanism described above exist as well (42, 43).

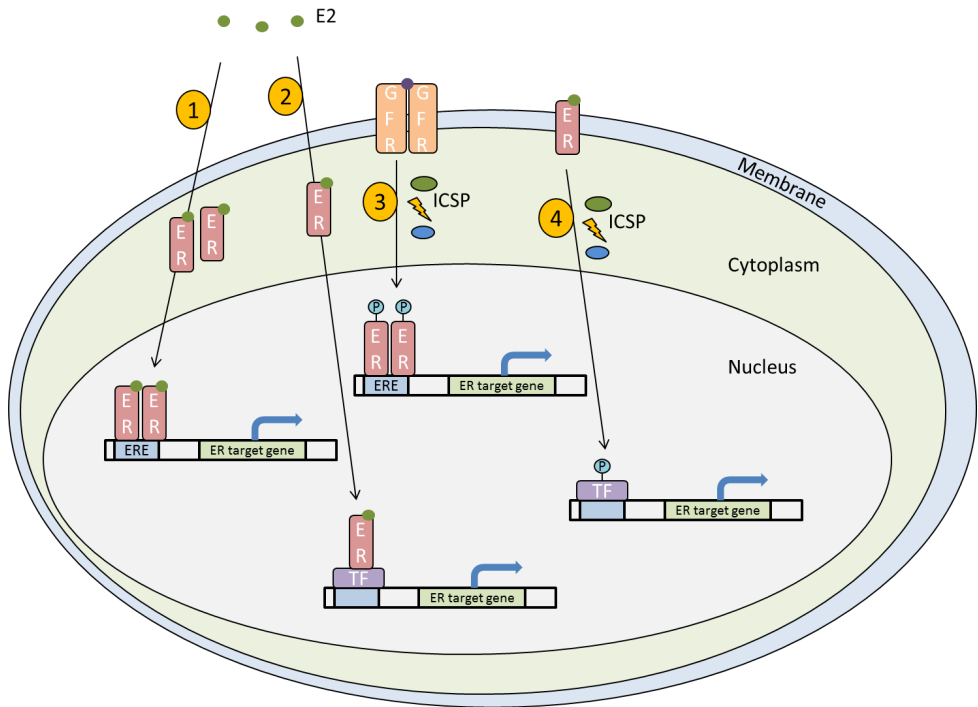


**Figure 5. Structural and functional domains in nuclear receptors.** The A/B domain contains the activating function (AF-1) domain; the C domain contains the DNA-binding domain and a dimerization domain; the D domain consists of the hinge region; the E domain contains the ligand-binding domain; and nuclear receptors may or may not contain the F domain, which is of unknown function and a region with variable sequences among the NRs. Modified from (44).

### 1.3.3 The estrogen receptor and its transcriptional mechanisms

The ER is a nuclear steroid hormone receptor that exists in two subtypes, ER $\alpha$  and ER $\beta$ . ER $\alpha$  was first discovered in 1967 by Elwood Jensen (45), and the gene (*ESR1*) encoding the receptor was later described in 1986 (46, 47), while ER $\beta$  was first described in 1996 (48). In addition, splice variations lead to existence of several isoforms of both ER $\alpha$  (n=7) and ER $\beta$  (n=5) (49-51). ER $\alpha$  and ER $\beta$  are highly conserved in structure in the DBD (97%) and less conserved in the LBD (55%), and expression levels of the two subtypes vary among tissue types. Both ER subtypes are expressed in luminal cancers and may form homodimers (ER $\alpha$ /ER $\alpha$ , ER $\beta$ /ER $\beta$ ) or heterodimers (ER $\alpha$ /ER $\beta$ ) upon binding of ligands. The role of ER $\beta$  in breast cancer development and progression is unclear, and its potential clinical value remains to be determined (52). ER $\alpha$  is, in contrast, overexpressed in over 70% of breast cancers, and luminal breast cancer progression is associated with deregulation of the ER $\alpha$  target genes involved in differentiation, proliferation, and migration (53). ER $\alpha$  transcription is activated through the classical NR activation pathway or three non-classical pathways (Fig. 6). The classical pathway involves the binding of 17 $\beta$ -estradiol (E2) to cytoplasmic ER $\alpha$ , followed by a conformational change causing the ER $\alpha$  to dissociate from inactivating chaperons. After separating from the chaperons, ER $\alpha$  form dimers with other ligand-bound ERs and translocate to the nucleus, binding to cis-regulatory elements on DNA called estrogen response elements (EREs). Importantly, the binding of E2 to ER induces the rotation of helix-12, which exposes binding sites, allowing for recruitment of co-activators (CoA) (54, 55) (Fig. 8A). The coactivators facilitate

recruitment of the transcriptional machinery to instigate transcription. ER $\alpha$  may also regulate the transcription of genes that do not have EREs (56, 57). In this non-classical pathway, E2-activated ER $\alpha$  bind to transcription factors associated with their target promoters and facilitate the transcription of genes that lack ERE. In a second non-classical pathway, growth factor stimulation and the consequent activation of their downstream signaling pathways may activate ER $\alpha$  in a ligand-independent manner through phosphorylation of ER $\alpha$  by down-stream kinases (58). The third non-classical pathway involves ER $\alpha$  transcriptional regulation in a non-genomic manner. In this scenario, membrane-bound ER $\alpha$  can be activated by E2 and activate proteins of other signaling pathways, thereby indirectly regulating transcription of the pathway's downstream targets (58). Adding to the complexity, the transcriptional activity of ERs can be further regulated by interaction with co-regulators (corepressors and coactivators) (59) and post-translational modifications including SUMOylation, phosphorylation, acetylation, and ubiquitination (60).



**Figure 6. Activation of ER transcriptional regulation.** 1) Classical NR activation pathway. 2) ERE-independent pathway. 3) Ligand-independent pathway. 4) Non-genomic pathway. ICSP, intracellular signaling pathway; TF, transcription factor; ER, estrogen receptor; ERE, estrogen response element; E2, 17 $\beta$ -estradiol; GFR, growth factor receptor; P, phosphate.

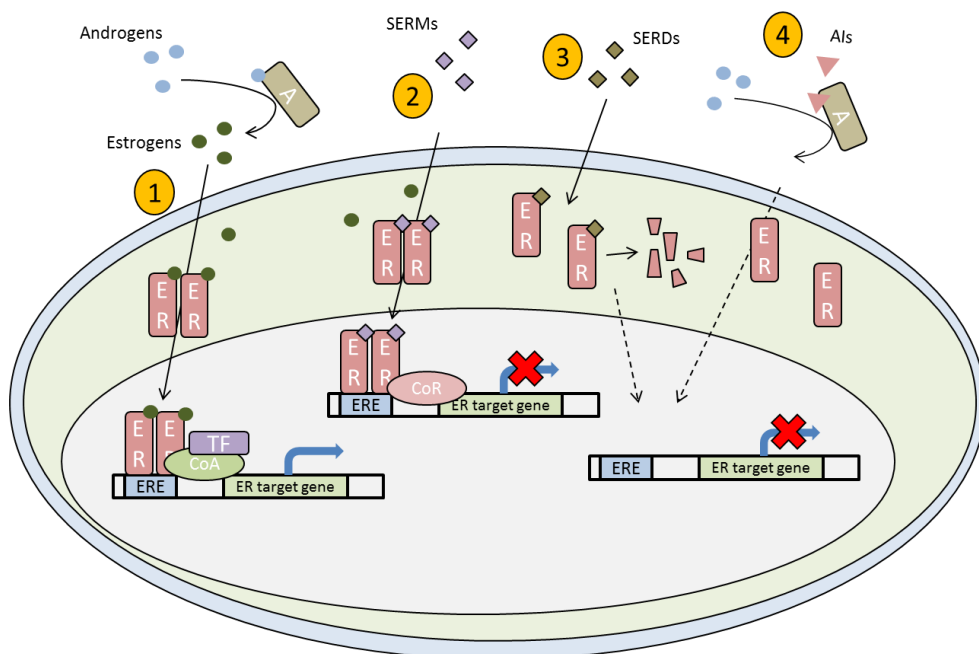
## 1.4 Endocrine treatment

### 1.4.1 Concept and history of endocrine treatment of breast cancer

The rationale for endocrine treatment of breast cancer is to inhibit the growth stimulatory effect of estrogen on tumor cells. This can be achieved through ablation of estrogen or by inhibition of the ER. Today, three classes of drugs dominate endocrine treatment of luminal breast cancer: aromatase inhibitors (AIs), selective estrogen receptor modulators (SERMs) and selective estrogen receptor degraders (SERDs) (Fig. 7). AIs and SERMs are used by millions of breast cancer patients throughout the world

and their long-term use in the adjuvant setting is one of the main reasons for the significant improvement in survival rates for luminal breast cancer patients (61, 62).

However, the road to that improvement was long. It began in 1896 when George T. Beatson discovered that ovarian ablation reduced tumor progression (63). He had unknowingly inhibited the growth stimulatory effect of estrogen before estrogen and its receptor had been characterized. Subsequently, castration by surgery or radiation became a frequent part of the palliation of advanced breast cancer, and the concept of estrogen ablation was further introduced to post-menopausal therapies in the 1950s through adrenalectomy and hypophysectomy (64). During the same time period, a rather counterintuitive concept of endocrine treatment involving administration of high-dose synthetic estrogens to patients with advanced diseases was developed (65). Tumor regression after high dose estrogens or androgens was demonstrated in several clinical trials, and the treatment became standard palliative care from the early 1960s (66). However, both synthetic estrogens and surgical estrogen ablation had rather negative safety images, and the search for more targeted drugs continued. The first AI, aminoglutethimide, was introduced in the 1970s as a “medical adrenalectomy” (67), although it was later discovered that the drug actually inhibited the aromatase enzyme and not adrenal androgen synthesis (68). Less toxic second-generation AIs soon followed. The first SERM, tamoxifen was also developed during the late 1960s. This antiestrogen drug showed less toxicity than high-dose synthetic estrogen in clinical trials and soon became the preferred palliative drug. However, the real revolution in endocrine treatment came when the long-term anti-tumor effects of tamoxifen were demonstrated, which subsequently led to its use in long-term adjuvant treatment (69), a concept later transferred to third generation AIs (70). The first SERD was introduced in the early 1990s after further development of a steroidal compound first known as ICI 164,384. This pure antiestrogen, named fulvestrant, displayed a unique mechanism of ER inhibition that could inhibit growth in tamoxifen-resistant tumors (71). It later became an important drug for treatment of endocrine-resistant metastatic breast cancer (72).



**Figure 7. Basic mechanisms of SERMs, SERDs, and AIs.** 1) Classic ER activation pathway. Estrogens activates ER, after dimerization of ER association with ERE occurs. Recruitment of coactivators (CoA) and transcriptional machinery (TF) leads to transcription. 2) ER bound by SERM. Binding of SERM promotes dimerization and association to ERE. SERM-bound ER recruits corepressors (CoR) and transcription is inhibited. 3) SERD-bound ERs cannot dimerize and therefore do not bind EREs; also, SERDs promote proteasomal degradation of ERs. 4) AIs prevent the conversion of androgens to estrogens by binding aromatase (A) leaving no estrogens available for activation of ERs. Modified from (72).

#### 1.4.2 Aromatase inhibitors

The principle of AIs is to inhibit ER transcriptional activity by suppressing its ligand, E2. This is facilitated by inhibiting the aromatase enzymes that aromatize androgens to estrogens (androstenedione  $\rightarrow$  estrone (E1), testosterone  $\rightarrow$  estradiol (E2)). E1 does not activate the ER, and its circulating levels exceed E2 levels by a factor of 4-5, caused by androstenedione being the major substrate for aromatase. However, E1 is

readily converted to E2 by dehydrogenases (73). Two classes of AIs exist and differ from one another in how they interact and inhibit the aromatase enzyme. Steroidal AIs (exemestane) are derivatives of androstenedione that irreversibly bind the substrate-binding pocket of aromatase, causing inactivation. In contrast, non-steroidal AIs (letrozole, anastrozole) form a reversible bond to the heme moiety of the substrate-binding site, causing inhibition (73). In the adjuvant setting, AIs are mainly used in postmenopausal women. After menopause, ovarian production of estrogen ceases, and estrogen synthesis is restricted to the enzymatic conversion of androgens to estrogens in peripheral tissues. While estrogens are produced at lower concentrations in postmenopausal women, they continue to activate the ER and promote tumor growth, and AIs have proven to be effective compounds for inhibition of this process in postmenopausal patients (62). When given to pre-menopausal women, AIs instigate a feedback-loop involving increased secretion of gonadotropin and increased synthesis of estrogen in the ovaries. Therefore, AIs must be given in concert with gonadotropin-releasing-hormone agonists in pre-menopausal patients (74). In postmenopausal women, AIs taken from the outset for at least for 2 years (23) have been shown to be more effective than tamoxifen at increasing relapse-free survival (62). Thereafter, patients may continue with AIs or tamoxifen for another 3 years, for a total of 5 years. In addition, 5 to 10 years on tamoxifen is a viable option for these patients, in particular for node-negative patients (6, 75) (see also Fig. 13). AIs have also been shown to have greater efficacy in the treatment of patients with postmenopausal metastatic breast cancer and are therefore the treatment of choice for such patients (23).

#### 1.4.3 Selective estrogen receptor degraders

SERDs inhibit ER transcriptional activity by binding to the ligand-binding pocket of ER and preventing dimerization of the receptors (Fig. 7). More specifically, the protruding sidechain of the SERD obstructs helix 12 from interaction with the LBD, and the receptor is not able to adopt antagonistic or agonistic forms (72). In addition, as its name implies, SERDs induce degradation of the ER; however, this mechanism has not yet been established as an essential contribution to its ER inhibitory effect

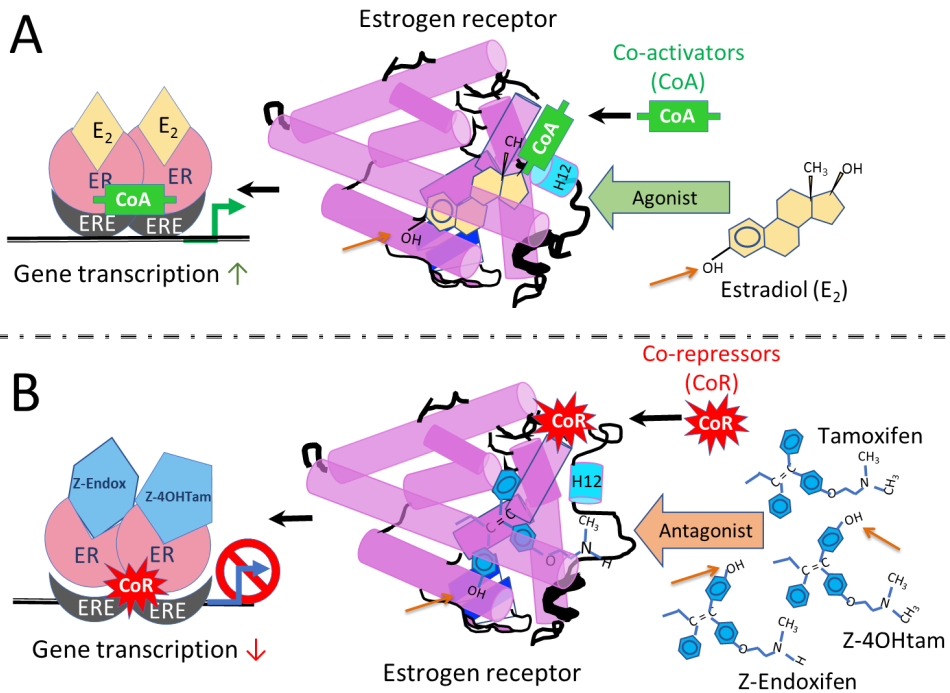
(76). Although several SERDs have been developed, fulvestrant is the only SERD approved for clinical use at this time. Fulvestrant has not been shown to be superior to tamoxifen in reducing proliferation; however its unique mechanism of ER inhibition represents an important treatment option for patients that have progressed on prior endocrine treatment such as AIs or tamoxifen (72). It has consequently become a useful drug in the metastatic setting, where it is approved as a first- and/or second-line treatment option alone or in combination with AIs or the CDK 4/6 inhibitor palbociclib (72).

#### 1.4.4 Selective estrogen receptor modulators

SERMs bind the ER and induce the recruitment of corepressors instead of the coactivators that are recruited when the ER is bound by E2 (Fig. 8). More specifically, the binding of E2 to the ER ligand-binding pocket promotes conformational change to the receptor in which the ligand becomes covered by helix 12 in the LBD, which allows coactivators to bind to the hydrophobic groove in the AF-2 region that is exposed during this process (Fig. 8A). In contrast, when a SERM binds to the ligand-binding pocket of ER, the protruding side chain of the SERM obstructs helix 12 in covering the molecule thus forcing a conformational change that results in the helix 12 occupying the site of coactivator binding. In this situation, the SERM-bound ER is not able to recruit coactivators and instead associates with corepressors, repressing transcription (54, 55) (Fig 8B). The non-steroidal SERM tamoxifen was the first SERM to be approved for clinical use in breast cancer and remains the only SERM used in neoadjuvant, adjuvant, and metastatic settings. Due to variations in levels of coactivators and corepressors between various tissues, the antagonistic behavior of tamoxifen in tumor tissue is contrasted in other tissues such as the uterus, where it has an agonistic effect (69, 77), and tamoxifen use is consequently associated with a higher risk of endometrial cancer (78). Several SERMs have subsequently been developed in an effort to reduce the agonistic effect on the endometrium while preserving the anti-ER effect on tumor tissue (72). Raloxifene is the only SERM that meet these two criteria and has thus been approved for use in the prevention setting for high-risk pre-menopausal women (79).



Tamoxifen may also be used in a prevention setting, where it has been shown to reduce breast cancer incidence by 33% (79). In the adjuvant setting tamoxifen can reduce relapse by 50% (80) and mortality by 31% (12). Current guidelines for adjuvant tamoxifen use in Norway (6) recommend up to 10 years of treatment based on the findings of the ATLAS and aTTom trials, which showed improved long-term survival after extending treatment time from 5 to 10 years (81, 82). Depending on menopause status, tamoxifen may also be used in combination with ovarian suppression and in sequence with AIs (6, 23). In the metastatic setting, tamoxifen is the preferred endocrine drug in treatment of pre-menopausal patients and is often used in combination with ovarian ablation or suppression (23). Depending on the previous treatment regimens a patient has undergone, tamoxifen may also be useful as a late-line treatment in the metastatic setting.



**Figure 8. Mechanism of agonism and antagonism of E2 and SERM on ER.** Binding of E2 leads to conformational shift of Helix 12 (H12) revealing binding motifs for coactivators (CoA), which activate the transcription process. In contrast, SERMs obstruct H12 rotation and thus binding of CoA. However, SERMs allow the binding of corepressors (CoR) with repression of gene transcription as a result. The phenol bound OH-groups (orange arrows) on the Z-4OHTam and Z-Endoxifen molecules resemble the phenol bound OH group in E2, which leads to a deeper and stronger binding in the ligand-binding domain of ER. Thus, active metabolites have up to a 100-fold increase in affinity to ER compared to tamoxifen itself. Adapted from (54, 55).

### 1.4.5 Endocrine resistance

Resistance to endocrine treatment can occur from the onset of treatment (de novo resistance) or after a defined duration of treatment (acquired resistance). The mechanisms that underlie endocrine resistance have been linked to alterations in the ER by mutations, epigenetic modifications, and amplifications of the ESR1 gene. Further, over-expression or activation of membrane-bound receptor tyrosine kinases (RTKs) such as EGFR, HER2, HER3, insulin receptor and the insulin like growth factor receptor-1 are known to be involved in endocrine resistance (83). Activation of the RTKs leads to alterations in the PI3K/Akt/mTOR pathway, a pathway that may crosstalk with ER signaling, has also been identified in endocrine-resistant breast tumors. Importantly, perturbation of coactivators in the ER pathway, such as increased levels of coactivators or decreased levels of corepressors are often observed in endocrine resistance (84). Abnormalities in the regulation of the cell cycle is another factor that plays a part in endocrine resistance through abnormal activation, expression, or phosphorylation of the cyclin D/CDK/Rb pathway (72). Several promising agents that can be administered in concert with standard endocrine agents to combat endocrine resistance have been and are being developed, including multiple mTOR inhibitors and CDK4/6 inhibitors such as palbociclib. The latter, used in combination with an AI, was shown to double progression-free survival when compared to AI alone in a trial including patients with advanced metastatic disease, consequently leading to swift approval of the drug by the US food and drug administration (85, 86). In addition to the tumor-associated resistance mentioned above, patient characteristics may also cause resistance to the endocrine drug, tamoxifen. This is referred to as metabolic resistance and involves interpatient variability in the ability to bio-activate tamoxifen and is discussed in detail in the sections below.

## 1.5 Tamoxifen metabolism

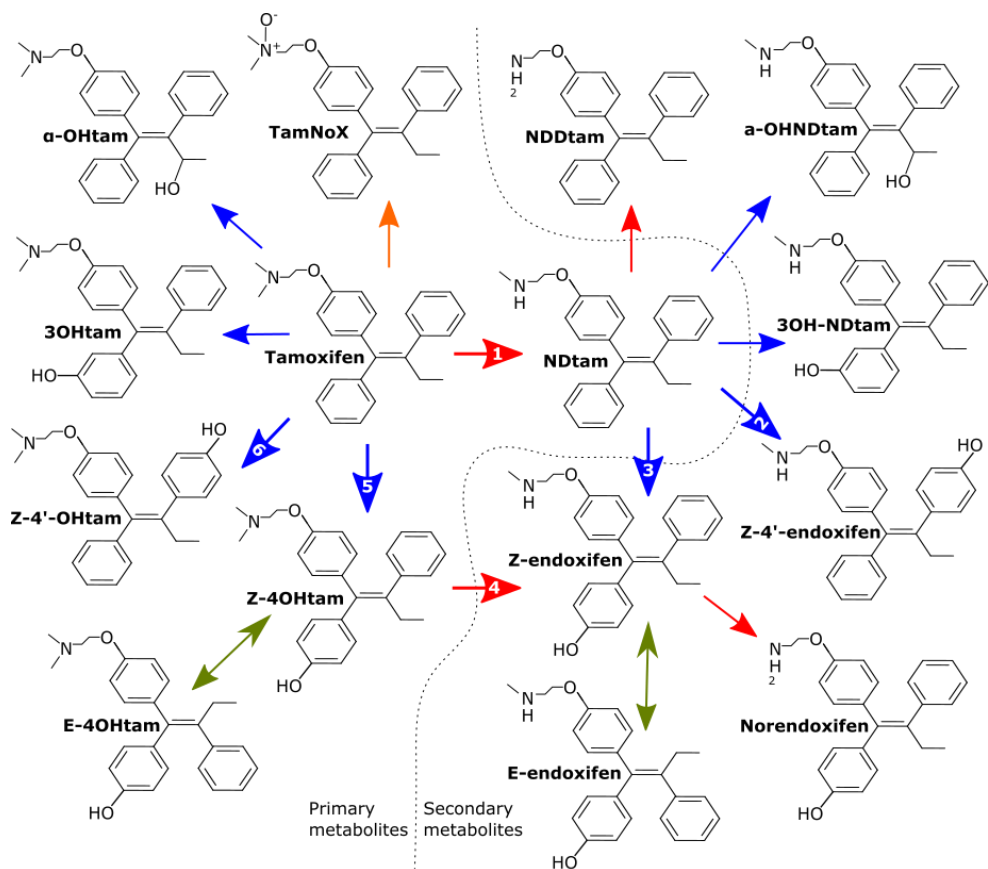
### 1.5.1 Tamoxifen pharmacodynamics

The non-steroidal compound tamoxifen, first known as ICI 46,474, is a trans isomer of p- $\beta$ -dimethylaminoethoxy-1,2-diphenylbut-1-ene. Tamoxifen is a pro-drug that undergoes extensive first phase metabolism, resulting in the formation of two metabolites, 4-hydroxy-tamoxifen (4OHTam) (87) and 4-hydroxy-N-desmethyl-tamoxifen (4OHNDtam/endoxifen) (88) that exhibit affinity to the ER up to 100 times higher than does tamoxifen itself (89, 90). The more potent anti-estrogenic action of these *two active metabolites* compared to tamoxifen itself is caused by the presence and position of a phenolic hydroxyl in their structures. The phenolic A ring ensures “deeper” binding to the binding pocket in the LBD of the ER, causing conformational changes in the LBD and “correct” positioning of the bulky side chain for antagonistic conformation of the ER (54) (Fig. 8B). The two active metabolites have similar affinities to the ER and similar anti-estrogenic effects on breast cancer cell proliferation (91). However, endoxifen is present at concentrations up to 10 times higher than 4OHTam and is therefore regarded as the more important active metabolite. In contrast to 4OHTam, endoxifen has been shown to induce proteasomal degradation of the ER (92) and to exhibit distinct and highly concentration-dependent effects on gene expression (93). However, another gene expression study comparing 4OHTam and endoxifen at the same concentrations (100 nM) found the two active metabolites have similar effects on global gene expression (94). It is important to note that although other metabolites such as 3-hydroxylated metabolites have been reported to show anti-estrogenic activity, their concentrations *in vivo* are generally too low to demonstrate any noteworthy antagonistic effect on the ER, and to this point no other highly active metabolites have been described (95).

### 1.5.2 Tamoxifen pharmacokinetics

Upon oral administration, tamoxifen will follow the classic phases of metabolism of which the main outcome is to make the foreign molecule more water-soluble so it can be excreted from the body. Steady-state levels are reached within 4-8 weeks (96). Although some first-pass metabolism may occur in the small intestine (97), the main site of tamoxifen metabolism is the liver, and the focus here will be on the hepatic metabolism. Once tamoxifen has reached the liver, it first undergoes phase I metabolism involving N-oxidation, hydroxylation and demethylation by various members of the cytochrome P450 (CYP) family of enzymes (98) (Fig. 9). The major route of phase I tamoxifen metabolism is the demethylation of tamoxifen to N-desmethyl-tamoxifen (NDtam) catalyzed primarily by CYP3A4/5, in addition to other CYP enzymes including CYP2D6 (98). In plasma, NDtam is found at approximately twice the concentrations of the mother-drug tamoxifen at steady state (99, 100). An alternative and minor route involves hydroxylation of tamoxifen to 4OHTam catalyzed by CYP2D6, CYP2C9, CYP2C19, CYP2B6 and CYP3A4 (101). Tamoxifen may also be N-oxidized to tamoxifen-N-oxide (tamNoX) (102) by flavin-containing monooxygenases and reduced back to tamoxifen by various CYP enzymes (103). As NDtam, 4OHTam, and tamNoX are metabolized directly from tamoxifen, they are referred to as primary metabolites. Other primary metabolites include 3-hydroxy-tamoxifen and  $\alpha$ -hydroxy-tamoxifen (98). Secondary metabolites are formed by further metabolism of the primary metabolites and include hydroxylation of NDtam to endoxifen catalyzed by CYP2D6 and demethylation of 4OHTam to endoxifen by CYP3A4, CYP3A5, CYP2C19, and CYP2D6 (98). Secondary metabolites such as N-didesmethyl-tamoxifen (N,N-DDtam) and 3-hydroxy-N-desmethyl-tamoxifen (3-OHTam) are also formed from the primary metabolites. A minor metabolite known as norendoxifen that is formed by demethylation of endoxifen interestingly exhibits an aromatase inhibition function (104); however, the clinical impact of this metabolite has not been determined. Phase I metabolites are further conjugated by glucuronic acids (88, 102) and sulfates (105) in hepatic phase II metabolism to increase the hydrophilic properties. These reactions are catalyzed by glucuronidases and sulfotransferases (106). The conjugated metabolites are subsequently excreted via bile

or urine; however, as much as 69% of the metabolites in the bile are reabsorbed by enterohepatic recirculation (107, 108). In addition, tamoxifen has a highly lipophilic structure and has been shown to be 98% albumin-bound, which contributes to its long half-life (97). Another aspect to consider in tamoxifen metabolism is the formation of different geometric isomers (E- and Z-isomers), in which the Z-isomers exert the most potent anti-estrogenic activity (Fig. 9). Adding to the complexity, the position of the hydroxyl group on the rings of hydroxylated metabolites may also vary. For example, when the hydroxyl group in 4OHTam is on the secondary structure, it is expressed as 4-prime-OHTam or 4'OHTam (Fig. 9). The Z-4'-isomers exhibit reduced binding affinity to the ER compared to the Z-isomers (109). Notably, in most studies on active tamoxifen metabolites the Z- isomer (= the active isomer) has not been separated from the E- and Z'-isomers. Consequently, the collective notation 'endoxifen' and '4OHTam', which includes all isomers (i.e. endoxifen = Z-endoxifen + E-endoxifen + Z'-endoxifen) have been used. Thus, the notations endoxifen and 4OHTam are generally used in this thesis. Importantly, in the two clinical papers (paper II and III) of the present thesis the active isomers Z-endoxifen and Z-4OHTam were selectively measured.



**Figure 9. Overview of tamoxifen metabolites formed during first phase hepatic metabolism.** Only isomers of 4OHtam and endoxifen are shown; other metabolites may also be subject to isomerism. Blue, red, orange and green lines represent hydroxylation, demethylation, N-oxidation, and non-enzymatic isomerization, respectively. Numbered arrows represent the following enzymes involved in generation of active metabolites and their less active isomers: **1)** CYP3A4/5, CYP1A1/2, CYP2C9/19, CYP2D6 **2)** Unknown **3)** CYP2D6 **4)** CYP3A4/5, CYP2C19, CYP2D6. **5)** CYP2C9/19, CYP2D6, CYP3A4, CYP2B6 **6)** CYP2B6, CYP2D6. Modified from (95).

### 1.5.3 Tamoxifen pharmacogenomics

The rationale behind most pharmacogenomic studies of tamoxifen metabolism is the assumption that allelic variants of tamoxifen-metabolizing enzymes affect concentration levels of active tamoxifen metabolites.

The majority of studies regarding the pharmacogenomics of tamoxifen have focused on the CYP2D6 isoform as this enzyme is pivotal in the formation of the active metabolite endoxifen. CYP2D6 catalyze the hydroxylation of the major metabolite NDTam to endoxifen and are also involved in hydroxylating tamoxifen into the other active metabolite 4OHTam. The *CYP2D6* gene is located on a highly polymorphic locus, chromosome 22q13.1, and over 100 allelic variants of *CYP2D6* have been identified (110). The polymorphisms in the *CYP2D6* gene include deletions, duplications, and single nucleotide polymorphisms (SNPs), which may affect the function or expression of the CYP2D6 enzyme. A classification system is used to translate the polymorphisms (genotypes) into the functional activity of the enzyme observed in humans (phenotype) (111). In short, the system assigns an activity score for each allele based on the functional consequence of the polymorphism and then sums the score of two alleles to assign a phenotype. The phenotypes are commonly distinguished into four groups; poor, intermediate, normal, and ultra-rapid metabolizers. The allelic frequency of genes resulting in a poor metabolizer phenotype is reported to be as high as 5-10% in Caucasians (112), and significant differences in allele frequencies between racial, ethnical, and geographical groups have been reported (113).

The association between CYP2D6 phenotypes and concentration levels of endoxifen is well established, and it is recognized that *CYP2D6* genotype can explain up to 34-52% of endoxifen generation from NDTam (100, 114-116). In addition, it has been reported that CYP2D6 contributes up to 45% of 4OHTam formation from tamoxifen in human liver microsomes and that *CYP2D6* genotype was significantly associated with concentrations of the metabolite (117). Significant associations between CYP2D6 phenotype and concentrations of 4OHTam have also been reported in breast cancer patients using tamoxifen (118, 119).



Several other polymorphic phase I CYP enzymes contribute in the metabolic pathway leading to the formation of the two active metabolites (95, 120), and the pharmacogenetics of these enzymes has also been linked to steady-state levels of active metabolites. Polymorphisms in *CYP2C9*, an enzyme involved in the formation of both 4OHTam and NDtam (the precursor of endoxifen), have been associated with systemic concentrations of both of these active metabolites in patients receiving tamoxifen treatment (95, 116). CYP3A4 is involved in the same metabolic pathways as CYP2C9 in addition to the demethylation of 4OHTam to endoxifen. CYP3A4 polymorphism has been shown to be a predictor of endoxifen concentrations (121) and tamoxifen plasma levels (122). The latter study also demonstrated a significant association between 4OHTam concentrations and CYP3A4 phenotype in patients with impaired CYP2D6 activity. CYP3A5 is an enzyme similar in structure and function to CYP3A4, and several activity-impairing polymorphisms have been identified in CYP3A5. However, no studies have shown *CYP3A5* polymorphisms to be associated with tamoxifen metabolite concentrations *in vivo*. It is believed that functional defects in CYP3A5 may be compensated for by CYP3A4, given their very similar functions (123). CYP2C19 is an enzyme involved in all metabolic steps leading to formation of active metabolites, and the formation of 4OHTam from tamoxifen has been significantly associated with *CYP2C19* genotypes in breast cancer patients from two separate studies (114, 124).

The effect on steady-state metabolite levels by polymorphisms in enzymes responsible for phase II metabolism of tamoxifen has not been fully determined. Inconsistent results have been reported; however, two recent studies demonstrated associations between systemic levels of active metabolites and genetic variants of *SULT1A2* (125) and levels of glucuronidated endoxifen and 4OHTam in plasma with allelic variants of Uridine 5'-diphospho-glucuronosyltransferase (UGT) enzymes (126). Significant association between *SULT1A1* genotypes and metabolic ratios between NDtam/tamoxifen and NNddtam/NDtam has been shown (127).

Finally, transporter proteins such as ATP-binding cassette sub-family B member 1 (ABCB1) have been shown to export active metabolites out of cells (113). Although some modest associations between endoxifen levels and SNPs in these transporter

proteins have been reported (115), the impact of pharmacogenetics in transporter proteins has not been determined.

## **1.6 Tamoxifen metabolism as a predictor of response to treatment**

### **1.6.1 Pharmacogenetic evidence**

In 2005 Goetz and colleagues provided the first evidence for a link between *CYP2D6* genotype and breast cancer outcome when they demonstrated that patients using tamoxifen with two non-functional alleles of *CYP2D6* had a significantly higher risk of relapse and lower risk of hot flashes after a median follow-up time of 11.4 years (128). The link between outcome and the *CYP2D6* genotype was replicated in a study two years later, demonstrating that several activity-inhibiting allelic variants of *CYP2D6* were associated with adverse outcomes, and patients with high-activity variants of *CYP2C19* had improved outcomes (129). More studies reporting a positive association between *CYP2D6* phenotypes and clinical outcome followed (130, 131). However, studies reporting no association (132, 133) and, surprisingly, improved outcomes among patients with non-functional *CYP2D6* alleles (134, 135) also emerged during these years. Concern was expressed over the lack of attention given to the studies reporting “negative results.” Epidemiologists claimed that all genotype-outcome association studies performed at the time were susceptible to several biases and that their results must be interpreted with caution (136). At the present time, more than 40 articles evaluating the *CYP2D6*-outcome association have been published (111). Among the 40 articles, four studies are regarded as having potential to resolve the issue. These four studies include a comprehensive meta-analysis (137) and three retrospective analyses of the large prospective tamoxifen trials ATAC (138), BIG1-98 (139) and ABCSG8 (140). The meta-analysis showed a positive association only when applying strict selection criteria, however no significant outcome association was found when using less strict or no inclusion criteria (137). The analyses of ATAC and BIG1-98 showed no association and ABCSG8 applied a case-control design that demonstrated a positive association only during the period of tamoxifen administration (5 years). Based on the discrepancies between these high impact studies, ASCO

Clinical Practice Guidelines currently do not recommend the use of *CYP2D6* polymorphisms as a biomarker for guiding adjuvant endocrine treatment selection (141). Associations between clinical tamoxifen efficacy and polymorphisms in other enzymes involved in the generation or elimination of the active metabolites have also been reported; however, these results are also divergent. In any case, since the contributions of the other enzymes to active metabolite concentrations are minor compared to *CYP2D6*, it is not plausible that these enzymes can function alone as biomarkers for tamoxifen efficacy.

The lack of consistent evidence for an association between tamoxifen pharmacogenetics and clinical efficacy of tamoxifen may be caused by multiple factors. First, all clinical studies on the association between pharmacogenetics and outcome have been performed retrospectively on studies in which this association was never the primary hypothesis. Importantly, adherence to tamoxifen can be as low 50% over a 5-year treatment period (142-144) and adherence has not been accounted for in many of the pharmacogenetic studies. Co-medications commonly prescribed to breast cancer patients such as SSRIs have been shown to inhibit *CYP2D6* and decrease endoxifen concentrations (99), and this effect has not been accounted for in many of the studies. Loss of heterozygosity is found in 40% of ER-positive breast cancers, and tumor DNA is not recommended for determination of germline polymorphisms unless special methodological considerations are taken into account (145). Several trials have used tumor DNA to determine *CYP2D6* phenotype, while others have used germline DNA, and this may have added to discrepancies. Also, *CYP2D6* genotype can only explain 50-60% of variability in endoxifen concentrations, and prediction models including other enzymes and factors that contribute to steady-state concentrations of endoxifen should also be accounted for (121). Further, the systems used to classify *CYP2D6* phenotypes from genotype is not substrate specific for endoxifen, and the phenotypes produced do not predict actual concentration levels between phenotype groups precisely enough (146). A final reason results may differ is that there is no association between outcome and tamoxifen metabolism. Some models have indicated that tamoxifen and its metabolites saturate the receptors and out-compete estrogen by a

factor of 1,558 in activity at the receptor (147) in postmenopausal women, regardless of *CYP2D6* genotype (136).

### 1.6.2 Pharmacokinetic evidence

Using pharmacogenetics is an indirect approach that aims to predict levels of active metabolites. Obviously, the direct measurement of active metabolite levels would circumvent many of the issues discussed in the section above. However, the main obstacle for this analysis has been that very few prospective tamoxifen trials have collected samples allowing for measurement of steady-state tamoxifen metabolite levels. Therefore, the investigation of this association in the adjuvant setting has to this point been limited to three retrospective analyses of tamoxifen treated patients and a recently commenced prospective trial.

Madlensky et al. measured tamoxifen metabolite concentrations in a cohort of 1,370 patients using tamoxifen adjuvantly originally recruited through the women's healthy eating and living (WHEL) study, a randomized controlled trial with dietary intervention. The study had an average follow-up time of 7.3 years, and they reported that patients with endoxifen levels representing the lowest quintile of endoxifen ( $\leq 16$  nM) had a higher risk of metastatic events (118). A similar threshold effect of endoxifen was demonstrated in a retrospective analysis of 306 pre-menopausal patients included in the prospective study of outcomes in sporadic versus hereditary breast cancer (POSH). This study included patients adjuvantly treated with tamoxifen and had a median follow-up time of 6.4 years. The patients were separated into quartiles based on their endoxifen levels, and a significant difference in distant relapse-free survival was identified when comparing the lowest quartile ( $< 14.15$  nM) with the highest quartile ( $> 35$  nM) (114). An explorative study, including 48 patients, used a nested case-control design to show that patients with high levels of endoxifen ( $> 187$  nM) were at greater risk for recurrence (148). An additional finding was a J-shaped relationship between endoxifen concentrations and recurrence, meaning that patients with very low levels (no threshold was specified by the authors) also had a higher risk. However, the results of this explorative hypothesis-generating study should be interpreted with caution due to the low number of patients. A recent

prospective trial has investigated the impact of endoxifen concentrations in the neoadjuvant and metastatic setting (149). The authors reported that no relationship between endoxifen concentrations and objective response rates, clinical benefit, or progression-free survival was identified in the neoadjuvant and metastatic setting. The first results of the CYPTAM trial, a prospective trial evaluating the association of endoxifen levels on breast cancer outcome in the adjuvant setting was recently presented at the ASCO conference in which no threshold effects could be observed (150). However, the results from this prospective trial may be premature as follow-up time is currently limited. Updates from this trial will certainly be interesting to follow in the coming years as the trial represent the only prospective trial investigating both genotype and active metabolite concentration association in adjuvantly tamoxifen-treated breast cancer patients.

The association between tamoxifen metabolite concentrations and tamoxifen efficacy has also been investigated in the prevention setting, in which no association could be demonstrated (151). However, the LC-MS/MS method used to measure tamoxifen metabolites in this study was later criticized for producing inaccurate and over-estimated endoxifen levels (152).

## **1.7 Discontinuation of per-oral tamoxifen treatment**

### **1.7.1 Challenges with tamoxifen treatment**

Tamoxifen treatment is administered orally by the patients themselves on a daily basis. The main challenge of this unsupervised long-term treatment (5-10 years) is low adherence and/or discontinuation of therapy. Adherence refers to a patients compliance to follow the prescription guidelines and non-adherence is generally set at taking less than 80% of the pills during a given period (153). The cut-off is based on the fact that missing a dose or two is not believed to affect treatment efficacy of tamoxifen due to its relatively long half-life of 2-4 weeks. Persistence is the length of time from start to discontinuation of therapy, and non-persistence is a synonym to discontinuation, which means stopping the treatment prior to the recommended

treatment time. Thus, it is important to distinguish between non-adherence and discontinuation.

In general terms, the adherence rates for tamoxifen are between 45% and 93.4%, and the rates for discontinuation are between 15% and 60%; patients tend to be more adherent at the start of the treatment compared to later (144). A meta-regression analysis, including more than 85,000 patients from 26 studies, on tamoxifen adherence showed a discontinuation rate of 47.1% at 5 years (154). However, the studies vary in terms of how adherence and discontinuation is measured (self-reported/prescription databases), both of which are susceptible to biases. Self-reported adherence represents the method most susceptible to bias, but also using the patients refill records to calculate the medication possession ratios may also not represent the actual adherence, as it is not given that patients withdrawing drugs from the pharmacy actually take the medication. Further, publications studying adherence in patients not included in clinical trials are generally believed to represent a more accurate picture of adherence compared to studies that included patients enrolled in clinical trials. This is most likely due to the fact that patients involved in clinical trials receive closer follow-up and usually represent a selection of patients that are highly motivated to participate (155).

Two large studies using prescription databases to determine discontinuation rates among patients not included in clinical trials (142, 156) found that 35.2% and 38% of the patients had discontinued their tamoxifen treatment at 3.5 and 4.5 years, respectively. Similar results were obtained in a study relying on self-reported persistence from 435 patients not included in clinical trials; in this study 31% of the patients had discontinued before the end of the treatment at 5 years (157).

It is plausible that early discontinuation of tamoxifen could represent a higher risk of disease recurrence, which has been shown in patients with shorter duration of treatment (158). Other studies have found that patients who discontinued or were non-adherent to endocrine therapy (AIs and tamoxifen) had more early breast cancer events (159) and significantly poorer overall survival (160) compared to patients completing the treatment and remaining adherent. Increased risk of all cause of death for women with shorter time on tamoxifen (161) has also been demonstrated.

Importantly, the results of the ATLAS (81) and aTTom (82) trials showing improved outcome after prolonging tamoxifen treatment from 5 to 10 years, should encourage more focus on adherence and persistence to treatment.

### 1.7.2 Risk factors for discontinuation

The reasons for discontinuation are multifactorial and complex as several factors have been linked to endocrine treatment discontinuation. Comorbidities and concurrent treatments, financial issues, patient's perception of risk and psychological factors such as anxiety and depression are factors that have been associated with risk of discontinuation (155, 162). The most commonly reported risk factors for quitting tamoxifen treatment prematurely are side effects and age. Age has repeatedly been significantly associated with adherence to tamoxifen therapy in which the youngest and the oldest patients are the ones who exhibit the poorest adherence (142, 163). Side effects have also been associated with non-persistence in several independent cohorts (143, 157, 164). Further, a recent study by Kwan et al. found that the occurrence of adverse events was the second most common reason (most common reason was menopausal status) for switching from tamoxifen to an AI among 1,143 patients with early breast cancer and that 22% of the patients who had switched eventually discontinued the treatment (165). However, data from a prospective placebo-controlled tamoxifen trial showed that patients using placebo were more likely to discontinue than patients using tamoxifen, and high portions of the placebo group reported experiencing side effects commonly associated with tamoxifen use such as hot flashes and vaginal discharge (166).

Substantial inter-individual variability exists in the experience of side effects among tamoxifen users, and tamoxifen metabolism has been suggested as predictor of side effects. Few studies have investigated the role of tamoxifen metabolite concentrations (167-169) and CYP2D6 (128, 170-172) in relation to adverse events, and the results remain inconsistent.

## 1.8 Methodology

### 1.8.1 Cell model

To study the effects of tamoxifen metabolites on gene expression *in vitro*, we used the breast cancer cell line MCF-7, short for Michigan Cancer Foundation-7, the cancer institute where the cell line was first isolated in 1973 (173). This adenocarcinoma cell line originates from a metastatic site (pleural effusion) of a 69 year old female and has molecular characteristics similar to the luminal A subtype of breast cancer (174), and the cell line is positive for ER and PR and negative for HER2 expression. Despite its metastatic origin, MCF-7 cells exhibit the characteristics of differentiated mammary epithelium with low ability for invasion and migration. The MCF-7 breast cancer cell line was chosen since it is a well-established *in vitro* model for studying the effects of anti-estrogens on proliferation and ER gene regulation (94, 175, 176). The cell line was purchased from the American Type Culture Collection (ATCC<sup>®</sup>) and cultured as recommended by the supplier. We chose to use tamoxifen metabolite concentrations found in tissue, and MCF-7 cells were treated with 1000 ng/mL NDtam, 100 ng/mL 4Ohtam and 1000 ng/mL endoxifen to elucidate the gene regulatory roles of these tamoxifen metabolites.

### 1.8.2 Gene expression analyses

#### 1.8.2.1 Microarray

To characterize the effect of tamoxifen metabolites on MCF-7 global gene expression, Illumina BeadArray Microarray Technology was utilized at the Norwegian Microarray Consortium core facility at the University of Bergen. This microarray technology uses silica beads that have thousands of oligonucleotides attached to them that are randomly arranged in microwells on glass or silica slides. Each oligonucleotide contained the complementary sequence of a specific RNA transcript (probe) and an address sequence allowing for identification of the probe on the array. Each bead had thousands of copies of one unique probe attached, and an array contained up to 44,000 unique oligonucleotides. Total RNA from MCF-7 cells were converted to double stranded cDNA and further biotin-labelled to generate fluorescently labelled cRNA



transcripts. The labelled transcripts were then hybridized to the probes, and the signal intensity was read by the Illumina iScan System and calculated as the average of all beads with the same probe. The identity of each bead was determined by decoding the address-sequence. The signal intensity corresponded to the quantity of the specific gene transcript in the sample.

Differential gene expression between the differently treated samples was analyzed using significance analysis of microarray (SAM) (177) method in J-express gene expression analysis software (178). The SAM analysis provided a ranked gene list of the differential gene expression between different samples. The list was ranked according to the  $\Delta[i]$ , which calculated the difference between the strength of the gene expression  $D[i]$  and the expected strength  $D_e[i]$  significance between treatment groups. Another important parameter in the SAM analysis is the false discovery rate (FDR) which corrects for multiple testing and is estimated through permutation and expresses the expected proportion of false positives in the list of genes. The difference in expression of a given gene is usually reported by the use of fold change (FC), and the significance of the finding is reported by the q-value, an FDR analog to the p-value. These measures are employed when determining cut-off values to generate a selection of differentially expressed genes that can further be studied by bioinformatics such as GSEA, gene ontology and pathway analyses. These measures were employed in paper I to generate a list of genes in which we characterized the gene ontology using the PANTHER database ([www.pantherdb.org](http://www.pantherdb.org)) to search for statistically over-represented ontology categories.

#### 1.8.2.2 *Quantitative real-time polymerase chain reaction (Q-rt-PCR)*

Q-rt-PCR using the LightCycler® 480 Probes Master system was applied to validate differential gene expression observed in microarray analysis, as well as to characterize differential gene expression after estrogen deprivation and knockdown of SRC-3. In short, total RNA isolated from cell samples was reversibly transcribed to cDNA, which formed the template for the Q-rt-PCR reaction. Primers allowing for amplification of complementary regions of target genes were designed *in silico* using the Universal ProbeLibrary (UPL) Assay Design Center (Roche®, Applied Science). The UPL system also selected primer-compatible probes that emitted fluorescent

signals when hybridized to target PCR products. The fluorescent signal of the probe increased in correlation with amount of target PCR product and after a certain number of PCR cycles the fluorescent signal became detectable, referred to as the crossing point (Cp). The Cp was inversely related to the expression level of the target gene, i.e. low Cps indicated high level of gene expression. To normalize for inter-sample variations, the expression level of the target gene was normalized to the expression level of a reference gene that was stably expressed under various treatment conditions. Differential gene expression between two treatment conditions was determined by relative quantification using the Cp for each sample and the efficiency (Eff) of each transcript ( $\text{Eff}_{\text{target gene}}^{\Delta\text{Cp}}/\text{Eff}_{\text{reference gene}}^{\Delta\text{Cp}}$ ) and was reported as FC.

### 1.8.3 Short hairpin knockdown

An MCF-7 cell line with stable SRC-3 knockdown and empty vector control previously generated through lentiviral transduction in our group (179) was used to investigate if the expression of *CK6* was regulated by ER. The basic principle of short hairpin RNA (shRNA) is sequence-specific degradation of a specific mRNA in a host cell of interest. ShRNA utilizes the cellular mechanism termed the RNA-induced silencing complex (RISC). After viral transduction the shRNA is integrated into the host cell's DNA and is diced to silencing RNA (siRNA) after transcription. The siRNA strand is further bound by proteins and guided to the RISC complex which identifies and binds the complementary strand of the target mRNA. The siRNA-target mRNA hybrid is consequently cleaved and degraded by endogenous nucleases (180) leading to down-regulation of the target gene. To validate successful knockdown of the target gene, mRNA and protein levels of the target gene were compared between knock-down and control using Q-rt-PCR and Western Blots, respectively.

### 1.8.4 Genotyping

DNA obtained from whole blood samples from patients included in paper II were screened for 15 common allelic variants of *CYP2D6* by the INFINITI® CYP450 2D6I Assay. In this assay, which applies microarray technology, DNA was amplified,

fluorescently labelled and hybridized to probes that are immobilized to a chip. The fluorescent signals were detected and measured. Results were validated using the Taqman drug metabolizing enzyme assay that is a Q-rt-PCR with genotype specific primers. Performing genotyping on tumor tissue may produce biased results caused by “loss of heterozygosity” (145). This was avoided in our study as we used DNA collected from blood. Although we chose to use this general assay that maps the most common polymorphism in the *CYP2D6* gene, there are several more allelic variants of *CYP2D6* that potentially could have been screened.

### 1.8.5 Quantitative measurements of tamoxifen metabolites in human serum

To measure concentrations of tamoxifen and eight metabolites in human serum (papers II and III), a liquid chromatography tandem mass spectrometry (LC-MS/MS) assay was developed and applied. Experimental design and standard analytical methods in regard to development, validation, and application of the LC-MS/MS method are described in detail in paper II. This section focuses on some of the basic concepts of LC-MS/MS (Fig. 10) and specific improvements in the method that was performed in the two studies (papers II and III).

Biological samples, in our case human serum, are complex and need efficient sample preparation (clean-up) protocols to remove interfering elements, prevent matrix effect, decrease ion suppression, and selectively extract the compounds of interest before the LC-MS/MS analysis. Sample clean-up was automated and performed using a Hamilton STAR pipetting robot and consisted of the addition of the internal standards to the sample, subsequent precipitation of the proteins with acetonitrile, and dilution in water and methanol to reduce ion suppression.

Tamoxifen and eight of its metabolites were chromatographically separated using an Aquity Ultra Performance LC system (Waters), with a phenyl C18 column as stationary phase and a gradient elution of water and methanol as mobile phase. The separation of tamoxifen and its metabolites occurs within the column and is based on the affinity of the compounds to the packing material particles of the column and the mobile phase. A compound with high affinity to the mobile phase and low affinity to the solid phase will travel quickly through the column. The opposite is true for a

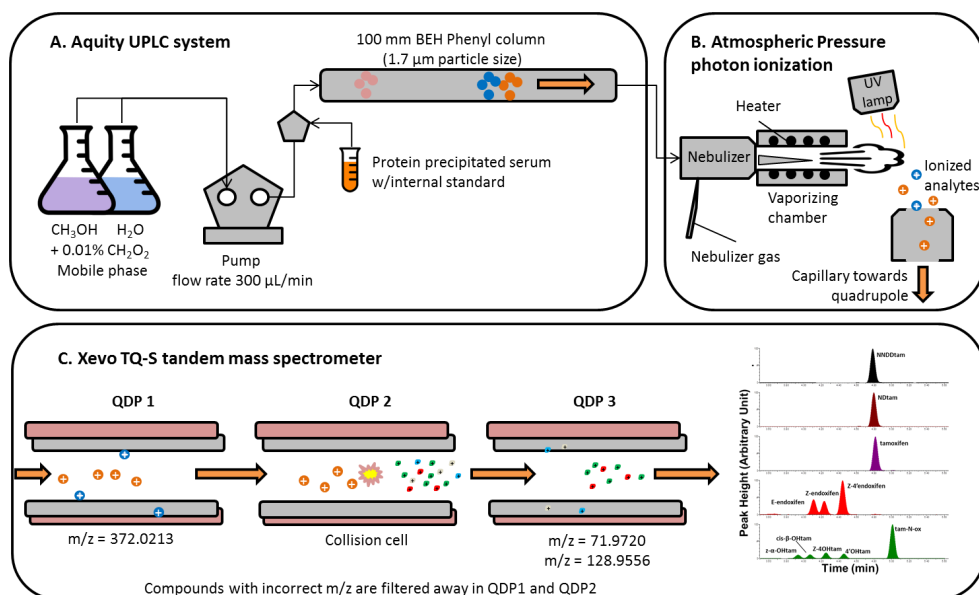
compound with low affinity to the mobile phase and high affinity to the column. This separates the compounds chemically and ensures that the compounds exit the column at different times where they are subjected to mass spectrometry for detection (181).

In order for the compounds to be detected by the mass spectrometer, the liquid from the mobile phase is vaporized to gas-phase and the molecules are ionized (182). In our assay, the tamoxifen metabolites were converted to positively charged ions using an atmospheric pressure photoionization source (APPI).

The LC system was coupled to a Xevo TQ-S tandem mass spectrometer (Waters). In short, this MS system contains three quadrupoles (QDP), and each QDP is made up of four parallel rods that function as “filters” detecting and separating ions based on their specific mass-to-charge ratio. In addition, each QDP has its specific role; the first QDP performs mass selection and at the same time accelerates the ions of interest towards QDP2 where the ions collide with neutral gas and undergo fragmentation. The fragmented ions are then accelerated further towards the third and last QDP (183).

The two fragmented ions (quantify and qualify) are recorded as peaks on a computer in which the area under the peak and peak-height vary in proportion to the concentration in the samples. Concentrations were calculated using a 7-point calibration curve in human serum and plotting the area of the analyte adjusted with the area of the internal standard against the nominal concentration.

In the time between our two studies (papers II and III) the method was modified to reduce sample volume requirements and improve sensitivity. The sample volume was decreased from 50 to 20  $\mu$ L and the lower the limit of quantification was decreased for several analytes, including the active metabolites Z-4OHTam and Z-endoxifen. Several Hamilton parameters (tips, dispense speed, tube format) and the dilution factor were also modified. Moreover, we optimized the chromatographic separation by modifying the mobile phase gradient and the column temperature.



**Figure 10. Basic concept of the LC-MS/MS assay used for measurements of tamoxifen metabolites in human serum.**  $m/z$  values shown are transitions for parent ion and daughter ion 1 and 2 for tamoxifen.  $m/z$ , mass-to-charge ratio; QDP, quadrupole; +, ionized compounds. Modified from (181-183).

### 1.8.6 Patient populations

The **OSLO1 cohort** originally consists of 920 breast cancer patients enrolled in an observational trial on the prognostic value of isolated tumor cells from bone marrow (184). The patients were recruited from five different hospitals in the Oslo region in Norway. Routine clinical follow up included blood sampling, clinical examination, mammography, and chest x-ray. Adjuvant chemotherapy regimens consisted of six cycles of intravenous cyclophosphamide 600 mg/m<sup>2</sup>, methotrexate 40 mg/m<sup>2</sup>, and fluorouracil 600 mg/m<sup>2</sup>, and adjuvant endocrine therapy was 20 mg/day tamoxifen for 5 years. Thirteen patients received high-dose chemotherapy. Three years after inclusion, serum was drawn from 356 relapse-free patients, and 99 operable patients treated with tamoxifen from this cohort were included in paper II. The fact that only patients that were relapse-free at 3 years were included in our study should be considered when interpreting the results from paper II.

**The Prospective Breast Cancer Biobank (PBCB) cohort** originally consists of approximately 1,300 patients included in a population-based regional biobank from patients with breast cancer at Haukeland University Hospital and Stavanger University Hospital in Western Norway. The biobank began collection in 2011, and recruitment is ongoing. The biobank comprises blood samples and patient reported outcome measures from breast cancer patients at all stages, and patients are treated according to national guidelines (6). A population of 220 ER-positive patients using tamoxifen and recruited between 2011 and 2016 were included in paper III. Out of these 220 patients, 124 patients routinely answered questionnaires every 6 months as part of their participation in the biobank, while the remaining 96 patients delivered one single questionnaire in 2016.

#### 1.8.7 Patient reported outcome measures (PROM)

To estimate side effects of tamoxifen (paper III), patients filled out the following instruments; the functional assessments of cancer therapy-endocrine subscale (FACT-ES) version 4 and subjective health complaints (SHC). The questionnaires were filled out at home without supervision from health personnel.

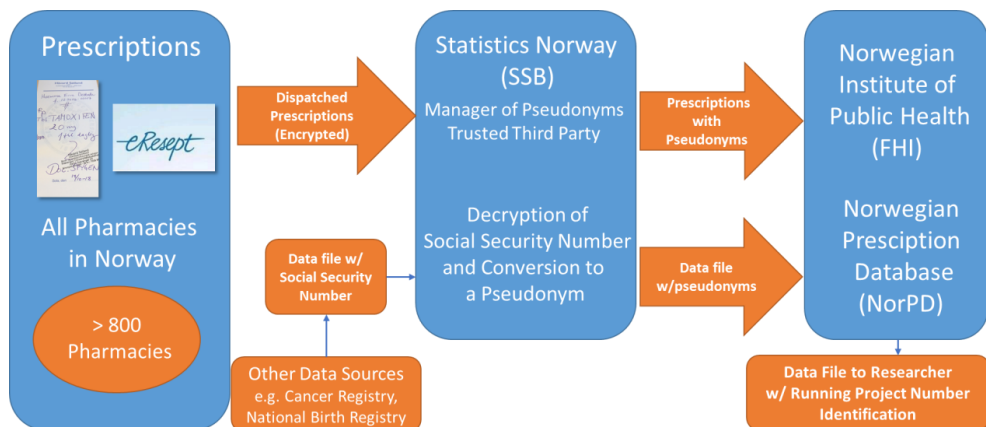
FACT-ES consist of the functional assessment of cancer therapy (FACT-B), a standardized quality of life measure for patients undergoing breast cancer treatment, with the addition of an 18-item endocrine subscale specifically developed to measure side effects and possible benefits of endocrine treatment for breast cancer patients (185). The instrument has been validated for use in breast cancer patients (185, 186) as well as specifically in Norwegian breast cancer patients (187). The items are presented as 5-point Likert-type response scales.

The SHC instrument measures severity of 29 subjective, somatic, and psychological health complaints during the last month, and the instrument has shown satisfactory validity and reliability (188). Severity is rated on a Likert scale between 0-4, where 4 represents the highest severity of a complaint. The instrument has recently been validated for use in breast cancer (189). The questionnaire was used as a supplement to FACT-ES to validate items similar between the two questionnaires. A maximum sum score of 80 can be achieved from this questionnaire, for which a score above 20 has

been reported as a cut off for experiencing more than normal health complaints (188, 190). We applied this cut-off value in our analyses of tamoxifen metabolite associations and as a predictor of discontinuation. Both for the FACT-ES and SHC, we applied dichotomization for most items, meaning responses of 0=not at all where coded as “no” and all other values as “yes.” We also dichotomized the variables to not severe/severe, in which 0=not at all, 1=a little bit and 2=somewhat were coded as “not severe”. 3=quite a bit and 4=very much were coded as “severe”.

### 1.8.8 The Norwegian Prescription Database

The Norwegian Prescription Database (NorPD) from the Norwegian Institute of Public Health was used to measure adherence, persistence, switch of endocrine treatment, and discontinuation among the 220 patients (Fig. 11) (paper III). Information on amount of pills and time points was used to calculate adherence by the use of medication possession ratio (sum of days covered by prescription/number of days patient was observed) and the cut-off for non-adherence was set to 80%. Patients with a lapse of tamoxifen treatment for more than 60 days were classified as discontinuers. As the NorPD does not provide information on patient’s consumption of pills, these measures are associated with uncertainty.



**Figure 11.** Data-flow for individual data from the pharmacies or other data sources, via Statistics Norway to the Norwegian prescription database and an additional data source, which provides data files to researchers. Figure reproduced with courtesy of Prof. Kari Furu at the Norwegian Institute of Public Health.

## 2 Aims

Substantial evidence for inter-individual differences in the ability to metabolize tamoxifen exists. However, it has not been determined if these inter-individual differences are predictive of tamoxifen efficacy and toxicity. The overall aim of this thesis was to determine the role of active and inactive tamoxifen metabolites in regard to gene regulation, clinical outcome, and side effects. The specific aims were as follows:

- 1) Elucidate the gene regulative roles of endoxifen, 4OHTam, and NDtam in MCF-7 breast cancer cells.
- 2) Determine the predictive value of *CYP2D6* genotyping and direct measurements of active tamoxifen metabolites in serum on clinical outcome in operable breast cancers.
- 3) Elucidate the use of serum tamoxifen metabolite concentrations as biomarkers for side effects.
- 4) Determine the validity of using pharmacy refill data to measure adherence through the use of tamoxifen drug monitoring.



### 3 Summary of results

Paper I: “*The active tamoxifen metabolite endoxifen (4OHNdtam) strongly down-regulates cytokeratin 6 (CK6) in MCF-7 breast cancer cells*”

In this study, global gene expression analyses were performed in MCF-7 breast cancer cells after treatment with three tamoxifen metabolites: 4Ohtam, endoxifen, and NDtam. When comparing expression patterns of genes in MCF-7 cells after treatment with endoxifen and 4Ohtam, endoxifen was the treatment that led to the most pronounced differences in gene expression. NDtam is a tamoxifen metabolite with low affinity to the ER, and our results showed that this metabolite caused no significant difference in gene expression compared to estradiol-treated cells, further verifying its role as an inactive metabolite. In addition, we identified a strong down-regulation of all three isoforms of *CK6* (*KRT6 A, B, C*) after endoxifen treatment compared to control. The three *CK6* isoforms were also down-regulated after estrogen-depletion of the MCF-7 cells and after knockdown of the ER coactivator steroid receptor coactivator 3 (SRC3/AIB1), which implied an ER-dependent regulation of *CK6* expression.

Paper II: “*Serum concentrations of active tamoxifen metabolites predict long-term survival in adjuvantly treated breast cancer patients*”

In this retrospective, observational study comprising 86 operable breast cancer patients with a median follow-up time of 13.9 years, we compared the predictive value of CYP2D6 phenotypes and concentrations of tamoxifen metabolites on long-term survival. Increased serum concentrations of the two active tamoxifen metabolites Z-4Ohtam and Z-endoxifen were significantly correlated to increasing CYP2D6 activity of the patients. None of the remaining metabolites were correlated to CYP2D6 phenotype. No association between the CYP2D6 phenotype and breast cancer outcome was identified. Cox regression analyses showed a log-linear decrease in hazard (0.25) for BCSS for each unit (1 nM) increase of Z-4Ohtam. There was no linear association between BCSS and the remaining metabolites. Using supervised cut-off values representing low concentrations for Z-4Ohtam (3.26 nM) and Z-endoxifen (9.00 nM), we performed univariable survival analyses. The Kaplan-Meier analysis showed that

patients with Z-4OHTam levels  $\leq 3.26$  nM and Z-endoxifen levels  $\leq 9.00$  nM had significantly poorer outcomes compared to patients with levels above these thresholds. Multivariable models adjusting for age, tumor size, nodal status, histological grade, ER and PR status, and chemotherapy did not change the results. A Kaplan-Meier total log-rank test of patients re-grouped into low, intermediate, and high serum concentrations of Z-4OHTam and Z-endoxifen, respectively, demonstrated significant survival difference between these three subgroups for both metabolites. No BCSS events for patients with high concentrations of Z-4OHTam ( $>8.1$  nM) and Z-endoxifen ( $>59.6$  nM) were found. The same differences were also observed in the overall survival analysis.

Paper III: *“Drug monitoring of tamoxifen metabolites predicts vaginal dryness and verifies a low tamoxifen discontinuation rate from the Norwegian Prescription Database (NorPD) in a real-world data set of breast cancer patients.”*

In this prospective longitudinal observational study comprising 220 breast cancer patients treated with adjuvant tamoxifen between 2011 and 2017, we investigated the value of tamoxifen metabolite concentrations as predictors for side effects and adherence. Hot flashes, vaginal problems, joint pain, and decreased libido were the most common side effects in our patient cohort. There was no difference in severity or prevalence for any side effects between years one to three. Patients that reported experiencing vaginal dryness had significantly higher serum concentrations of tamoxifen, tam-N-ox, and Z-4'-OHTam. This was shown using the concentrations as quartiles and as a continuous variable. In addition, patients who reported severe vaginal dryness had significantly higher levels of tamoxifen compared to patients not experiencing severe vaginal dryness. Pharmacy refill data from the NorPD showed a discontinuation rate of 37% after 6.1 years and 97.2% adherence. Using drug monitoring, we could only identify 6% of patients who did not have measurable tamoxifen concentrations during a period covered by a pharmacy refill of tamoxifen. Kaplan-Meier analyses of the two approaches showed strikingly similar curves. Reporting more than normal SHC, vaginal dryness, and not using chemotherapy were significant predictors of tamoxifen discontinuation.

## 4 General discussion

### 4.1 Tamoxifen metabolites in regulation of gene expression

Tamoxifen itself is regarded as a weak antiestrogen and is dependent on the enzymatic biotransformation to form the potent antiestrogens endoxifen and 4OHTam. Large variations in steady-state concentrations of these metabolites, in particular endoxifen, are found between breast cancer patients. A central hypothesis is that certain levels of these metabolites must be reached to achieve the full therapeutic benefit of tamoxifen. Both metabolites have similar affinity to the ER, but endoxifen may be found at up to 10-fold higher concentrations compared to 4OHTam and is therefore regarded as the main effector of anti-estrogenic activity. However, several studies have reported that it is not only the concentration difference that separates the metabolites in terms of anti-estrogenic effects. Unique effects on gene expression have been identified for endoxifen (93), and some reports have even suggested endoxifen may have an ER degradation function (92). In the first study (paper I), we investigated the effects of 4OHTam and endoxifen on gene regulation using the 1:10 concentration ratios that mimicked the clinical setting. When applying a 1.5-fold change compared to control cut off, we found that 57% of the genes regulated by 4OHTam were also regulated in the same direction by endoxifen. The genes that were commonly regulated by both active metabolites were, in general, more strongly regulated by endoxifen. This observation probably reflected the 10-fold concentration difference between the metabolites. Gene ontology analyses indicated regulation of similar function of the two active metabolites, but a stronger effect from endoxifen was also observed here. A gene expression study using 4OHTam and endoxifen at the same concentration found the two metabolites had similar effects on gene expression (94). It has also been demonstrated that increasing doses of endoxifen leads to increasingly differential gene expression (93). We identified 183 unique endoxifen-regulated genes; however, we did not explore the uniquely endoxifen-regulated genes in separate GO analyses. This analysis should be performed in the future to uncover possible unique mechanisms of action between the metabolites in our dataset. The major metabolite NDtam, found in

high concentrations in breast cancer patients, was also included in our analyses. NDTam, similarly to tamoxifen itself, is regarded as a weak antiestrogen (89, 90, 92). Our global gene expression analyses confirmed this perception as we found NDTam to cause minimal change in gene expression compared to control.

In paper I, we also report on CK6 as a potential novel target for tamoxifen and suggest an ER-mediated regulation of all three isoforms of CK6. The potential clinical implication of this finding remains to be investigated. The possible down-regulation of CK6 as an unspecific response to decreased proliferation in breast cancer cells should be investigated. This investigation could be performed with proliferation-inhibiting agents that do not mediate their effect through the ER. Anti-proliferative experiments with breast cancer and/or normal cells that do not express ER could elucidate ERs putative involvement in CK6 regulation. Importantly, it should be determined if the effects we observed on mRNA levels translate to CK6 protein levels. Most importantly, experiments should be designed to examine whether down-regulation of CK6 is related to the anti-cancer effect of tamoxifen or if it is a potential marker for endocrine sensitivity.

#### **4.2 The value of CYP2D6 phenotypes and active metabolite concentrations as predictive factors of tamoxifen efficacy**

In the second study (paper II) we used breast cancer outcome endpoints to compare the value of tamoxifen metabolite concentrations and CYP2D6 phenotyping as predictors of tamoxifen efficacy. CYP2D6 phenotype can explain as much as 60% of the endoxifen concentrations (191-194), and as expected, we found concentrations of Z-endoxifen significantly increased with increased CYP2D6 activity. Z-4OHTam concentrations were also positively correlated to CYP2D6 phenotypes; however, the correlation was borderline significant ( $p=0.05$ ). Generation of 4OHTam from tamoxifen is less dependent on CYP2D6, and several additional enzymes known to be polymorphic contribute to its generation (Fig. 9). Polymorphism in CYP2D6 is therefore not regarded as a strong predictor of 4OHTam concentrations. Based on the premise that CYP2D6 phenotype is predictive of endoxifen concentrations, and

endoxifen concentrations being a putative predictor for tamoxifen efficacy, a large number of studies have investigated the association between CYP2D6 phenotypes and clinical outcomes (111). As outlined in section 1.6.1, the results of these studies have been heterogeneous, and CYP2D6 phenotype is currently not regarded as a valid biomarker for tamoxifen efficacy. Our data support this decision as we could not identify an association between CYP2D6 phenotypes and BCSS. Several factors may contribute to the lack of reproducibility in the association between CYP2D6 and outcome. As mentioned, the rationale for using CYP2D6 phenotypes to predict tamoxifen efficacy is based on the notion that CYP2D6 can predict endoxifen concentrations. However, several other factors contribute to steady-state endoxifen concentrations including the contribution of other polymorphic enzymes (115, 116, 125, 126), clinical factors such as age (195) and weight (116), and the co-administration of CYP2D6 inhibitory drugs (99). Disregarding all these factors and using CYP2D6 alone as a predictor for tamoxifen efficacy, therefore, seems to be a naïve approach. If one is to use an indirect approach to estimate endoxifen concentrations, an algorithm that takes CYP2D6 phenotypes and all these variables into account should be created (121, 196).

An alternative approach that circumvents these issues is to measure concentrations of active metabolites directly. We applied this approach and demonstrated an association between adverse breast cancer outcome and low concentrations of Z-endoxifen. This is in line with two former studies (114, 118) that also identified an association between low concentrations of Z-endoxifen and adverse long-term outcomes. Our results therefore suggest that direct measurement of active metabolites is a superior approach in prediction of tamoxifen efficacy compared to using the CYP2D6 phenotype. This notion is further strengthened by the observation that our Z-endoxifen and Z-4OHTam cut-off values for poor prognosis included patients from all CYP2D6 phenotypes. However, it should be taken into account that due to our low patient number, we had low power to detect differences in outcome between the CYP2D6 phenotypes.

An original finding in our second study (paper II) is the promising predictive power of Z-4OHTam, which has often been overshadowed by endoxifen. Despite exhibiting equally high binding affinity to the ER, endoxifen has earned its superior status over

4OHTam due the supposed 10:1 concentration ratio between the two metabolites. This is the reason we used the “biological concentration ratio” of 10:1 in paper I. Intriguingly, in paper II, we observed that the Z-endoxifen/Z-4OHTam concentration ratio among the patients varied from 2:1 to 9:1 (median 5:1) (unpublished data). Thus, the concept “biological concentration ratio” should be probably reconsidered. The diversity in concentration ratios of Z-endoxifen and Z-4OHTam in our study may explain their equal value in predicting breast cancer outcomes. In the clinical setting, both tamoxifen and all metabolites compete to bind to the ER simultaneously. Algorithms to calculate the total anti-estrogenic activity score (AAS) have been developed (109). However, use of AAS did not improve the ability to predict outcomes in paper II. Hence, we recommend using measurements of Z-endoxifen and Z-4OHTam in clinical monitoring of breast cancer patients.

Three studies, including ours, have reported three different Z-endoxifen cut-off values associated with adverse outcomes. Saladores et al. (114) separated the patients into Z-endoxifen level quartiles and demonstrated adverse outcome for patients in the lowest quartile (<14.15 nM) compared to the highest quartile (>35 nM). Madlensky et al. (118) identified patients with Z-endoxifen levels below 16 nM as being an at-risk subgroup by exploring dichotomized optimal cut-off points for a split-regression analysis. This concentration cut off (16 nM) corresponded closely to the lowest quintile in their dataset. We failed to validate Madlensky’s and Saladore’s cut offs, but instead identified a unique cut off for our patient population using a supervised approach to search for an optimal cut-off point based on the multivariate “highest Wald” method. Although discrepancies in the cut offs between the three studies are apparent, the studies all identified a sub-group of patients in the lower extremities of active metabolite concentrations that had adverse outcomes. As mentioned above, many factors influence Z-endoxifen concentrations, and as we show in paper III, the concentrations of Z-endoxifen may vary as much as 20% within the same patient at different time points. Therefore, a nanomolar-specific therapeutic threshold identified in one population will be challenging to replicate in an independent patient cohort. However, if more studies show similarly low concentrations to be associated to adverse outcome, i.e. providing clinical validity, a consensus threshold taking into

account all reported cut offs should be set. The consensus threshold should add a safety margin of a pre-determined amount of nanomolars to ensure capture of all patients at risk for under-treatment. The validated therapeutic threshold can then be utilized in the clinical setting by means of therapeutic drug monitoring.

### **4.3 Therapeutic drug monitoring**

The goal of therapeutic drug monitoring (TDM) is to personalize and optimize treatment by guiding a patient's dosing regimen based on drug concentration measurements. If the association between clinical outcome and steady-state tamoxifen metabolites could be validated, TDM would represent a promising approach to increasing the treatment efficacy of tamoxifen, avoiding unnecessary treatment of patients without response, and most importantly, avoiding under-treatment and reducing the number of recurrences among tamoxifen users. Further potential benefits of TDM may include increased compliance, dose adjustment in patients with renal or hepatic dysfunction (197), and detection of novel drug interactions.

When or if a consensus threshold for active metabolites is established, TDM can be applied in tamoxifen users by measuring the levels of active metabolites at steady state by LC-MS/MS (Fig. 12). Patients with active metabolite levels below the consensus-thresholds could be dose escalated and measured again to ensure the thresholds have been reached. For patients that do not reach the threshold after dose escalation, a switch to an alternative endocrine treatment could be advised. Another possible use of TDM is in safely reducing dosing in patients with severe side effects while ensuring threshold levels are maintained. Dose reductions may also be applicable in patients with very high concentrations of endoxifen, a finding indicated by Love et al. (148), to have a negative impact on recurrence-free survival.

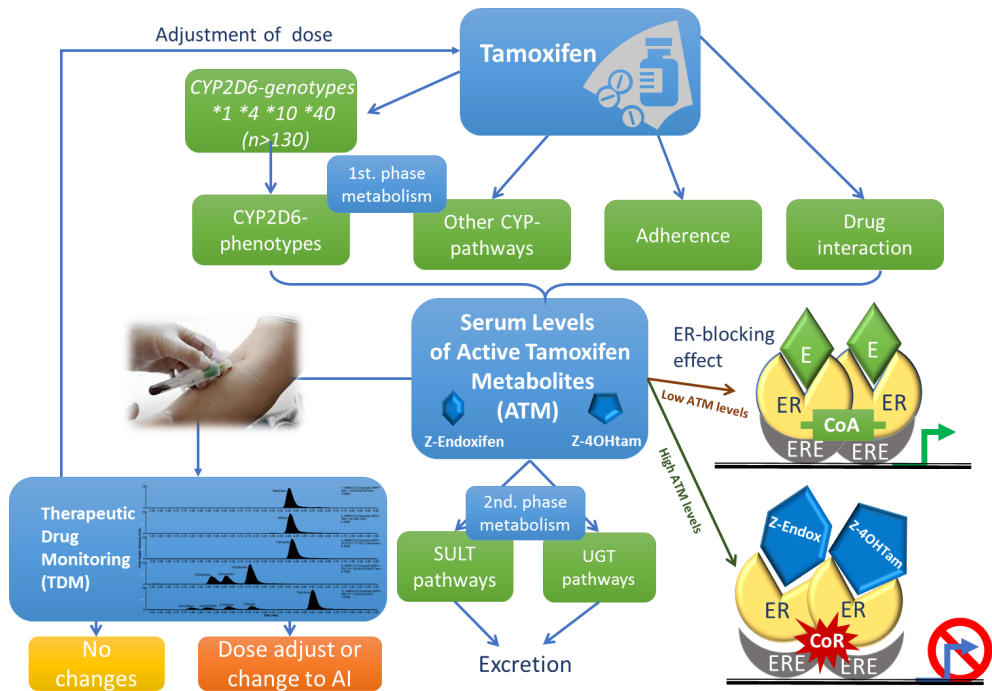
Several studies have indicated that dose escalation increases tamoxifen efficacy. A meta-analysis of 20 trials comprising patients with early breast cancer using tamoxifen for 5 years indicated a trend toward reduction of recurrence in patients using higher doses (30 mg or 40 mg versus 20 mg) (198). Dose relationships on anti-estrogenic activity have also been demonstrated *in vitro* (93, 199). Importantly, a positive

correlation between serum and tissue concentrations of tamoxifen metabolites have been shown, which ensures that blood sampling of systemic concentrations are representative for all tissues (200).

Recently, a TDM approach was piloted in 205 breast cancer patients using tamoxifen (201). Serum steady-state concentration levels of tamoxifen metabolites were measured, and the threshold suggested by Madlensky et al (16 nM) (118) was used as a putative therapeutic threshold. In this study, they found that 22% of the patients using 20 mg of tamoxifen had not reached the threshold and that dose escalation of these patients to 30 or 40 mg resulted in 96% of patients obtaining serum concentrations above the threshold. Importantly, no significant increase in toxicity was observed. Two other studies increasing doses from 20 to 30 and 40 mg based on CYP2D6 genotypes also showed significant increases in endoxifen steady-state concentrations (109, 202). Notably, the median concentration of poor metabolizers in one of the studies changed from 6.7 nM to 33.5 nM, thereby increasing from below to well above the Madlensky threshold of 16 nM, after switching from 20 mg to 40 mg (202). The study cohort was later expanded, and the significant endoxifen increase was further validated. Quality of life (QoL) parameters after dose escalation showed no significant move towards poorer QoL, further indicating that TDM can be safely practiced (171). The study had a 4-month follow-up period for evaluating side effects, so the findings must be validated in studies with longer follow-up times. Notably, a recent study also suggested that drug monitoring of tamoxifen would be cost-effective for health systems (203).

In conclusion, before TDM can be implemented for tamoxifen users, a clear relationship between systemic concentrations of active metabolites and tamoxifen efficacy must be validated.





**Figure 12. Illustration of therapeutic drug monitoring during tamoxifen treatment.** ER, estrogen receptor; CoA, coactivator; CoR, corepressor; ERE, estrogen response element; UGT, Uridine 5'-diphospho-glucuronosyltransferase; SULT, Sulfotransferase.

#### 4.4 Metabolite profiling and side effects

Tamoxifen treatment has been extended to 10 years based on results from the ATLAS and aTTom trials (81, 82) that showed improved outcomes after prolonged treatment. This is positive news, however up to 50% of patients outside clinical trials discontinues tamoxifen treatment before 5 years (142, 143). In the third study (paper III), we found a discontinuation rate of 37% using the NorPD, with a median follow-up time of 3 years. Longitudinal drug monitoring of tamoxifen confirmed these rates. High discontinuation rates among tamoxifen users are particularly alarming in light of the benefits of prolonging tamoxifen treatment to 10 years. Determining both clinical and biological predictors for discontinuation is therefore warranted. We identified vaginal dryness, chemotherapy-naïve status, poor adherence, and reporting more than

normal health complaints as significant predictors of tamoxifen discontinuation. Side effects have repeatedly been associated with tamoxifen discontinuation (143, 157, 204, 205), but biological markers for prediction of which patients will experience side effects are scarce. Some studies have suggested a link between high levels of tamoxifen metabolites and the prevalence or severe side effects (167-169). We demonstrated that high concentrations of tamoxifen, Z-4'-OHTam, and tamNoX at year 2 after surgery were predictive of vaginal dryness and further found vaginal dryness to be predictive of discontinuation. This finding may have clinical implications. Vaginal dryness among patients undergoing anti-hormonal breast cancer treatment is believed to be associated with vulvovaginal atrophy (VVA), a condition associated with decreased estrogenization of vaginal tissue. This is particularly relevant for AIs, which lower the systemic estrogen levels in a non-tissue specific manner. For tamoxifen, the situation is more complex as tamoxifen's anti-estrogenic effects are tissue specific, and for the vaginal epithelium, tamoxifen has been reported to have both estrogenic and anti-estrogenic effects (206). Still, vaginal dryness is a widely reported side effect of tamoxifen. In our data, tamoxifen concentration itself was the strongest predictor of vaginal dryness, not concentrations of the active metabolites, which are the main inducers of the anti-estrogenic effect. One putative explanation for this is that tamoxifen-induced vaginal dryness could be ER independent. Tamoxifen's lipophilic properties have been shown to allow for accumulation and disturbance of the function of phospholipid bilayer cell membranes (207, 208) and could potentially disturb the lubrication function of vaginal epithelial cells. Regardless of the mechanisms by which tamoxifen causes vaginal dryness, the symptom should be addressed as it is frequently noted as one of the most unpleasant side effects of hormonal treatment (209), and patients rarely discuss these problems with their healthcare providers (210). If the cause is lack of estrogen stimulation of the vaginal tissue, VVA symptoms can be effectively relieved by administration of vaginal estrogens. Although effective, oncologists do not consider this treatment to be safe for patients with hormone-sensitive breast cancer. Non-hormonal treatments such as lubricants are commonly prescribed, however these treatments are only considered effective by 30% of oncologists (211). Taken together, more attention should be drawn to vaginal problems

in order to prevent tamoxifen discontinuation and greater risk of relapse. Monitoring of tamoxifen metabolite concentrations may help in early detection of breast cancer patients at greater risk for vaginal dryness. However, as we only found this association at year 2 after surgery, more studies with a higher number of patients should be conducted.

## 5 Conclusions

The following conclusions can be drawn from the studies in this thesis:

- I. Based on biologically relevant concentration ratios, endoxifen was the tamoxifen metabolite that caused the largest effects on global gene expression in MCF-7 breast cancer cells when compared to NDtam and 4OHTam. This observation further confirms endoxifen as the most important active metabolite in the execution of the anti-ER effect of tamoxifen.
- II. The mRNA expression of *KRT6A*, *B* and *C* were down-regulated by active tamoxifen metabolites, estrogen deprivation, and SRC-3 knockdown in MCF-7 breast cancer cells, suggesting that the expression of *CK6* is regulated through the ER.
- III. Low levels of Z-endoxifen and Z-4OHTam were associated with adverse clinical outcome for patients using tamoxifen, while CYP2D6 phenotypes were not associated with breast cancer outcome. The therapeutic thresholds for these active tamoxifen metabolites must be probably be reached to achieve the full clinical anti-ER effect of tamoxifen. Considerable overlap of Z-endoxifen and Z-4OHTam concentrations between the CYP2D6 phenotype groups suggest that CYP2D6 is not a suitable biomarker for tamoxifen efficacy.
- IV. High levels of tamoxifen, tamNoX, and Z-4'OHTam serum concentrations were associated with vaginal dryness in breast cancer patients two years after surgery. TDM may be used to predict which patients will have a greater risk of experiencing vaginal dryness.
- V. Drug monitoring of tamoxifen metabolites confirmed the NorPD as a reliable source of adherence among breast cancer patients using tamoxifen.

## 6 Future perspectives

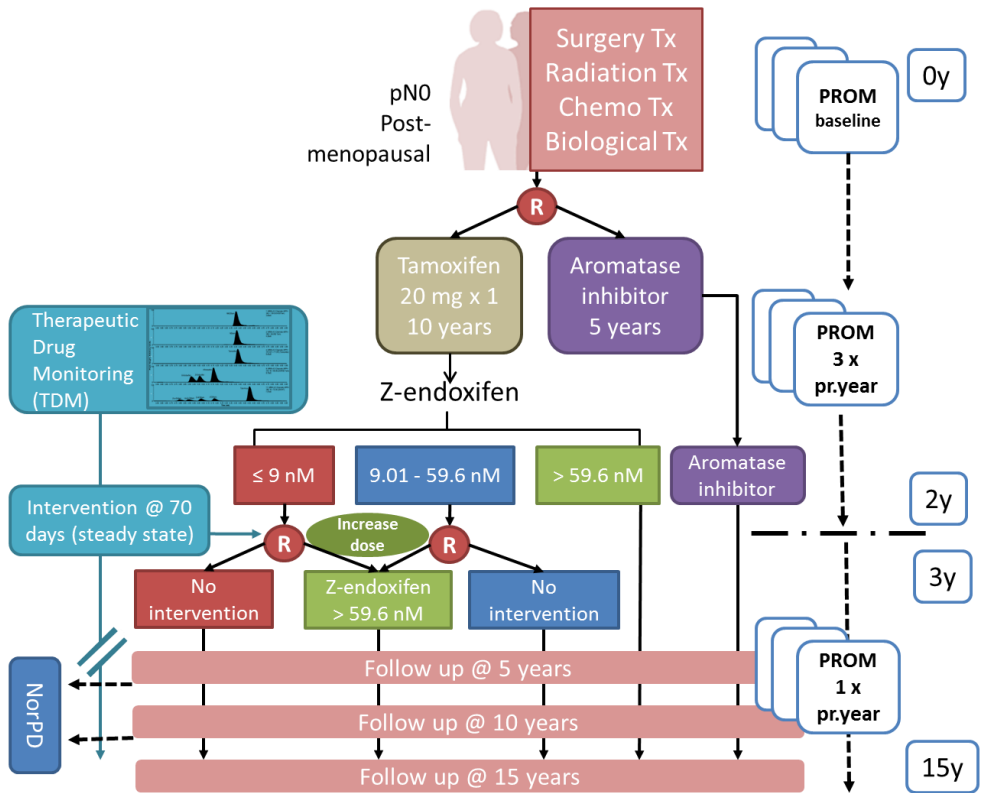
This thesis has shed light on several aspects of the functional and clinical roles of active tamoxifen metabolites. The pre-clinical global gene expression patterns induced by NDtam, 4Ohtam, and endoxifen showed that endoxifen uniquely regulated over 180 genes (paper I). The role and functions of these genes should be further explored by applying gene ontology, pathway or gene set enrichment analyses of genes uniquely regulated by endoxifen. Such analyses could be compared to similar analyses performed on genes uniquely regulated by 4Ohtam to compare the differences in the gene regulating effects of these two active metabolites. The possible functional effects of reduced *CK6* mRNA levels in breast cancer cells should also be further studied to ascertain if endoxifen-mediated down-regulation of *CK6* is part of the anti-proliferative effects of tamoxifen or other cellular processes. As *CK6* is part of the cytoskeleton in the cancer cell, we may speculate that such strong down-regulation induced by endoxifen may be a novel mechanism of the apoptotic process. We endorse performing an electron microscopy study of endoxifen-treated MCF-7 cells as this would provide more insight in the changes into the cytoskeleton structure before and after Z-endoxifen treatment. The strong down-regulation of *CK6* was observed after treatment of MCF-7 cells with high levels of endoxifen (1000 ng/mL), and the regulation of *CK6* may be dose-dependent. In paper II, we showed that patients with the highest Z-endoxifen levels had 100% breast cancer-specific survival. Consequently, studies on apoptosis and EMT processes in regard to *CK6* should be performed with increasing doses of Z-endoxifen to elucidate dose-response patterns in these important cellular programs. MCF-7 cells are classified as luminal A breast cancer cells. Among all luminal breast cancer patients two thirds have luminal A and one third have luminal B cancers. Luminal B cancers comprise increased proliferation and therefore increased benefit from chemotherapy, while luminal A cancers do not (212). Obviously, it will be of great interest to investigate the role of luminal A vs. B status on various concentrations of Z-endoxifen and Z-4Ohtam. A plausible hypothesis is that luminal B cancers will need higher serum concentrations of Z-endoxifen and Z-4Ohtam than luminal A cancers to achieve the same anti-tumor

effect on micro metastases. In this scenario, TDM will offer a feasible solution for clinicians to meet this challenge. To investigate this, dose-response proliferation experiments comparing increasing doses of active tamoxifen metabolites on luminal A and B cell lines, could be applied.

Before TDM of tamoxifen can be introduced to clinical situations, we need to validate our findings in independent patient materials. The optimal patient materials for validation are large randomized tamoxifen studies like ATAC (62) and BIG-1 98 (75) trials. However, these trials did not draw serum samples during follow-up. Therefore, validation must be done retrospectively in observational studies with adequate follow-up times. Through collaboration within the breast cancer research network in Norway, we have recently gained access to the Secondary Adjuvant Therapy with Taxotere (SATT) study (184). This is a reasonable large study comprising >500 patients treated adjuvantly with tamoxifen with a 12-year follow-up period. This unique material represents an excellent opportunity to validate the association between low levels of active tamoxifen metabolites and poor long-term survival.

Interestingly, among the breast cancer patients with the highest serum concentrations of Z-endoxifen ( $\geq 59.6$  nM) or Z-4OHTam ( $\geq 8.1$  nM), there were no breast cancer-related deaths (paper II). In order to validate the clinical relevance of such high serum concentrations of Z-endoxifen and/or Z-4OHTam, a new prospective randomized study is suggested. Notably, two aspects of adjuvant endocrine systemic treatment in postmenopausal patients allow for such a randomized trial. First, in the BIG 1-98 trial, there was no difference in relapse-free survival between the AI (letrozole) arm and the tamoxifen arm in postmenopausal node-negative breast cancer patients (75). Secondly, the high fracture rate in postmenopausal Scandinavian women (213) suggests choosing tamoxifen over AIs is wise in lymph node-negative patients who are at low risk for relapse. In such a study, postmenopausal lymph node-negative breast cancer patients would be randomized to either AI or tamoxifen after standard primary treatment according to treatment guidelines (Fig. 13). All patients should be followed for 15 years, as luminal breast cancer comprises late recurrences and deaths of breast cancer (27). In the tamoxifen arm, serum concentrations would be measured after 70 days, at steady state. Due to substantial intra-individual variation in serum concentrations, at

least two independent blood samples should be drawn. Based on an average of the two measurements of tamoxifen metabolites, the patients would be grouped into three phenotypes: “Green patients,” comprising high serum Z-endoxifen above 59.6 nM; “Blue patients,” comprising serum Z-endoxifen concentrations between 9.0 and 59.9 nM and “Red patients,” comprising low serum levels of Z-endoxifen below 9.0 nM. The blue and red subgroups should be further randomized into “No intervention” or “Incremental increase of tamoxifen dose” until Z-endoxifen > 60 nM (“Green group”). Final long-term survival analysis would take place at 15 years, with planned interim analysis at 2 years (oncological safety), 5 years (short-term survival) and 10 years (intermediate-term survival). Moreover, the study should be adequately powered and patients on tamoxifen should be regularly monitored to keep them at the desired serum concentration. Adherence to tamoxifen would be assessed by drug monitoring, while adherence to AI could be determined from NorPD (paper III). Even better, development of LC-MS/MS methods for AIs (anastrozole, letrozole and exemestan) would increase the quality of estimation of the discontinuation rate of AIs. Finally, all side effects should be structurally followed and reported through PROMs. Such a trial would also allow for verification of relationships between tamoxifen, Z-4’OHtam, and tamNoX metabolites and vaginal dryness (paper III) and other possible associations that might not have been identified in paper III. Notably, during the first 2 years we suggest more frequent monitoring of side effects (Fig. 13) in order to capture differences between patients during the first period of anti-estrogenic treatment.



**Figure 13. Randomized controlled trial of adjuvant endocrine treatment in node-negative postmenopausal breast cancer patients: aromatase inhibitor vs TDM adjusted Tamoxifen.** pN0, lymph node negative; Tx, treatment; R, randomization; nM, nanomolar; @, at; PROM, Patient Reported Outcome Measures, NorPD, Norwegian prescription database.

The patient cohort in paper III originated from the ongoing PBCB project, which is a population-based regional observational study at Haukeland University Hospital and Stavanger University Hospital (214). These patients are monitored for 10+ years using liquid biopsies (i.e. blood and urine) every 6 months. Long-term follow up of these patients will ensure insight into longstanding discontinuation rates among tamoxifen users. Long-term adherence data on tamoxifen use is of particular clinical value now that treatment time has been extended to 10 years for many patients (81, 82).



## **Closing remarks**

Tamoxifen has been in use for more than 50 years, but it still may not be the optimal treatment for some patients due to individual variation in its metabolism. This topic is the crux of this thesis and should not be ignored as 10% of the luminal patients will present with low serum concentrations of Z-endoxifen and Z-4Ohtam. Thus, a considerable number of luminal breast cancer patients using tamoxifen may become undertreated and at higher risk of relapse. In the coming years, it is therefore of critical importance to provide clinical validity for the association between active tamoxifen metabolite concentrations and tamoxifen treatment efficacy. From there, drug monitoring and individualized tamoxifen dosing for the largest subgroup of breast cancer patients can be implemented in the clinical setting. This would be a small step for the medical laboratory, but a giant leap towards the ultimate goal: a reduction in the mortality rate of luminal breast cancer.

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RESEARCH ARTICLE

# The Active Tamoxifen Metabolite Endoxifen (4OHNDtam) Strongly Down-Regulates Cytokeratin 6 (CK6) in MCF-7 Breast Cancer Cells

Thomas Helland<sup>1</sup>\*, Jennifer Gjerde<sup>1,2</sup>\*, Simon Dankel<sup>1,2</sup>, Ingvild S. Fenne<sup>1,2</sup>, Linn Skartveit<sup>1,2</sup>, Andreas Drangevåg<sup>1,2</sup>, Olivera Bozickovic<sup>1,2</sup>, Marianne Hauglid Flågeng<sup>1,2</sup>, Håvard Søyland<sup>2,3</sup>, Gunnar Mellgren<sup>1,2</sup>, Ernst A. Lien<sup>1,2</sup>\*

**1** Hormone Laboratory, Haukeland University Hospital, Bergen, Norway, **2** Department of Clinical Science, University of Bergen, Bergen, Norway, **3** Section for Breast and Endocrine Surgery, Stavanger University Hospital, Stavanger, Norway

☞ These authors contributed equally to this work.

\* [Ernst.Lien@k2.uib.no](mailto:Ernst.Lien@k2.uib.no)



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## Abstract

### Introduction

Tamoxifen is an anti-estrogen drug used in treatment of Estrogen Receptor (ER) positive breast cancer. Effects and side effects of tamoxifen is the sum of tamoxifen and all its metabolites. 4-Hydroxytamoxifen (4Ohtam) and 4-hydroxy-*N*-demethyltamoxifen (4OHNDtam, endoxifen) both have ER affinity exceeding that of the parent drug tamoxifen. 4OHNDtam is considered the main active metabolite of tamoxifen. Ndemethyltamoxifen (NDtam) is the major tamoxifen metabolite. It has low affinity to the ER and is not believed to influence tumor growth. However, NDtam might mediate adverse effects of tamoxifen treatment. In this study we investigated the gene regulatory effects of the three metabolites of tamoxifen in MCF-7 breast cancer cells.

### Material and Methods

Using concentrations that mimic the clinical situation we examined effects of 4Ohtam, 4OHNDtam and NDtam on global gene expression in 17β-estradiol (E<sub>2</sub>) treated MCF-7 cells. Transcriptomic responses were assessed by correspondence analysis, differential expression, gene ontology analysis and quantitative real time PCR (Q-rt-PCR). E<sub>2</sub> deprivation and knockdown of Steroid Receptor Coactivator-3 (SRC-3)/Amplified in Breast Cancer 1 (AIB1) mRNA in MCF-7 cells were performed to further characterize specific effects on gene expression.

### Results

4OHNDtam and 4Ohtam caused major changes in gene expression compared to treatment with E<sub>2</sub> alone, with a stronger effect of 4OHNDtam. NDtam had nearly no effect on the global

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gene expression profile. Treatment of MCF-7 cells with 4OHNDtam led to a strong down-regulation of the CytoKeratin 6 isoforms (*KRT6A*, *KRT6B* and *KRT6C*). The CytoKeratin 6 mRNAs were also down-regulated in MCF-7 cells after E<sub>2</sub> deprivation and after SRC-3/AIB1 knockdown.

## Conclusion

Using concentrations that mimic the clinical situation we report global gene expression changes that were most pronounced with 4OHNDtam and minimal with NDtam. Genes encoding CytoKeratin 6, were highly down-regulated by 4OHNDtam, as well as after E<sub>2</sub> deprivation and knockdown of SRC-3/AIB1, indicating an estrogen receptor-dependent regulation.

## Introduction

The Selective Estrogen Receptor Modulator (SERM) tamoxifen is used in breast cancer treatment and prevention. It may act as a full estrogen agonist, partial agonist or antagonist depending on the dose, species, or target organ [1]. Tamoxifen is regarded as a pro-drug since two of its metabolites, 4-hydroxytamoxifen (4OHTam) and 4-hydroxy-*N*-demethyltamoxifen (4OHNDtam, endoxifen), both have Estrogen Receptor (ER) affinity markedly exceeding that of tamoxifen itself [2–4]. 4OHNDtam is considered the main active metabolite of tamoxifen since it has 100-fold higher affinity for the ER than tamoxifen and its serum levels are 10-fold higher than that of 4OHTam [5–9]. During steady state tamoxifen treatment the concentrations of 4OHTam, 4OHNDtam and *N*desmethyltamoxifen (NDtam) are roughly present in serum in concentrations 5, 50 and 150% respectively compared to that of tamoxifen [10–12]. However in the clinical situation, these concentrations vary up to tenfold between patients using an identical daily dose, furthermore the concentrations increase by increasing age [9, 13].

Properties of tamoxifen metabolites may be studied in the ER positive human breast cancer cell line MCF-7. The majority of *in vitro* studies on effects of tamoxifen are using 4OHTam as single drug, whereas studies including 4OHNDtam as single drug are used only in few *in vitro* studies. Lim *et al* observed that 4OHNDtam and 4OHTam have similar effects on the global expression pattern in MCF-7 cells, especially on the estrogen-regulated genes [14]. Hawse *et al* also studying global gene expression in MCF-7 cells observed that 4OHNDtam molecular mechanism of action was concentration dependent and different than that of other anti-estrogens [15]. High but not low concentrations of 4OHNDtam resulted in induction of cell cycle arrest and markers of apoptosis [15]. Recently, effects of 4OHNDtam as single drug have been examined in animal studies [16, 17] and at present clinical studies using 4OHNDtam as single drug are underway [15]. NDtam is the major tamoxifen metabolite in serum, but due to a low affinity to the ER NDtam is believed not to influence tumor growth and little attention has been drawn to the compound.

The effects and side effects represent a summary of effects of tamoxifen and all its metabolites. In two earlier studies where the hydroxylated metabolites were not measured, it was observed that the proportion of tamoxifen and its demethylated metabolites NDtam and *N*-desdimethyltamoxifen (NDDtam) in serum was higher in patients with toxicity versus those not experiencing toxicity [18, 19]. More recent studies report that women with higher 4OHNDtam levels are more likely to report side effects [13, 20].

In the present explorative study we examined effects of the major demethylated tamoxifen metabolite NDtam and the hydroxylated potent metabolites 4OHTam and 4OHNDtam on global gene expression in MCF-7 cells using concentrations that are representative for the clinical situation [9, 21]. We also studied differences in effects between 4OHTam and 4OHNDtam which may influence tumor growth and searched for genes that had the most extensive changes in gene expression profile.

## Materials and Methods

### Cell culture

Michigan Cancer Foundation-7 (MCF-7) human breast adenocarcinoma cells [22] were grown at 37°C under 5% CO<sub>2</sub> in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 1% (vol/vol) penicillin/streptomycin solution and 4.5 g/liter glucose. The MCF-7 cell medium also contained 1 μM insulin. The MCF-7 cell line is a commercial cell line in which no ethical approval is required for experiments.

### Cell treatments

**Treatment of MCF-7 cells with tamoxifen metabolites.** The cells were preconditioned in phenol red-free DMEM (Invitrogen, Carlsbad, CA) containing charcoal-stripped fetal bovine serum (Hyclone™, Thermo Fischer Scientific, MA, USA) and the above supplements, for 2 days. The cells were seeded in six-well plates at a density of 300,000 cells/ml and then treated with 10nM E<sub>2</sub> alone or in combination with 4OHNDtam, 4OHTam or NDtam (Table 1) for three days. E<sub>2</sub> and 4OHTam (>70% Z isomer) were purchased from Sigma-Aldrich (Steinheim, Germany) and 4OHNDtam (Z/E isomers 1/1) from Sintef Materials and Chemistry (Oslo, Norway). NDtam was a gift from Imperial Chemical Industries, PLC Pharmaceutical divisions (Macclesfield, UK). Cells were harvested after 3 days of incubation for the microarray analysis. The growth medium from the incubated cell cultures was collected and the concentrations of tamoxifen and its metabolites determined by High Pressure Liquid Chromatography (HPLC)—Tandem Mass Spectrometry (MS/MS) [8, 23].

**Estrogen deprivation and knockdown of SRC-3 in MCF-7 cells.** MCF-7 cells were grown in alpha MEM (Lonza, Belgium) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units penicillin, 100 μg streptomycin and 1 μM insulin for one day and then grown in phenol red-free Alpha MEM supplemented with 5% charcoal-stripped FBS

**Table 1. Concentrations (ng/ml) of tamoxifen and its metabolites in growth media.**

	NDtam	4OHTam Concentration (ng/ml)	4OHNDtam
<b>Calculated</b>			
Day 0	1000	100	1000
<b>Measured</b>			
Day 1	939	72,6	609
Day 2	158	16	64,9
Day 3	119 (93–133)	6.0 (4.0–7.7)	36.6 (26.4–54.4)

Concentrations determined by High Pressure Liquid Chromatography (HPLC)—Tandem Mass Spectrometry (MS/MS). Three measurements performed at day 3. One measurement performed at day 1 and 2.

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for 3 days. MCF-7 cells grown for 3 days with the addition of 10 nM 17 $\beta$ -estradiol (Sigma) were used as control.

Short Hairpin RNA (shRNA) lentiviral transduction was used to generate MCF-7 cells containing stably integrated shRNA SRC-3/AIB1 mRNA as previously described [24]. 68% reduction in SRC-3/AIB1 mRNA expression was obtained after KD of SRC-3 (SRC-3 shRNA) compared to the control shRNA.

## Homogenization and RNA extraction

Lysates from cell samples were harvested in PBS and RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Samples were treated with the RNase-Free DNase Set (Qiagen). Amount and quality of the extracted RNA were measured by the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, ThermoScientific, Waltham, MA, USA) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

## Illumina iScan system

250 ng of RNA from the cell samples treated with E<sub>2</sub>, NDtam, 4OHTam and 4OHNDtam were biotin-labeled and amplified using the Illumina TotalPrep RNA amplification kit (Ambion, Austin, TX, USA) and the Eppendorf Mastercycler (Eppendorf Hamburg, Germany). This procedure involves RNA being reversely transcribed, amplified and biotin-16-UTP-labeled. The biotin-labeled cRNA was thereafter quality and quantity controlled using the Agilent 2100 Bioanalyzer and the NanoDrop ND-1000 spectrophotometer. 1500 ng of cRNA was hybridized to the humanWG-6 v.3.0 expression BeadChip (Illumina, San Diego, CA, USA) and the fluorescence of the biotin-labeled cRNA was detected using the Illumina iScan.

## Microarray data extraction and analysis

**Quality control and preprocessing.** After scanning in the iScan reader the microarray raw data was imported into GenomeStudio software (Illumina) which removed control probes and produced a text file that contained the signal and detection p-values per probe for all samples. This text file was further imported into J-Express 2009 (MolMine AS, Norway) where signal intensity values were quantile normalized and logarithmically transformed (base 2) [25]. Quantile normalization removes obscuring variations that arise from differences in the preparation of the microarray samples. A Correspondence Analysis (CA) and hierarchical clustering with Pearson Correlation as a distance measure were performed to visualize the differential expression between the four differently treated groups and analyze global trends in the data [26].

In adherence to the standards of the Microarray Gene Expression Data Society (mged) the microarray data is publicly available at ArrayExpress under the title "Tamoxifen treatment of MCF-7 breast cancer cells" and accession number (E-MTAB-2729).

**Analyses of differentially expressed genes.** To search for differentially expressed genes in MCF-7 breast cancer cells treated with E<sub>2</sub>, NDtam, 4OHTam or 4OHNDtam a Significance Analysis of Microarrays (SAM) [27] was applied. The SAM analysis calculates the significance of the gene expression based on the deviation between the actual signal intensity and the signal intensity expected by chance. To obtain manageable datasets, differentially expressed genes were defined by q-value = 0. A rank product analysis was set up to examine if there were genes that were more regulated by one metabolite than the other. By using this non-parametric statistical method it was possible to rank the genes according to fold change and compare several rank product lists against each other. The analysis was performed using J-express (Molmine) and Excel (Microsoft).

To analyze which Gene Ontology (GO) functional groups the differentially expressed genes belonged to Protein ANalysis THrough Evolutionary Relationships (PANTHER) (dated, 15. February, 2012) [28] was applied. PANTHER identifies which functional groups are over-represented among a selection of genes and expresses the degree of over-representation with p-values (binomial statistics). The integrated gene ontology analysis in J-Express 2012 (Molmine) software was used to validate the findings from PANTHER. Gene lists used for PANTHER were compared to the entire gene list from the microarray and an over-representation analysis was performed.

### Quantitative-real time-PCR

1  $\mu$ g or 350 ng RNA per reaction was transcribed to cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, GmbH, Mannheim, Germany) and further quantified using the LightCycler480 Probes Master kit (Roche) and the LightCycler480 rapid thermal cycler system (Roche). Each gene was quantified relative to reference genes TATA-binding protein (TBP), glucose-6-phosphate-1-dehydrogenase (G6PD) or peptidylprolyl isomerase A / Cyclophilin A (PPIA) to correct for variations occurring within samples and during sample preparations. The quantification of the genes was performed using specific Universal ProbeLibrary (UPL) probes and target-specific primers, designed at Universal ProbeLibrary (UPL) Assay Design Center (Roche, Applied Science), software version 2.45 (S1 Table). The relative quantification of the genes was performed according to manufactures protocol (Roche Applied Science).

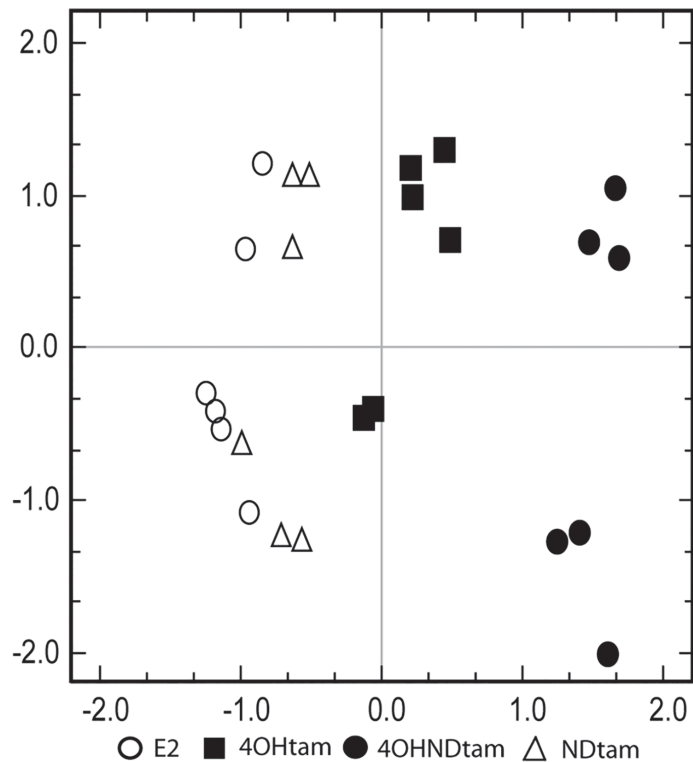
### Results

To elucidate the gene regulative roles of the three tamoxifen metabolites 4OHTam, 4OHNDtam and NDTam in breast cancer, we performed a microarray analysis on MCF-7 cells. The MCF-7 cells were treated with the respective tamoxifen metabolites and  $E_2$  for three days with concentrations intended to mimic physiological concentrations (Table 1). We used the concentration levels found in tissues which are approximately 10 times higher than the concentrations found in serum [29]. Although the inter-individual serum concentrations of tamoxifen and its main metabolites vary tenfold and increase by age [9], the ratio between 4OHNDtam/4OHTam is usually 10/1 in the individual patient [9, 21]. Therefore, we used a concentration of 4OHNDtam ten times higher than that of 4OHTam. To analyze the differential expression on a global level between the four compounds ( $E_2$ , 4OHTam, NDTam and 4OHNDtam), a Correspondence Analysis (CA) was performed (Fig 1). The CA, displaying global gene expression in a two-dimensional plot, showed that the samples treated with 4OHTam and 4OHNDtam were clearly separated from control ( $E_2$ ). 4OHTam and 4OHNDtam shifted gene expression in the same direction, but the shift was greater for 4OHNDtam (Fig 1). The samples treated with NDTam were clustered closely with the  $E_2$  control suggesting limited effect on gene expression in MCF-7 cells.

To further visualize gene expression trends in our dataset we performed a self-organizing map analysis. This clustering analysis was performed on high level mean normalized expression values for genes with a significant expression value ( $q$ -value = 0) when comparing treatment to control ( $E_2$ ). The results showed a stepwise regulation between 4OHTam and 4OHNDtam, where 4OHNDtam resulted in the most differential expression (Fig 2).

A Significance Analysis of Microarray (SAM) identified the differentially expressed genes for each metabolite compared to control ( $E_2$ ). Only six NDTam-regulated genes met the 1.5-fold change and  $q$ -value = 0 cut-off for differential expression, but half of these genes overlapped with one or both of the other treatment groups (Fig 3). Of the 251 genes regulated by



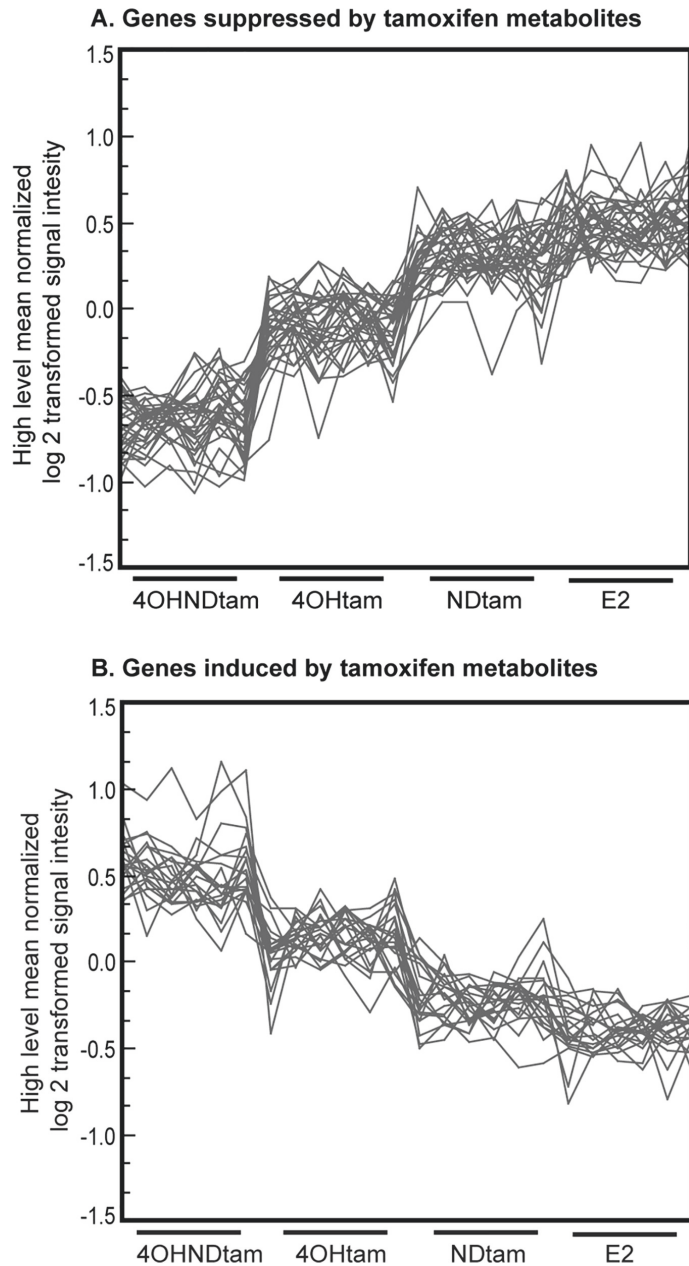


**Fig 1. Correspondence analysis showing projection of MCF-7 cell samples treated with E<sub>2</sub>, NDtam, 4OHTam and 4OHNDtam.** The x-axis displays the first principle component (variance: 8.01%) and y-axis displays the second principle component (variance: 6.959%). The MCF-7 cell samples; MCF-7 treated with only E<sub>2</sub> (blank circle), MCF-7 treated with E<sub>2</sub> and NDtam (blank triangle), MCF-7 treated with E<sub>2</sub> and 4OHTam (black square), and MCF-7 treated with E<sub>2</sub> and 4OHNDtam (black circle).

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4OHNDtam and 115 regulated by 4OHTam, there were 66 overlapping genes, i.e. 57% of the 4OHTam-regulated genes were also regulated in the same direction at least 1.5-fold by 4OHNDtam. Differential expression observed in the microarray (S2–S5 Tables) was confirmed using Q-rt-PCR on a selection of genes with high differential expression (Table 2).

In the gene list for 4OHNDtam treated cells, the cytoke­ratin 6 (KRT6) genes were strikingly down-regulated compared to control (E<sub>2</sub>). *KRT6A* was down-regulated 9.1-fold compared to control while *KRT6B* and *KRT6C* were down-regulated 3.3- and 4.1-fold, respectively (S3 Table). The strong down-regulation of these genes was confirmed by Q-rt-PCR (Table 2). To further explore whether the down-regulation of the KRT6s were regulated through estrogen receptor signaling we did two separate Q-rt-PCR analyses. Firstly, we cultured the cells in absence of E<sub>2</sub> for 3 days and examined the expression levels of the three KRT6s by Q-rt-PCR (Fig 4). Estrogen deprivation of the MCF-7 cells for 3 days nearly abolished the mRNA expression of KRT6A and B. Estrogen deprivation also down-regulated the KRT6C mRNA levels, but to a less extent than KRT6A and B. Secondly, we knocked down the ligand-dependent ER-coactivator Steroid Receptor Coactivator 3 (SRC-3), also known as Amplified in Breast Cancer 1 (AIB1)



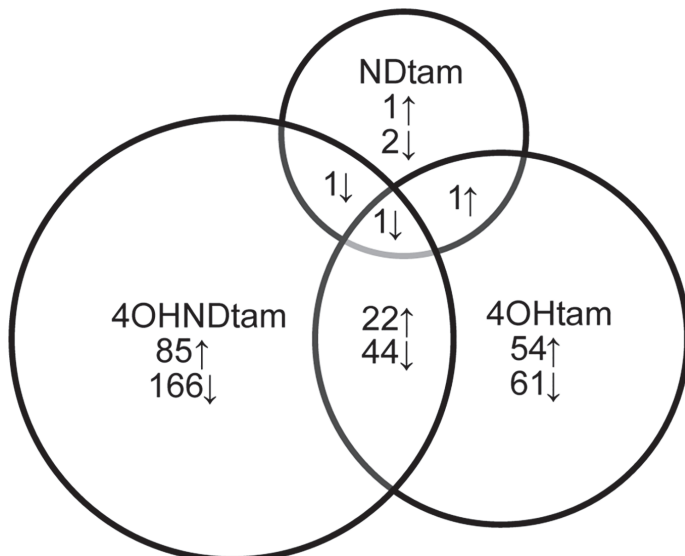
**Fig 2. Self-organizing maps displaying stepwise regulation between 4OHNDtam and 4OHtam.** Self-organizing map displaying the high level mean normalized log<sub>2</sub> transformed signal intensities (y-axis) across different treatments (x-axis). Each treatment has 5 parallel samples (x-axis). Figure A displays a clustering of

down-regulated genes in a stepwise regulation where 4OHNDtam is the strongest down-regulated. Figure B shows a clustering of up-regulated genes regulated in a stepwise regulation with 4OHNDtam being the most up-regulated.

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(Fig 5). SRC-3/AIB1 knockdown also repressed the expression of all three KRT6s. Based on these data, we conclude that the KRT6s are positively regulated by E<sub>2</sub>.

To inform on functions of the differentially expressed genes after treatment with the three different metabolites (fold change  $\geq 1.5$ , q-val = 0) we performed the Gene Ontology (GO) analysis with PANTHER. Differentially expressed genes after treatment with 4OHNDtam and 4OHtam were largely enriched in the same GO categories (Fig 6). However, the categories are more over-represented after treatment with 4OHNDtam compared to 4OHtam. This may reflect the fact that there are more genes that met the cut of criteria for the clinical applicable concentration (1000 ng/mL) of 4OHNDtam (82 $\uparrow$ , 142 $\downarrow$ ) in contrast to the lower concentration (100 ng/mL) of 4OHtam (53 $\uparrow$ , 58 $\downarrow$ ). NDtam on the other hand, has no representation in PANTHER because the fold change of 1.5 only resulted in 3 differentially expressed genes despite having a concentration of 1000 ng/mL. Studying specific categories in PANTHER we observed genes belonging to the category cell cycle to be down-regulated after both treatments (4OHtam and 4OHNDtam), while genes involved in cell adhesion were up-regulated. In addition, treatment of MCF-7 cells with 4OHtam resulted in a clear down-regulation of genes involved in apoptosis, while treatment with 4OHNDtam resulted in both up- and down-regulation of the apoptosis related genes. Of particular note, only 4OHNDtam showed a significant effect on genes related to antigen processing and presentation (up-regulation). To validate our findings in the PANTHER analysis we performed an alternative GO over-representation analysis using



**Fig 3. Venn diagram showing genes uniquely regulated and regulated in the same direction after treatment with tamoxifen metabolites.** Genes included have a fold change  $\geq |1.5|$  and q-value = 0 when comparing tamoxifen metabolites to E<sub>2</sub>. The numbers in the overlapping circles shows the number of genes regulated in the same direction by different treatments.

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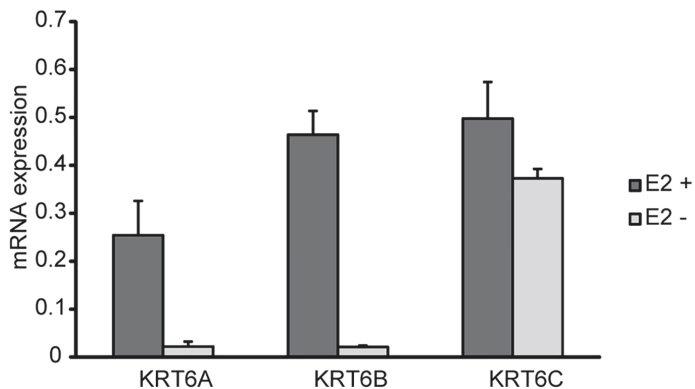
**Table 2. Validation of microarray fold change values using Q-rt-PCR.**

Target gene		NDtam	4OHtam	4OHNDtam
KRT6A	Microarray	0.99	0.93	0.73
	Q-rt-PCR	0.84 (0.52–1.07)	0.49 (0.23–1.06)	0.06 (0.05–0.08)
KRT6C	Microarray	0.99	0.94	0.78
	Q-rt-PCR	1.08 (0.7–1.32)	0.69 (0.24–1.40)	0.11 (0.05–0.12)
CXCR4 <i>tv2</i>	Microarray	0.9	0.694	0.63
	Q-rt-PCR	0.68 (0.51–0.90)	0.45 (0.37–0.54)	0.35 (0.27–0.47)
SERPINA3	Microarray	0.73	0.3	0.12
	Q-rt-PCR	0.64 (0.58–0.69)	0.21 (0.18–0.24)	0.07 (0.05–0.08)
GPER TV4	Microarray	0.92	0.39	0.27
	Q-rt-PCR	0.85 (0.76–0.94)	0.34 (0.26–0.43)	0.24 (0.15–0.35)
CTGF	Microarray	1.14	1.1	2.24
	Q-rt-PCR	1.05 (0.82–1.28)	1.54 (1.26–1.83)	1.15 (0.97–1.32)
COL3A1	Microarray	1.49	2.427	2.56
	Q-rt-PCR	2.03 (1.36–2.70)	5.86 (3.89–7.81)	6.11 (3.46–8.77)
IRX2	Microarray	0.91	0.64	0.43
	Q-rt-PCR	0.88 (0.80–0.97)	0.48 (0.44–0.52)	0.47 (0.42–0.53)
IRX3	Microarray	0.95	0.59	0.55
	Q-rt-PCR	0.85 (0.75–0.97)	0.43 (0.38–0.48)	0.42 (0.35–0.52)
IRX5	Microarray	0.97	0.64	0.57
	Q-rt-PCR	0.94 (0.92–0.97)	0.55 (0.52–0.58)	0.59(0.55–0.64)

Median values of fold change ( $E_2$ /treatment). Numbers in brackets are min-max values. Fold changes calculated from signal intensity (microarray) and concentration (Q-rt-PCR). N = 6 for each treatment.

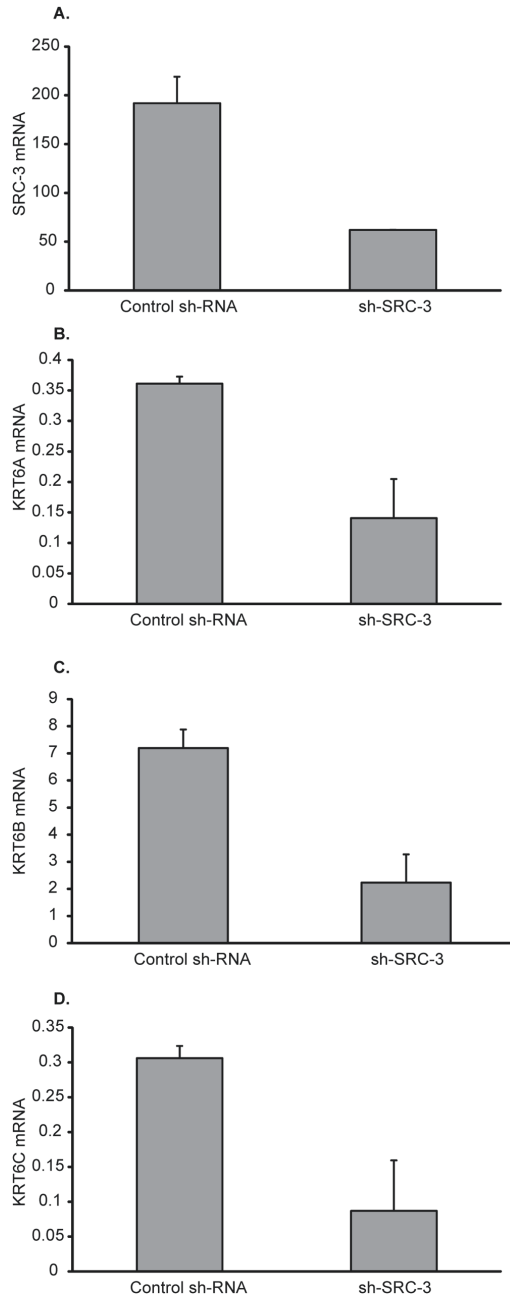
doi:10.1371/journal.pone.0122339.t002

the integrated GO analysis software in J-Express 2012. This analysis resulted in predominantly the same over-represented categories as in PANTHER ([S6 Table](#)).



**Fig 4. KRT6 mRNA expression after estrogen deprivation in MCF-7 cells.** MCF-7 cells were grown in phenol red-free Alpha MEM supplemented with 5% charcoal-stripped FBS for 3 days. Cells grown for 3 days in presence of 17 $\beta$ -estradiol (10 nM) were used as control. The mRNA expression was measured by Q-rt-PCR and the relative expression levels of each gene were related to TBP mRNA. The results presented are mean values with SEM from 3 biological replicates.

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**Fig 5. KRT6 mRNA expression after knockdown of SRC-3 in MCF-7 cells.** **A:** Quantification of SRC-3 mRNA expression in MCF-7 cells infected with shRNA targeting SRC-3 (SRC-3 shRNA) and Control shRNA. **B-D:** mRNA expression of KRT6A, KRT6B and KRT6C in SRC-3 shRNA- and Control shRNA MCF-7 cells. The expression level of each gene is relative to TBP mRNA. The results presented are mean values with SEM from 6 biological replicates.

doi:10.1371/journal.pone.0122339.g005

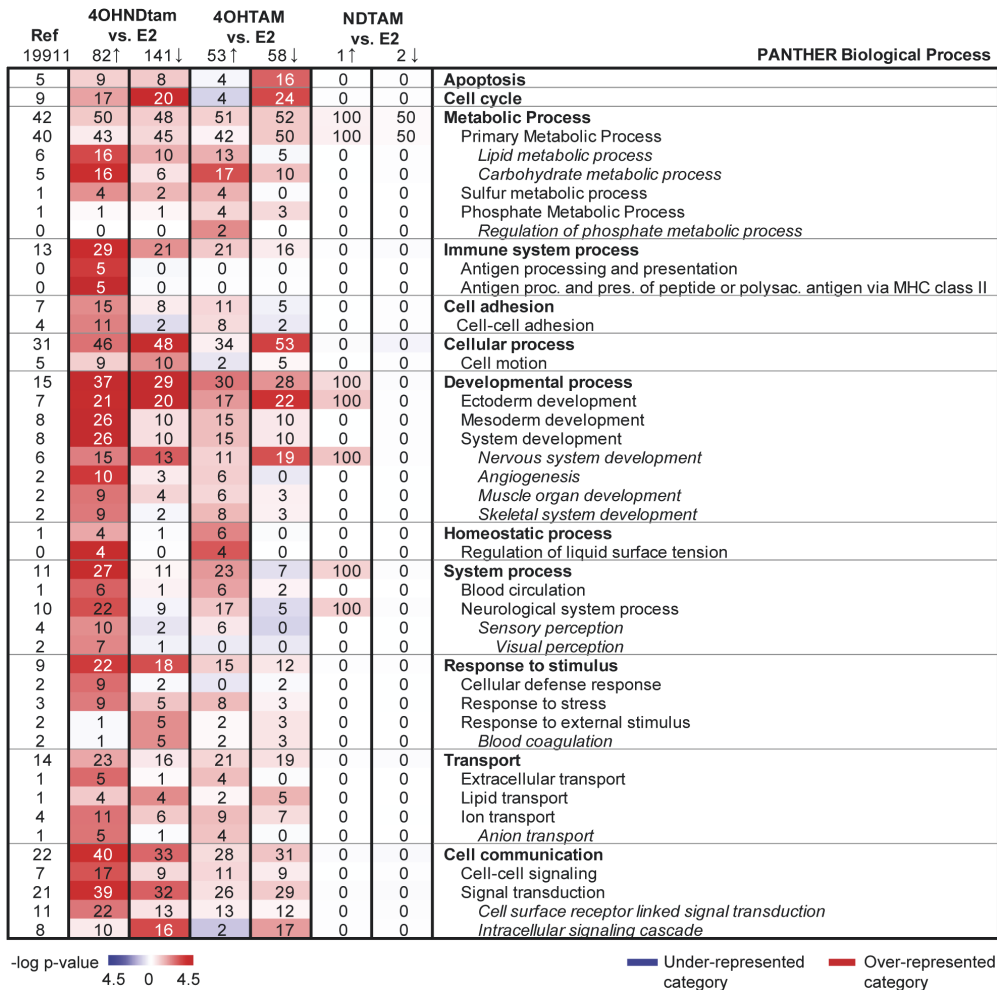
To further analyze the metabolite-specific potency to regulate gene expression, we searched for genes that were differentially expressed after treatment with 4OHTam and at the same time more differentially expressed after treatment with 4OHNDtam (rank product analysis, fold change  $\geq 1.5$  for each step and  $q$ -value  $\leq 0.2$ ). These cut-off values resulted in 20 up-regulated genes and 81 down-regulated genes, that we subjected to PANTHER analysis to evaluate their function (S1 Fig). We found that the categories developmental processes and signaling, including G-protein coupled receptor protein signaling pathway, were over-represented among the down-regulated genes (fold change  $\leq 0.05$ , S7 Table). Genes involved in neurological processes such as sensory perception were found to be over-represented among the up-regulated genes (S8 Table). The cancer related category apoptosis was over-represented with both up- and down-regulated genes, represented by two up-regulated genes (*TNS3*, *EMP1*) and five genes down-regulated (*DNASE1L2*, *CXCR4*, *Gal-7*, *FKBP5*, *SGK1*) (Table 3).

## Discussion

In this paper, we investigated changes in global gene expression in response to three tamoxifen metabolites: 4OHTam, 4OHNDtam and NDtam. The main findings are that 4OHNDtam and 4OHTam strongly altered global gene expression in  $E_2$  treated MCF-7-cells compared to treatment with estrogen alone. The effects of 4OHNDtam and 4OHTam largely overlapped, with an overall stronger response for 4OHNDtam. NDtam had nearly no effect. We moreover identified specific genes that responded selectively to either 4OHNDtam or 4OHTam, providing new molecular insight into metabolite-specific effects with tamoxifen treatment. Our study, using physiological metabolite ratios, improves our understanding of how tamoxifen may mediate its positive and adverse effects *in vivo*.

Effects of NDtam and tamoxifen have previously been studied in MCF-7 cells. Reddel and Sutherland [30], using much higher concentrations than those observed in man (7.5–10  $\mu$ M), observed that NDtam was much more potent than tamoxifen in inhibiting growth. This inhibition of cell proliferation was only partially reversed by  $E_2$  or not reversed at all. The results may be due to direct toxic effects of NDtam and not promoted via the influence on the ER. Hawse *et al* examined effects of 4OHTam, 4OHNDtam and NDtam in MCF-7 cells in an extensive study, however they did not report on results when NDtam was used as a single drug [15]. Even with high concentrations of NDtam, mimicking that observed in man during steady state treatment, our microarray analysis clearly demonstrated that NDtam had little impact on global gene expression in  $E_2$ -treated MCF-7 cells. This indicates that 4OHTam and 4OHNDtam are the main contributors to the anti-estrogenic effects of tamoxifen. However, NDtam, as the major tamoxifen metabolite, may still contribute to toxic effects such as crystalline retinal deposits, macular edema, and corneal changes that have been observed previously [31, 32].

In line with others [15, 33], we observed that treatment with 4OHTam and 4OHNDtam resulted in differential gene expression in  $E_2$  treated MCF-7 cells when compared to MCF-7 cells treated with  $E_2$  alone. A clear shift in gene expression was seen in the CA (Fig 1) after the treatment with 4OHTam and 4OHNDtam, however the shift was greater for 4OHNDtam than with 4OHTam. The CA showed a shift in gene expression in the same direction for 4OHTam and 4OHNDtam. When comparing the GO of the two metabolite effects separately (Fig 6), we found that genes in categories related to cancer processes such as intracellular signaling cascade



**Fig 6. Functional categorization of differentially expressed genes in MCF-7 cells after treatment with tamoxifen metabolites.** PANTHER was used to search for over-represented categories in the ontology class Biological Process. A significance of microarray analysis (SAM) was applied to search for genes that were differentially expressed after treatment with the three different tamoxifen metabolites. Genes with  $q\text{-val} \leq 0$  and fold change  $\geq 1.5$  were selected from the SAM analysis. A  $p\text{-value} \leq 0.05$  was used as inclusion criterion for categories. The reference column at the left of the table displays the percentage of genes that belongs to a specific category when analyzing the whole human NCBI genome (19,911 genes), e.g. 5% of the 19,911 genes belong to the "Apoptosis" category. The same principle goes for the other numerical columns, e.g. in the 4OHNDtam vs. E<sub>2</sub> column there are 82 up-regulated genes and 9% of these belonged to the "Apoptosis" category. The color intensity scales are based on the statistical significance (-log p-value) of over- and under-represented PANTHER functional categories. Red illustrates "over-represented category" where more genes than expected were found in a specific category. Blue color illustrates "under-represented category" where less genes than expected were found. Ref, Reference genes. Arrow up, up-regulated genes. Arrow down, down-regulated genes.

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were significantly down-regulated. Categories such as cell adhesion were up-regulated by both metabolites, whereas genes involved in cell cycle were down-regulated. In addition, genes in the cell motion category were down-regulated by 4OHNDtam, however not significantly by

**Table 3. Genes with differential expression after treatment with 4OHTam and more differential expression after treatment with 4OHTdam belonging to biological process category (PANTHER).**

	Up-regulated	Down-regulated
<b>Developmental process</b>	<i>VGLL1, BASP1, EMP1, KRT4</i>	<i>KRT6A, CXCR4, TUBA3D, EGR3, EGR2, MGP, KRT17, PRLR, IRX2, HEY2, SIAH2, OLFM1, KRT6C, IL27RA, TUBA3D, KRT6B</i>
System development	<i>BASP1</i>	<i>CXCR4, EGR3, EGR2, PRLR, MGP, IRX2, HEY2, SIAH2, OLFM1, IL27RA</i>
Nervous system development	<i>BASP1</i>	<i>CXCR4, EGR3, EGR2, IRX2, HEY2, SIAH2, OLFM1</i>
Ectoderm development	<i>BASP1, EMP1, KRT4</i>	<i>KRT6A, CXCR4, EGR3, EGR2, IRX2, HEY2, SIAH2, OLFM1, KRT6C, KRT6B</i>
<b>Cellular process</b>	<i>TNS3, EMP1, KRT4, INHBB, CAV1, GABRP</i>	<i>KRT6A, GPR68, RERG, CXCR4, TUBA3D, TGFA, CCBP2, Gal-7, FXYD4, FKBP5, EGR3, EGR2, GPER, MGP, RAB31, KRT17, PRLR, CCBP2, SGK1, Gal-7, GEM, PPP2R5A, ANXA8, OLFM1, KRT6C, IL27RA, TUBA3D, KRT6B, OXTR, PKIB</i>
Cell communication	<i>TNS3, INHBB, CAV1, GABRP</i>	<i>GPR68, RERG, CXCR4, TGFA, CCBP2, FXYD4, GPER, FKBP5, MGP, RAB31, PRLR, SGK1, GEM, CCBP2, PPP2R5A, ANXA8, OLFM1, IL27RA, OXTR, PKIB</i>
Signal transduction	<i>TNS3, INHBB, CAV1, GABRP</i>	<i>GPR68, RERG, CXCR4, TGFA, CCBP2, FXYD4, GPER, FKBP5, MGP, RAB31, PRLR, SGK1, GEM, CCBP2, PPP2R5A, ANXA8, OLFM1, IL27RA, OXTR, PKIB</i>
Intracellular signaling cascade		<i>RERG, RAB31, PRLR, FKBP5, SGK1, GEM, PPP2R5A, IL27RA, OXTR, PKIB</i>
Apoptosis	<i>TNS3, EMP1</i>	<i>DNASE1L2, CXCR4, Gal-7, FKBP5, SGK1, Gal-7</i>
Cell surface receptor linked signal transduction	<i>INHBB, CAV1</i>	<i>RERG, GPR68, CXCR4, TGFA, CCBP2, PRLR, GPER, CCBP2, GEM, OLFM1, IL27RA, OXTR</i>
Cytokine-mediated signaling pathway		<i>CXCR4, TGFA, CCBP2, PRLR, CCBP2, IL27RA</i>
G-protein coupled receptor protein signaling pathway	<i>CAV1</i>	<i>RERG, GPR68, CXCR4, CCBP2, GPER, CCBP2, GEM, OXTR</i>
<b>Reproduction</b>		<i>STARD5, OXTR</i>
Gamete generation		<i>STARD5, OXTR</i>
Female gamete generation		<i>STARD5, OXTR</i>
<b>Metabolic process</b>	<i>HSD17B11, GALNT12, TNS3, TGM2, DIO1, SPINK4, CAV1</i>	<i>DNASE1L2, SERPINA3, NXNL2, EGR3, EGR2, FKBP5, STARD5, LRRFIP2, SGK1, IRX2, KLK5, HEY2, PPP2R5A, ISG20, ANXA8, SIAH2, C5orf4, NAB2, SOX3, SOX9, SERPINA5, NT5DC3, ALDH3B2, ABCA12</i>
Primary metabolic process	<i>GALNT12, TNS3, TGM2, SPINK4, CAV1</i>	<i>DNASE1L2, SERPINA3, EGR3, EGR2, FKBP5, STARD5, LRRFIP2, SGK1, IRX2, KLK5, HEY2, PPP2R5A, ISG20, ANXA8, SIAH2, C5orf4, NAB2, SOX3, SOX9, SERPINA5, NT5DC3, ABCA12</i>
Lipid metabolic process	<i>TNS3, CAV1</i>	<i>STARD5, ANXA8, C5orf4, ABCA12</i>
Steroid metabolic process		<i>C5orf4, ABCA12</i>
Cholesterol metabolic process		<i>C5orf4, ABCA12</i>
Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process		<i>DNASE1L2, EGR3, EGR2, LRRFIP2, IRX2, HEY2, ISG20, NAB2, SOX3, SOX9, NT5DC3</i>
DNA metabolic process		<i>DNASE1L2</i>
DNA catabolic process		<i>DNASE1L2</i>
<b>System process</b>	<i>BASP1, SCNN1A, EMP1, GABRP, EYA2</i>	<i>RERG, FKBP5, OXTR</i>
Neurological system process	<i>BASP1, SCNN1A, EMP1, GABRP, EYA2</i>	<i>RERG, FKBP5, OXTR</i>
Sensory perception	<i>SCNN1A, EYA2</i>	<i>OXTR</i>
Sensory perception of chemical stimulus	<i>SCNN1A</i>	
Sensory perception of taste	<i>SCNN1A</i>	
<b>Regulation of biological process</b>	<i>SCNN1A</i>	
Regulation of vasoconstriction	<i>SCNN1A</i>	

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4OHTam. Further, we found an up-regulating effect on genes related to antigen processing and presentation, a pathway associated with the emerging hallmark of cancer [34]: evasion of immune surveillance. This up-regulation was observed after treatment with 4OHNDtam, but not with 4OHTam. It is believed that cancer cells that are weakly immunogenic, and therefore harder to identify by the immune system, are the cells which eventually will form a solid tumor [34]. There are several theories surrounding how the weakly immunogenic cancer cells are able to avoid being recognized by the immune system, and how the cells became weakly immunogenic. For breast cancer cells this may involve down-regulation of components of the major histocompatibility complex [35] or, as recently proposed, by releasing proteins associated with MHC-I through exosomes [36]. Our results show an up-regulation of genes associated with antigen processing and presentation of peptide or polysaccharide antigens via MHC class II. This might represent an effect of 4OHNDtam that counteracts the evasion of immune-surveillance. However, further studies are needed to explore this hypothesis.

The differential expression observed was generally stronger during 4OHNDtam treatment. These results are in line with earlier studies suggesting that 4OHNDtam is the main compound promoting the clinical effects of tamoxifen treatment [2, 37]. We further studied the stronger regulation observed by treatment with 4OHNDtam by selecting genes that were regulated by both metabolites, but more strongly by 4OHNDtam. In the PANTHER analysis performed on these genes (S1 Fig, Table 3), the apoptosis category was of particular interest. There was an over-representation of both up-regulated genes and down-regulated genes in this category. On closer inspection the up-regulated genes (*TNS3*, *EMP1*) were positive regulators of apoptosis [38] and 4 out of 5 down-regulated genes (*CXCR4*, *Gal-7*, *FKBP5*, *SGK1*) were negative regulators of apoptosis [39–42].

An interesting finding is the strong down-regulation by 4OHNDtam of genes expressing the different keratin 6 isoforms (*KRT6a*, *KRT6b* and *KRT6c*). *KRT6a* was the most down-regulated gene in our analysis (9.1-fold), and the effect on this gene was weaker after treatment with 4OHTam (1.8-fold decrease). The two-step validation by removal of the ligand (E2) (estrogen deprivation) (Fig 4) and knockdown of an important ER-coactivator (*SRC-3/AIB1*) (Fig 5) also suggest that the KRT6s are positively regulated by ER. It should be noted that *SRC-3/AIB1* is overexpressed in 31–64% of human breast tumors [43, 44], and has been shown to increase the agonist properties of tamoxifen [45].

The keratins, also called cytokeratins (CKs), belong to the intermediate filament proteins that create an insoluble dense meshwork through the cytoplasm giving structural support to the epithelial cell. Recently, however, it was shown that CKs play a more active role in various internal cellular survival processes (e.g. proliferation and apoptosis). These proteins may undergo phosphorylation and are also part of the bridging contact between the epithelial cell and its microenvironment [46]. CK6a is the dominant isoform in the mammary gland [47] and is up-regulated in the proliferative basal cells in healing wound edges of the skin, indicating that this CK is involved in cellular proliferation and migration [48]. Knockout of CK6 is associated with reduced proliferation in the murine mammary epithelium [47]. These genes are highly up-regulated in the basaloid molecular subtype of breast cancer demonstrated by Perou and Sorlie [49]. Interestingly, co-expression of CK 5/6 in ER positive breast cancer tumors seems to define a subset of patients with a more adverse prognosis [50, 51]. Therefore, the down-regulation of CK6 by 4OHNDtam should be further explored in appropriate designed 'bench-to-bed' studies since it may represent a new insight in understanding of the anti-cancer action of this active tamoxifen metabolite in ER positive breast tumors.

The present study has limitations. The standard tamoxifen metabolites used were not pure *z*-isomers. In the clinical situation both isomers are present although the *z*-isomers dominate [52]. Furthermore some isomerization may occur in the cultures during the study. Accordingly,

Katzenellenbogen *et al* observed a facile geometric isomerization of anti-estrogens which happened in tissue cultures as well as cell free medium [53]. They found that the MCF-7 cells mainly accumulated the trans-isomer and at the nuclear ER mainly the trans-isomer was located. Further limitations of the study are that we studied the effects of the metabolites only at one given concentration. It must also be taken into account that the concentration of 4OHNDtam was 10 times higher than that of 4OHTam in order to mimic the clinical situation, so the stronger regulation seen by 4OHNDtam might be a reflection of the concentration differences.

## Conclusions

Conclusively, the global gene expression changes caused by 4OHNDtam treatment of estrogen treated MCF-7 cells are stronger than those of 4OHTam when using concentrations that mimic the clinical situation. NDtam caused only minimal effects. Genes encoding CytoKeratin 6 were highly down-regulated by 4OHNDtam, as well as after E2 deprivation and knockdown of SRC-3/AIB1, indicating an estrogen receptor-dependent regulation. Further studies are warranted to elucidate possible clinical applications of this finding.

## Supporting Information

**S1 Fig. Functional categorization of genes differentially expressed genes after 4OHTam treatment and more differentially expressed after 4OHNDtam treatment.** PANTHER was used to search for over-represented categories in the ontology class Biological Process. To search for genes that were differentially expressed after treatment with 4OHNDtam and more differentially expressed after treatment with 4OHTam two subsequent rank product analyses were performed. First a rank product analysis for 4OHNDtam vs 4OHTam followed by a rank product analysis between the latter and “E2 vs 4OHTam”. Genes with rank product  $q\text{-val} \leq 0.2$  were selected from the rank product analysis. A  $p\text{-value} \leq 0.05$  was used as inclusion criterion for categories. The numbers inside the table are percentage values of the numbers above the columns. E.g. 11% of 19911 genes in the reference column can be found within the developmental process. The color intensity scales are based on the statistical significance ( $-\log p\text{-value}$ ) of over- and under-represented PANTHER functional categories. Red illustrates “over-represented category” where more genes than expected were found in a specific category. Blue color illustrates “under-represented category” where less genes than expected were found. Ref, Reference genes. Arrow up, up-regulated genes. Arrow down, down-regulated genes. (TIF)

**S1 Table. Primers used in RT-PCR**  
(DOC)

**S2 Table. Genes with increased expression after treatment with 4OHNDtam relative to E2 treatment in MCF-7 cells.**  
(DOC)

**S3 Table. Genes with decreased expression after treatment with 4OHNDtam relative to E2 treatment in MCF-7 cells.**  
(DOC)

**S4 Table. Genes with decreased expression after treatment with 4OHTam relative to E2 treatment in MCF-7 cells.**  
(DOC)

**S5 Table. Genes with increased expression after treatment with 4OHTam relative to E2 treatment in MCF-7 cells.**

(DOC)

**S6 Table. Gene Ontology analysis J-Express 2012**

(DOCX)

**S7 Table. Genes with decreased expression in 4OHTam and more decreased expression in 4OHNDtam**

(DOC)

**S8 Table. Genes with increased expression in 4OHTam and more increased expression in 4OHNDtam**

(DOC)

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**Author Contributions**

Conceived and designed the experiments: JG AD GM EAL MHF. Performed the experiments: TH JG AD ISF LS OB. Analyzed the data: TH AD GM JG EAL HS SND LS ISF OB MHF. Contributed reagents/materials/analysis tools: EAL HS GM JG MHF. Wrote the paper: TH AD GM JG EAL HS SND. Created sh-MCF-7 cell lines: ISF. Performed estrogen deprivation experiments: LS OB MHF.

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RESEARCH

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# Serum concentrations of active tamoxifen metabolites predict long-term survival in adjuvantly treated breast cancer patients

Thomas Helland<sup>1,2</sup>, Nina Henne<sup>2,3</sup>, Ersilia Bifulco<sup>2,3</sup>, Bjørn Naume<sup>4,5</sup>, Elin Borgen<sup>6</sup>, Vessela N. Kristensen<sup>7</sup>, Jan T. Kvaløy<sup>8,9</sup>, Timothy L. Lash<sup>10</sup>, Grethe I. G. Alnæs<sup>7</sup>, Ron H. van Schaik<sup>11</sup>, Emiel A. M. Janssen<sup>8,12</sup>, Steinar Hustad<sup>2,3</sup>, Ernst A. Lien<sup>1,2</sup>, Gunnar Mellgren<sup>1,2†</sup> and Håvard Søiland<sup>2,13\*†</sup>

## Abstract

**Background:** Controversies exist as to whether the genetic polymorphisms of the enzymes responsible for the metabolism of tamoxifen can predict breast cancer outcome in patients using adjuvant tamoxifen. Direct measurement of concentrations of active tamoxifen metabolites in serum may be a more biological plausible and robust approach. We have investigated the association between *CYP2D6* genotypes, serum concentrations of active tamoxifen metabolites, and long-term outcome in tamoxifen treated breast cancer patients.

**Methods:** From an original observational study comprising 817 breast cancer patients, 99 women with operable breast cancer were retrospectively included in the present study. This cohort of patients were adjuvantly treated with tamoxifen, had provided serum samples suitable for measuring tamoxifen metabolites, and were relapse-free at 3 years after the primary treatment commenced. The median follow-up time from this entry point to breast cancer death was 13.9 years. Patients were *CYP2D6* genotyped and grouped into four *CYP2D6* phenotype groups (Ultra rapid, extensive, intermediate, and poor metabolizers). Tamoxifen and nine metabolites were quantified in serum ( $n = 86$ ) and compared with *CYP2D6* phenotype groups and outcome.

**Results:** Breast cancer patients with low concentrations of Z-4-hydroxy-tamoxifen (Z-4OHTam;  $\leq 3.26$  nM) had a breast cancer-specific survival (BCSS) of 60% compared to 84% in patients with Z-4OHTam concentrations  $> 3.26$  nM ( $p = 0.020$ , log-rank hazard ratio (HR) = 3.56, 95% confidence interval (CI) = 1.14–11.07). For patients with Z-4-hydroxy-N-desmethyl-tamoxifen (Z-endoxifen) levels  $\leq 9.00$  nM BCSS was 57% compared to 84% for patients with concentrations  $> 9.00$  nM ( $p = 0.029$ , HR = 3.73, 95% CI = 1.05–13.22). Low concentrations of Z-4OHTam and Z-endoxifen were associated with poorer survival also after adjusting for clinically relevant variables (HR = 4.27, 95% CI = 1.35–13.58, and HR = 3.70, 95% CI = 1.03–13.25, respectively). Overall survival analysis showed similar survival differences for both active metabolites. The Antiestrogen Activity Score showed comparable effects, but did not improve the prognostic information.

**Conclusions:** Patients with Z-4OHTam and Z-endoxifen concentrations lower than 3.26 nM or 9.00 nM, respectively, showed an adverse outcome. Our results suggest that direct measurement of active tamoxifen metabolite concentrations could be of clinical value. Validation in larger study cohorts is warranted.

**Keywords:** Tamoxifen, Adjuvant, Metabolism, Survival, *CYP2D6*, Endoxifen, 4OHTam, Breast cancer, Prognosis

\* Correspondence: hsoiland@gmail.com  
Gunnar Mellgren and Håvard Søiland are equal senior authors.

†Equal contributors

<sup>2</sup>Department of Clinical Science, University of Bergen, Bergen, Norway

<sup>13</sup>Department of Surgery, Section of Breast and Endocrine Surgery, Stavanger University Hospital, Stavanger, Norway

Full list of author information is available at the end of the article



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## Background

Tamoxifen is a selective estrogen receptor modulator used for adjuvant treatment of luminal (estrogen receptor (ER)-positive and/or progesterone receptor (PR)-positive) breast cancer (BC) subtypes. Tamoxifen is the oldest and most prescribed endocrine BC drug and has been shown to reduce BC mortality by 31% [1] and BC recurrence by 50% [2]. Tamoxifen is a widely used endocrine adjuvant treatment option among pre-menopausal BC patients, with therapy durations of up to 10 years [3, 4]. Post-menopausal BC patients are mainly given aromatase inhibitors (AIs) for 5 years, in combination with tamoxifen for a 3–5 year period, or tamoxifen monotherapy for 10 years if the side effects from AIs are too bothersome [5]. Hence, tamoxifen is still an important drug in the management of BC. However, interpatient variability in the anti-ER response and adverse effects are common. Within 15 years of primary surgery one-third of BC patients receiving tamoxifen will have relapsed [1].

The interpatient variability in the clinical response to tamoxifen has been suggested to be connected to its enzymatic conversion into active metabolites. Several of these activating enzymes are polymorphic, including cytochrome P450 2D6 (CYP2D6), as combinations of the *CYP2D6* alleles have been related to various kinetic activity levels of the enzyme. CYP2D6 is a key enzyme in the formation of the two active metabolites, Z-4-hydroxy-N-desmethyl-tamoxifen (Z-4OHNDtam, also known as Z-endoxifen) and Z-4-hydroxy-tamoxifen (Z-4OHTam) [6], and concentrations of these two active metabolites have been found to be associated with *CYP2D6* genotypes [7, 8]. Z-endoxifen and Z-4OHTam are 30- to 100-fold more potent anti-ER inhibitors than the mother drug tamoxifen [9]. Endoxifen is present at up to 10 times higher plasma concentrations than 4OHTam and is therefore regarded as the most powerful metabolite [6]. After Goetz et al. in 2005 reported an association between the *CYP2D6* poor metabolizer (PM) phenotype and higher risk of relapse among tamoxifen users [10], several reports have been published on *CYP2D6* genotype and outcome. However, the various studies have reported contradictory results and more knowledge is required in order to make any conclusions [11–14].

An alternative approach would be to measure the concentrations of the active metabolites directly in serum and associate them with breast cancer outcomes. As the active metabolites are strong ER ligands, their serum levels may better reflect the functional anti-estrogenic effects in patients treated with tamoxifen. Recently, methods to separate the Z-isomers (Z-endoxifen and Z-4OHTam) from the less active or inactive isomers have been developed [15]. The additive anti-ER effect from tamoxifen metabolites and isomers with various affinity to the ER may also be of importance to estimate the

resultant effect of tamoxifen itself and all active tamoxifen metabolites [16].

In the present study, we have determined the *CYP2D6* genotypes and serum concentrations of tamoxifen and nine metabolites in 99 BC patients with a long-term follow-up. Our aim was to investigate the predictive value of direct measurements of active serum tamoxifen metabolites in patients with operable breast cancers and to compare these results with the *CYP2D6* genotyping method. We hypothesized that the genotype approach is inferior to direct measurement of tamoxifen metabolites regarding prediction of prognosis, and that patients with low serum levels of active tamoxifen metabolites will have poorer prognosis.

## Methods

In this retrospective observational study the primary objective was to compare the prognostic value of direct measurements of tamoxifen metabolites in serum with *CYP2D6* genotyping in 99 operable breast cancer patients. The secondary objective was to investigate the associations between concentrations of active tamoxifen metabolites and *CYP2D6* phenotypes.

## Patients

Between May 1995 and December 1998, 817 patients were studied in a population-based observational micro-metastasis study [17] in Oslo, Norway. The patients were treated according to the national guidelines at the time. All patients with hormone receptor-positive tumors received 20 mg tamoxifen daily for 5 years. The tumor was defined as hormone receptor positive if  $\geq 10\%$  of the cells were positive for ER or PR by immunohistochemistry analysis.

From this original study population, serum was drawn from 356 relapse-free patients 3 years after inclusion. Of these, 99 operable BC patients comprising T1/T2 tumors were adjuvantly treated with tamoxifen and included in the present study. The demographic and clinical characteristics are presented in Table 1. The median follow-up time for breast cancer death from this entry time was 13.9 years (range 0.6–16.5 years). The present study population of 99 patients did not differ from the relapse-free cohort [17] with regard to clinical and tumor biological variables other than the treatment selection (Table 1).

## *CYP2D6* genotyping and classification of *CYP2D6* phenotype groups

DNA was isolated from the blood or bone marrow using the Gentra Puregene Blood kit (Qiagen, Hilden, Germany) or an automated phenol-chloroform procedure. The *CYP2D6* genotype determination was performed at the Expert Center for Pharmacogenetics, Department of

**Table 1** Patient demographics and characteristics

Characteristics	Present study population (n = 99)	Relapse free at 3 years (n = 356)	Differences between the groups (P values)
Age at diagnosis (years)			
Mean (median)	58 (56)	57 (56)	
Range	34–78	28–85	0.380
Menopause status, n (%)			
Pre (< 55 years)	40 (40%)	151 (42%)	
Post (≥ 55 years)	59 (60%)	205 (58%)	0.710
Histology, n (%)			
IDC	74 (75%)	251 (76%)	
ILC	24 (24%)	69 (19%)	
Other infiltrating cancer	1 (1%)	16 (5%)	0.109
Tumor size, n (%)			
pT1	50 (51%)	253 (71%)	< 0.001*
pT2	49 (49%)	87 (25%)	
pT3	–	12 (3%)	
pT4	–	0 (0%)	
pTx	–	3 (1%)	
Tumor grade, n (%)			
G1	18 (18%)	110 (31%)	
G2	67 (68%)	184 (52%)	
G3	12 (12%)	56 (15%)	
Not reported	2 (2%)	6 (2%)	0.009
Node status, n (%)			
Positive	57 (58%)	93 (27%)	< 0.001
Negative	41 (41%)	257 (73%)	
Not reported	1 (1%)	6 (2%)	
HER2/neu status, n (%)			
HER2 <sup>+</sup>	7 (7%)	36 (10%)	
HER2 <sup>-</sup>	89 (90%)	298 (84%)	0.193
Unknown	3 (3%)	22 (6%)	

Comparison of the demographic and clinical characteristics between the patients in the present study population and 356 relapse-free patients from the original population [17]

The present study population comprises more patients with pT2 tumors, higher grade, and node-positive status due to treatment selection

\*The present study population only included operable breast cancer patients; therefore p value of tumor size comparison is between pT1 and pT2 populations  
IDC invasive ductal carcinoma, ILC invasive lobular carcinoma, pT pathological tumor size

Clinical Chemistry, Erasmus University Medical Center, Rotterdam, The Netherlands, using the CE-IVD approved INFINITI<sup>®</sup> CYP450 2D6I Assay (Autogenomics, Carlsbad, CA, USA) and verified using the Taqman DME assay (Thermo Fisher Scientific, Waltham, MA, USA) according to validated standard operating procedures in an ISO15189-certified laboratory. INFINITI detects 15 variant alleles (Additional file 1: Table S1) and the *CYP2D6* genotypes of the patients were determined based on the combination of wild-type (wt) and variant-type (vt) alleles and translated into four predicted *CYP2D6* phenotype groups: ultra-rapid metabolizers (UM; gene duplication

positive, no inactive variants), extensive/normal metabolizers (EM; no variants or only one decreased activity allele), intermediate metabolizers (IM; two decreased activity alleles or one active and one inactive allele), and poor metabolizers (PM; two inactive alleles).

#### Determination of tamoxifen metabolites by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

We developed a LC-MS/MS method to quantify tamoxifen and nine of its metabolites in human serum. All metabolites and four deuterated internal standards were obtained commercially (Additional file 2: Table S2).

Calibrators were created from pooled human serum of three male and six female non-tamoxifen users to which tamoxifen metabolites were added at seven concentrations (Additional file 3: Table S3).

Serum samples (50 µl) containing tamoxifen metabolites were processed using a Hamilton STAR pipetting robot (Bonaduz, Switzerland). Serum proteins were precipitated by adding 500 µl acetonitrile containing internal standards to the samples; 350 µl of the supernatant was evaporated to dryness under a nitrogen flow and subsequently reconstituted in 500 µl methanol and diluted 1 to 25 in water:methanol (20:80, v:v) before being subjected to LC-MS/MS analysis.

An Aquity UPLC system from Waters (MA, USA) with a thermostated column oven set at 50 °C was used to chromatographically separate the compounds; 25 µl of sample was injected onto a 100-mm BEH Phenyl column with a 2.1 mm internal diameter and 1.7 µm particle size (Waters, Milford, MA, USA). The column was developed by a weak mobile phase (A) consisting of water, and a strong mobile phase (B) consisting of methanol, both buffered with 0.01% formic acid. All gradient steps were linear, and the flow rate was 300 µl/min. The following gradient was used: 0–0.5 min: 95% A and 5% B; 1 min: 65% A and 35% B; 4 min: 10% A and 90% B; 4.5–8 min: 100% B; 8.1–9 min: 90% A and 10% B.

The LC system was coupled to a Xevo TQ-S tandem mass spectrometer (Waters, Milford, MA, USA) equipped with an atmospheric pressure photoionization source (APPI). All compounds were analyzed in the

positive mode. Additional file 4: Table S4 shows retention times and compound-dependent settings for the tamoxifen metabolites.

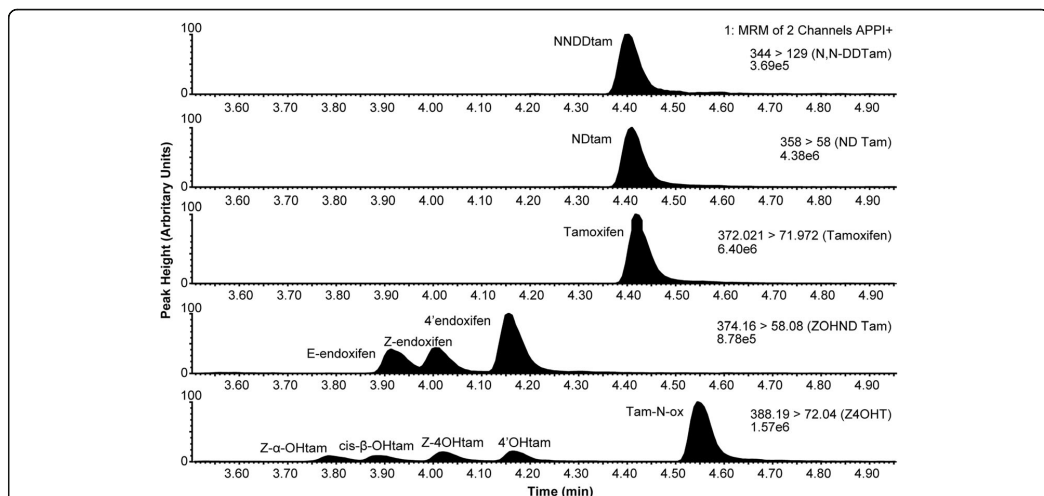
**Validation of the LC-MS/MS method**

The selectivity of the method is demonstrated in Fig. 1 as it separated the active Z-isomers of 4OHTam and endoxifen from its less active Z'-isomers and inactive E-isomers. Total analytical run-time was 9 min and the sample volume of serum was 50 µl. Accuracy and imprecision was well within the acceptance criteria defined by regulatory guidelines (Food and Drug Administration (FDA), Rockville, MD, USA; 2002). The method was linear for all analytes (Additional file 5: Table S5). For medium concentrations, imprecision (intra- and inter-day CV %) was within 9% and accuracies were in the range 95–106% (Additional file 3: Table S3) for all metabolites except cis-β-OHTam and z-α-OHTam, which had imprecision within 15% accuracies in the range 87–109%. Cis-β-OHTam and z-α-OHTam were not detected in patient samples.

**Data analyses and statistics**

SPSS statistical software, version 23 (SPSS, Inc., Chicago, IL, USA), and MedCalc for Windows, version 16.4.3 (MedCalc Software, Ostend, Belgium), were used for the basic statistical calculations.

Supervised cut-off values for Z-endoxifen (9.00 nM), Z-4OHTam (3.26 nM), and Antiestrogenic Activity Score (AAS) (16.7) were identified by multivariable Cox approach as described in Additional file 6.



**Fig. 1** MRM transitions of tamoxifen and its metabolites. The chromatograms are obtained by analyzing the second point of the calibration curve. The chromatographic separation of isomers of 4OHTam and endoxifen are shown in the lowest and second lowest panels, respectively. APPI atmospheric pressure photoionization, MRM multiple reaction monitoring

The present study included patients that had survived the first three 3 years after surgery without experiencing any relapse. The analysis is thus conditional on 3 year relapse-free survival, and in the survival analysis 3 years after surgery is used as the time origin to partially address the pitfall of immortal person time bias [18–20]. Breast cancer-specific survival (BCSS) was defined as the time from the primary surgery until death from breast cancer. Cause of death was provided from the hospital records, and in a few cases also by information from the patient's general physician.

Survival estimates were calculated by the Kaplan-Meier method. Univariable tests for survival differences in categorical variables were performed by the log-rank test or the log-rank test for trend as appropriate. Multivariable regression analysis for clinically relevant variables was performed using the Cox proportional hazards method. Chi-square, Mann-Whitney *U* test or Kruskal-Wallis were used for comparisons between groups as needed. Fisher's exact test was used when appropriate. Two-tailed *P* values < 0.05 were considered statistically significant.

To estimate the resultant ER blockade of tamoxifen itself and the various active tamoxifen metabolites we used the AAS as previously described [16]. In short, the estimation of the AAS was based on the serum concentrations of the various active tamoxifen metabolites and their relative affinity to the ER by the following algorithm:  $0.01 \times [\text{Tamoxifen}] + 1 \times [\text{Z-endoxifen} + \text{Z-4OHTam}] + 0.1 \times [\text{Z'-endoxifen} + \text{Z'-4OHTam}]$ .

## Results

### *CYP2D6* genotyping and quantification of tamoxifen metabolites in serum

*CYP2D6* allele frequencies are shown in Additional file 7: Table S6 and the most frequent genetic variants \*1, \*2, \*4 and \*41 were in Hardy-Weinberg equilibrium (HWE). The frequency of the remaining 5 alleles (\*3, \*5, \*6, \*9, and \*10) were too rare in our study cohort to perform a HWE calculation. Ninety-one patients were successfully *CYP2D6* genotyped and the phenotypes were distributed as follows: 4 (4.4%) ultra-rapid metabolizers, 43 (47.3%) extensive/normal metabolizers, 36 (39.6%) intermediate metabolizers, and 8 (8.8%) poor metabolizers. Eight patients were excluded from *CYP2D6* analysis due to inadequate volumes of blood/bone marrow for DNA extraction or because of poor quality of DNA.

Concentrations of tamoxifen and nine metabolites were measured using LC-MS/MS. All patient serum samples were run in duplicate (Table 2). The mean and median concentrations of tamoxifen and the nine metabolites for the 86 patients are shown in Table 2. Cis- $\beta$ -OHTam and *z*- $\alpha$ -OHTam were included for separation of the hydroxylated metabolites [21] and were not detectable in patient samples. As shown before [22], large

inter-individual variations in the concentrations of tamoxifen metabolites were observed between patients (Table 2). Thirteen patients had concentrations below the lower limits of quantification (LLQ) for tamoxifen and all the nine metabolites. These patients were regarded as non-adherent and excluded from the present study, leaving 86 patients for further analyses.

### Associations between *CYP2D6* phenotype groups and concentrations of tamoxifen metabolites

The median values for all metabolite concentrations stratified by *CYP2D6* phenotype groups are shown in Additional file 8: Table S7. An association between declining concentration levels and decreased *CYP2D6* function was observed for Z-4OHTam and Z-endoxifen ( $p = 0.05$  and  $p < 0.001$ , respectively; Kruskal-Wallis) (Fig. 2). Notably, there is a wide spread of levels of active metabolites within each *CYP2D6* phenotype group and also a considerable overlap between them; for example, use of the 3.26 nM (red line) and 8.13 nM (green line) cut-off values for Z-4OHTam will include patients from three *CYP2D6* phenotype groups (Fig. 2). None of the other metabolite concentrations showed an association with *CYP2D6* phenotype.

### Breast cancer outcome in association with *CYP2D6* phenotype and active tamoxifen metabolite concentrations

To investigate the association between *CYP2D6* phenotype and survival, a Kaplan-Meier linear trend analysis comparing the survival of the four *CYP2D6* phenotype groups (UM, EM, IM, and PM) was performed (Fig. 3). No significant association was observed ( $p = 0.966$ , total log-rank). However, using EM as a reference we achieved 80% power to detect hazard ratios (HRs) of 3.3, 10, and 35 for IM, PM, and UM, respectively. Therefore, the result should be interpreted with care. Notably, the excluded non-adherent patients ( $n = 13$ ) were evenly distributed among the various *CYP2D6* phenotype groups, and including them did not change the results.

A Cox log-linear trend analysis controlling for age, tumor size, grade, node status, ER, PR, and chemotherapy was performed to investigate the association between concentrations of tamoxifen metabolites and outcome. We identified a log-linear relationship between Z-4OHTam and BCSS ( $p = 0.044$ , HR = 0.75, 95% confidence interval (CI) = 0.56–0.99), indicating a 0.25 reduction in hazard for each unit (1 nM) increase in Z-4OHTam. There was no log-linear association between Z-endoxifen or the remaining metabolites and breast cancer outcome. We further wanted to explore the possibility of an association between survival and concentration thresholds for the active metabolites Z-4OHTam and Z-endoxifen. We identified supervised cut-off values representing low

**Table 2** Concentrations of tamoxifen and nine metabolites in 86 breast cancer patients

Analyte	Mean (median) serum concentration (nM)	Analytical CV %	Interpatient variability CV %
Tamoxifen	322.2 (287.5)	6.61	45.46
NDtam	723.2 (689.0)	9.55	44.75
Z-4OHNDtam	30.11 (28.15)	6.46	59.01
4'OHNDtam	30.08 (28.13)	8.18	38.09
Z-4Ohtam	5.67 (5.30)	6.03	42.32
4'Ohtam	7.64 (7.20)	7.54	39.34
Tam-N-ox	119.6 (97.52)	11.04	60.12
NNDDtam	92.69 (81.17)	11.14	50.80
cis-β-Ohtam	ND	–	–
z-α-Ohtam	ND	–	–

Samples were run in duplicate

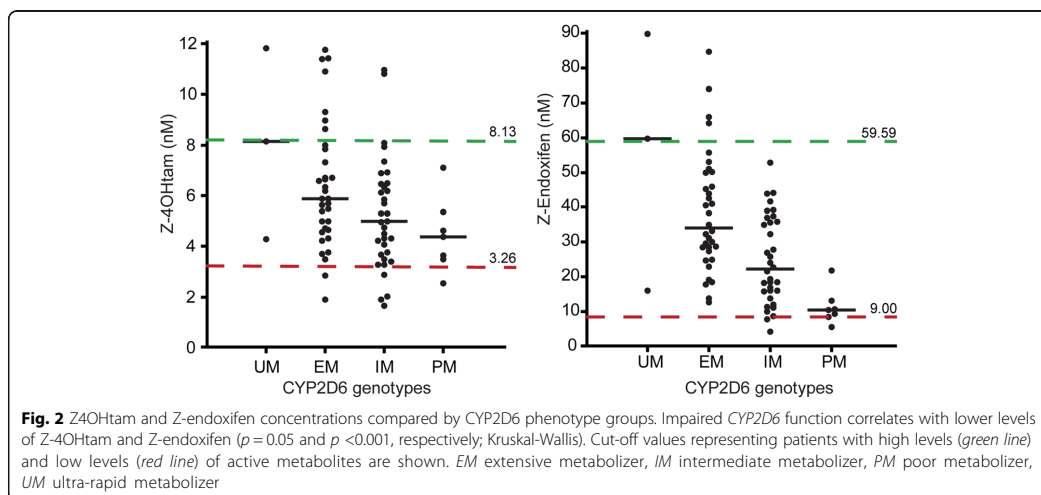
Thirteen patients with metabolite levels below the limit of detection are not included in the calculations, leaving 86 patients for further analysis

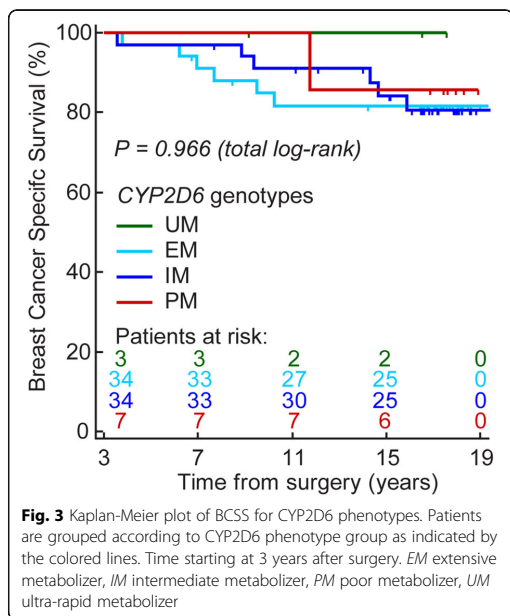
Analytical CV % indicates average CV between two replicate samples for all patients

CV coefficient of variation, ND not detected

concentrations for Z-4Ohtam (3.26 nM) and Z-endoxifen (9.00 nM) as described in the Methods section and performed univariable survival analyses (Fig. 4a and b). For Z-4Ohtam the BCSS was 60% vs. 84% for the ≤ 3.26 nM and > 3.26 nM groups, respectively ( $p = 0.020$ ; log-rank HR = 3.56, 95% CI = 1.14–11.07). For Z-endoxifen we observed a BCSS of 57% vs. 84% for the ≤ 9.00 nM and > 9.00 nM groups, respectively ( $p = 0.029$ ; log-rank HR = 3.73, 95% CI = 1.05–13.22). Adjustment for age, tumor size, nodal status, histological grade, ER and PR status, and chemotherapy given left Z-4OH tam and Z-endoxifen as the only factors in the final models with HR = 4.27 (95% CI = 1.35–13.58) and HR = 3.70 (95% CI = 1.03–13.25), respectively.

The Z' isomers of the active metabolites also have anti-estrogenic activity and, since our LC-MS/MS was able to measure the Z and Z' isomers of 4Ohtam and endoxifen separately, we were able to calculate the AAS score (as described in Additional file 6). We further identified threshold values representing patients with low and high AAS and showed a BCSS of 57% for patients with AAS ≤ 16.7 compared to 84% in patients with AAS > 16.7 ( $p = 0.026$ , HR = 3.81, 95% CI = 1.07–13.56) (Additional file 9: Figure S1). Adjusting for the same variables as mentioned above, AAS was the only factor associated with BCSS ( $p = 0.041$ , HR = 3.80, 95% CI = 1.06–13.64). We also investigated the possible effect on outcome from tamoxifen itself, the two Z'-isomers alone,





and the various non-active metabolites. No significant thresholds were identified.

In the analysis for overall survival (OS), Z-4OHTam, Z-endoxifen, and AAS were all significant in the univariable analysis (Table 3). When adjusting for the various clinico-pathological variables, tumor size, nodal status, and chemotherapy were added to the final models (Table 4).

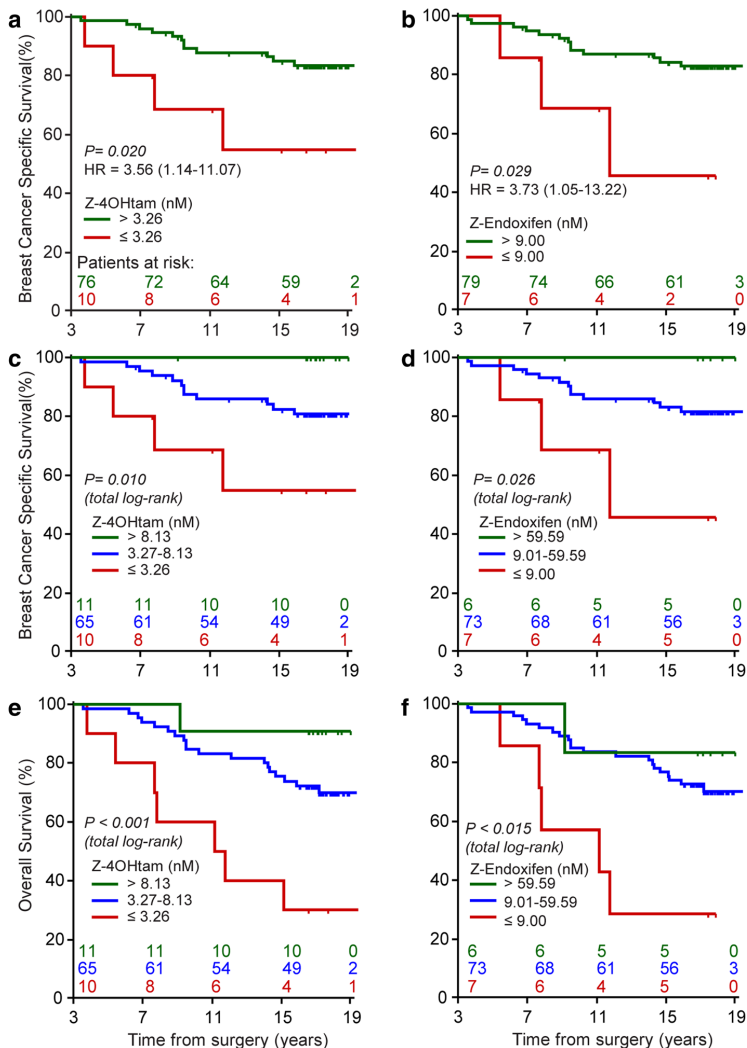
The significant linear trend observed for Z-4OHTam encouraged us to also assess survival effects for very high levels of active metabolites. We therefore arbitrarily used the concentrations corresponding to the median concentrations for Z-4OHTam and Z-endoxifen ultra-rapid metabolizers (UM) as cut-off values, i.e., 8.13 nM and 59.59 nM, respectively (Fig. 2). Hence, patients were re-grouped into low, intermediate, and high serum concentrations of Z-4OHTam and Z-endoxifen, respectively (Fig. 4c and d). The Kaplan-Meier log-rank trend test demonstrated significant survival differences between these three subgroups for both metabolites (Z-4OHTam,  $p = 0.010$ ; Z-endoxifen,  $p = 0.026$ ) with no BCSS events for patients with high concentrations of active metabolites (Fig. 4c and d). The same differences were also observed in the overall survival analysis (Z-4OHTam,  $p = 0.002$ ; Z-endoxifen,  $p = 0.014$ ; log-rank trend) (Fig. 4e and f). Notably, the distribution of all the adjusted clinico-pathological variables were equal between the low and the high serum concentration subgroups.

## Discussion

In the present study we identified an association between CYP2D6 phenotype groups and the serum levels of active metabolites (Z-4OHTam and Z-endoxifen). However, we did not find an association between CYP2D6 phenotypes and breast cancer outcome (Fig. 3). The low power to detect a relevant survival difference between the CYP2D6 phenotype groups (i.e., HR between 1.5 and 2.5) is a possible explanation for its absent prognostic value in the present study. We further investigated the association between concentrations of active metabolites and breast cancer outcome, and this is to our knowledge the first study to report a relationship between low levels of the active tamoxifen metabolites and higher risk of breast cancer death (Fig. 4). The long follow-up time in our study allowed the use of breast cancer-specific survival as the clinical endpoint. We identified threshold values representing low and high levels of active metabolites. Notably, these cut-off values included patients from all CYP2D6 phenotype groups suggesting that the genotype approach results in grouping of patients with heterogeneous serum levels of active metabolites (Fig. 2).

To our knowledge, only three studies have analyzed the association between tamoxifen metabolite concentrations and relapse of breast cancer [23–25]. Madlensky et al. found a 30% higher risk of relapse in patients with low endoxifen levels (<16 nM) in patients grouped according to endoxifen quintiles [23]. In a recent study, a higher risk of distant relapse was observed in patients with low (<14.15 nM) vs high (>35 nM) Z-endoxifen levels when splitting the patients into endoxifen quartiles [24]. Both studies reported that the lowest quintile/quartile had the worst outcome, whereas the highest quintile/quartile had the best outcome. Thus, it seems that the use of active metabolite thresholds creates reproducible results in survival analyses probably due to grouping of patients that are homogeneous regarding the anti-ER effect. This is in line with our results since we also observed a favorable survival in breast cancer patients with high serum metabolite levels. Our high cut-off value is equal to the median concentrations of active metabolites in the UM group (Fig. 2), and other studies have shown that UM groups are often reported to be in the best prognostic range in the subgroup analyses [26]. In a third study, no association was found between endoxifen levels and breast cancer outcome in patients receiving low doses of tamoxifen (1 mg, 5 mg, and 10 mg) [25]. However, the authors speculate that sensitivity issues for detecting differences at very low concentrations may have clouded the results. In addition, preliminary results presented at ASCO 2016 [27] showed no association between endoxifen concentrations and BC outcome. However, this study included





**Fig. 4** Kaplan-Meier plots of BCSS and overall survival for concentrations of active tamoxifen metabolites. Patients are grouped according to concentrations of active metabolites as indicated by colored lines. Time starting at 3 years after surgery. **a,b** BCSS for Z-4OHTam and Z-endoxifen at concentrations above and below 3.26 nM and 9.00 nM, respectively. **c,d** BCSS for Z-4OHTam and Z-endoxifen at three serum concentrations: low, intermediate, and high levels, as shown in the figure. **e,f** Overall survival for Z-4OHTam and Z-endoxifen at the same three concentrations as shown in **c** and **d**. HR hazard ratio

patients receiving 20 mg tamoxifen in the metastatic setting or as neoadjuvant treatment, a very different context often with developed endocrine resistance. Hence, this patient group is difficult to compare with the operable patients undergoing adjuvant tamoxifen treatment in our study. Interestingly, in a phase I study administering oral Z-endoxifen 160 mg daily in

endocrine refractory metastatic breast cancer patients [28] the response rate on the tumor was 26% and the side effects were tolerable, with endoxifen concentrations up to 5200 nM maintained over 28 days. This study suggests that the concentrations of the active metabolites may be important for the apoptotic effect on breast cancer cells [28].

**Table 3** Univariable survival analyses of breast cancer-specific survival and overall survival

Factor	Breast cancer-specific survival				Overall survival			
	Event/at risk	HR	95% CI	<i>P</i>	Event/at risk	HR	95% CI	<i>P</i>
Tumor size								
pT1	7/44	1			11/44	1		
pT2	9/42	1.47	0.55–3.96	0.439	16/42	1.70	0.79–3.67	0.169
Node status								
pN0	4/33	1			9/33	1		
pN+	12/52	2.00	0.64–6.19	0.222	18/52	1.33	0.60–2.96	0.484
Histological grade								
1	3/16	1			6/16	1		
2	10/57	0.94	0.26–3.42	0.924	16/57	0.76	0.30–1.94	0.564
3	3/11	1.48	0.30–7.35	0.631	5/11	1.30	0.40–4.28	0.661
Histological grade								
1 + 2	13/73	1			22/73	1		
3	3/11	1.56	0.44–5.46	0.335	5/11	1.61	0.61–4.25	0.335
Age								
< 55 years	7/35	1			7/35	1		
≥ 55 years	9/51	0.93	0.35–2.51	0.890	20/51	2.09	0.89–4.95	0.085
ER								
≥ 10%	15/75	1			26/75	1		
< 10%	1/7	0.63	0.08–4.73	0.646	1/7	0.36	0.05–2.65	0.294
PR								
≥ 10%	11/55	1			17/55	1		
< 10%	4/27	0.72	0.23–2.25	0.566	9/27	0.98	0.43–2.19	0.951
Chemotherapy*								
Yes	6/30	1			6/30	1		
No	10/56	0.93	0.34–2.57	0.893	21/56	1.97	0.80–4.89	0.135
AAS								
> 16.7	13/79	1			21/79	1		
≤ 16.7	3/7	3.81	1.07–13.56	0.026	6/7	5.37	2.14–13.51	< 0.001
Z-4OHTam								
> 3.26 nM	12/76	1			20/76	1		
≤ 3.26 nM	4/10	3.56	1.14–11.11	0.020	7/10	4.05	1.70–9.64	0.001
Z-endoxifen								
> 9.00 nM	13/79	1			22/79	1		
≤ 9.00 nM	3/7	3.73	1.05–13.23	0.029	5/7	4.03	1.51–10.74	0.003

\* Did the patients receive chemotherapy according to the treatment guidelines at the time

AAS antiestrogenic activity score, CI confidence interval, ER estrogen receptor, HR hazard ratio, pN pathologic node status, PR progesterone receptor, pT pathologic tumor size

Here, we report for the first time an association between Z-4OHTam and BC outcome. Although endoxifen is present at higher serum concentrations than Z-4OHTam, their affinity to the ER is the same. Cross tabulation between Z-endoxifen (cut-off 9.0 nM) and Z-4OHTam (cut-off 3.26nM) shows that 50% of patients below the Z-4OHTam threshold were not identified by the Z-endoxifen threshold (Additional file 10: Table S8).

This implies that measuring Z-4OHTam may be of clinical value. The Z'-isomers of the active metabolites also have a certain antiestrogenic effect. After calculation of tamoxifen and all active metabolites by means of the AAS score, we observed a significant association between low AAS score and worse BCSS (Additional file 9: Figure S1). However, using the AAS score was not superior to the use of Z-endoxifen and Z-4OHTam

**Table 4** Overall survival; multivariable analysis including Z-4Ohtam, Z-endoxifen, and AAS

Continuous variables				Categorical variables			
Factor	HR per unit*	95% CI	P	Factor	HR	95% CI	P
Z-4Ohtam				Z-4Ohtam	> 3.26 nM	1	
Adjusted <sup>†</sup>	0.81	0.66–0.99	0.040		≤ 3.26 nM	4.86	1.88–12.54
Unadjusted	0.85	0.70–1.02	0.077	pT	1	1	
					2	2.59	1.11–6.05
				pN	Negative	1	
					Positive	2.89	1.12–7.49
				Chemotherapy	Yes	1	
					No	2.34	0.90–6.11
Z-endoxifen				Z-endoxifen	> 9.00 nM	1	
Adjusted <sup>†</sup>	0.99	0.96–1.02	0.365		≤ 9.00 nM	5.65	2.00–16.00
Unadjusted	0.99	0.97–1.02	0.580	pT	1	1	
					2	2.44	1.08–5.49
				pN	Negative	1	
					Positive	2.40	0.99–5.80
				Chemotherapy	Yes	1	
					No	3.41	1.24–9.39
AAS				AAS	> 16.7	1	
Adjusted <sup>†</sup>	0.99	0.97–1.01	0.313		≤ 16.7	8.39	2.90–24.26
Unadjusted	0.99	0.98–1.10	0.532	pT	1	1	
					2	2.60	1.15–5.91
				pN	Negative	1	
					Positive	3.40	1.26–9.15
				Chemotherapy	Yes	1	
					No	2.67	1.03–6.94

\* Change in hazard ratio (HR) per 1 nM increase in serum concentration of Z-4Ohtam, Z-endoxifen, change in HR per 1 unit Antiestrogenic Activity Score (AAS; dimensionless)

<sup>†</sup> Adjusting variables: pT, pN, histological grade, estrogen receptor, progesterone receptor, age, and chemotherapy  
CI confidence interval, pN pathologic node status, pT pathologic tumor size

concentrations, strengthening previous observations that Z-endoxifen and Z-4Ohtam are the most active tamoxifen metabolites. In line with our results, a recent study showed an aggregate effect of tamoxifen and three metabolites on breast cancer relapse [29] without providing additional prognostic information compared to the use of endoxifen levels alone.

Our supervised threshold for low concentrations of endoxifen identified in the present study (9.00 nM) is slightly lower compared to the un-supervised thresholds identified in previous studies (16 nM and 14.15 nM) [23, 24]. Using these cut-off values in the present study, we observed the same pattern with poorer survival for the lower concentration groups; however, significance was not reached. Thresholds will vary depending on the number of patients included in a study, the statistical methods to determine cut-off values [30], the underlying patient distribution [31], and the assay used to quantify the metabolites.

Moreover, the threshold of a single metabolite in a clinical study (in-vivo setting) may be influenced by the relative concentrations of all the other metabolites present in the same environment. Thus, they will compete on the same binding site of the ER and contribute to the numeric difference in cut-off values.

Intriguingly, all the above three cut-off values identify a clinical relevant patient group with poor outcome in the lower concentration extremities of Z-endoxifen and Z-4Ohtam. Admittedly, our supervised thresholds may also have inflated the *P* values [32] and exaggerated the survival differences between subgroups in the present study. Hence, the threshold values in this learning set must be interpreted with caution and validation of the thresholds in a larger independent material is warranted [33]. Importantly, consensus on the “correct clinical threshold” should aim to characterize patients with low benefit of tamoxifen with a certain safety margin to avoid under-treatment.

There are some limitations to the present study. First, our patient population of 86 patients is small. Despite the low number of patients, we were able to identify significant associations between Z-4OH tam and Z-endoxifen levels and outcome probably due to our long follow-up time (median 13.8 years). The low number of events in each subgroup calls for caution in interpreting the results and may explain the lack of statistical power to determine prognostic information from the CYP2D6 phenotype groups. Therefore, validation in larger study cohorts is warranted. Furthermore, information on long-term adherence (5 years) and co-medication, such as CYP2D6 inhibitors, would have strengthened our study. Entry of patients after a 3-year relapse-free period post-surgery has created loss of early endpoints occurring during the first 3 years of follow-up. This might have contributed to the observed loss of prognostic information of the proliferation-related variables such as pT, pN, and histological grade in these luminal breast cancer subtypes. This selection bias will favor patients with late events in the natural course of their disease. In patients with luminal breast cancers, approximately 75% of the breast cancer-related deaths occur after 3 years [34]. As the present study comprises only patients with this tumor type with a long-term follow-up (i.e., up to 16.5 years) we believe that our findings are of value for evaluating the 3-year conditional survival in this patient group.

## Conclusions

Although tamoxifen has been on the market for several decades and is the most used drug against breast cancer, its use may still be improved. The present study shows that tamoxifen metabolism may predict breast cancer outcome by measuring serum concentrations of active tamoxifen metabolites. Our results imply that patients with serum Z-endoxifen levels lower than 9.00 nM or Z-4OHTam levels lower than 3.26 nM have poorer long-term BCSS and OS compared to patient with levels above these thresholds. The results may translate into clinical practice by means of therapeutic drug monitoring, which represents a direct and applicable method to identify breast cancer patients with poor tamoxifen metabolism regardless of genotype and inhibiting drug interactions on the CYP enzymes [35]. Dose adjustment or a switch to an alternative endocrine treatment could avoid under-treatment of such patients [36]. Our findings need to be verified in larger studies, preferable in randomized trials with a long follow-up time.

## Additional files

**Additional file 1: Table S1.** Alleles analyzed by INFINITI®. (DOCX 14 kb)

**Additional file 2: Table S2.** Suppliers and catalog numbers for tamoxifen metabolites. (DOCX 14 kb)

**Additional file 3: Table S3.** Imprecision and accuracy. (DOCX 16 kb)

**Additional file 4: Table S4.** Retention times, molecular weights, and compound-dependent instrument settings. (DOCX 16 kb)

**Additional file 5: Table S5.** Linear dynamic range of the assay. (DOCX 14 kb)

**Additional file 6:** Additional methods. Description of methods to determine cut-off values. (DOCX 17 kb)

**Additional file 7: Table S6.** CYP2D6 allele frequencies. (DOCX 13 kb)

**Additional file 8: Table S7.** Concentrations of tamoxifen metabolites stratified by metabolizer group. (DOCX 14 kb)

**Additional file 9: Figure S1.** Kaplan-Meier plot of breast cancer-specific survival according to Antiestrogenic Activity Score. (DOCX 109 kb)

**Additional file 10: Table S8.** Distribution of patients with high and low Z-endoxifen (cut-off 9.0 nM) among patients with high and low Z-4OHTam (cut-off 3.26 nM). (DOCX 13 kb)

## Abbreviations

AAS: Antiestrogenic Activity Score; AI: Aromatase inhibitor; BC: Breast cancer; BCSS: Breast cancer-specific survival; CI: Confidence interval; CYP2D6: Cytochrome P450 2D6; EM: Extensive/normal metabolizer; ER: Estrogen receptor; HR: Hazard ratio; HWE: Hardy-Weinberg equilibrium; IM: Intermediate metabolizer; LC-MS/MS: Liquid chromatography-tandem mass spectrometry; LLQ: Lower limits of quantification; OS: Overall survival; PM: Poor metabolizer; PR: Progesterone receptor; UM: Ultra-rapid metabolizer; vt: Variant type; wt: Wild-type; Z-4OHTam: Z-4-hydroxy-tamoxifen; Z-endoxifen: Z-4-hydroxy-N-desmethyl-tamoxifen

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## Availability of data and materials

The data that support the findings of this study are available from OSBREAC research group but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are, however, available from the authors upon reasonable request and with permission of OSBREAC research group.

## Authors' contributions

TH contributed to the laboratory analyses, statistical analyses, interpretation of data, and drafted the manuscript. NH was responsible for the development of the LC-MS/MS analyses along with EB and SH. BN contributed the clinical samples from the OSBREAC group and was principal investigator of the OSLO1 study. EB performed the assessment of the pathological parameters and scorings. VNK provided data from the OSBREAC group and DNA for the CYP2D6 genotyping. JTK gave expert advice on the statistical analyses. TLL gave expert advice on several aspects of the study and interpretation of data. GIGA performed purification of DNA from bone marrow and whole blood samples. RHvS performed the CYP2D6 genotype analyses. EAMJ and EAL contributed to the concept of the study and interpretation of data. GM contributed to the concept of the study, the interpretation of the data, and funding of the study. HS contributed to the concept of the study, statistical analyses, interpretation of data, and funding of the study. All co-authors contributed to the writing of the manuscript and gave their final approval of the final version to be published.

**Ethics approval and consent to participate**

All participants have given written consent and the original OSLO1 study was approved by the Norwegian Regional Ethical Committee (2015/1216).

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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**Author details**

<sup>1</sup>Hormone Laboratory, Haukeland University Hospital, Bergen, Norway. <sup>2</sup>Department of Clinical Science, University of Bergen, Bergen, Norway. <sup>3</sup>Core Facility for Metabolomics, University of Bergen, Bergen, Norway. <sup>4</sup>Department of Oncology, Division of Cancer Medicine, Oslo University Hospital, Oslo, Norway. <sup>5</sup>Institute of Clinical Medicine, Faculty of Medicine, University of Oslo, Oslo, Norway. <sup>6</sup>Pathology Department, Radium Hospital, Oslo University Hospital, Oslo, Norway. <sup>7</sup>Department of Cancer Genetics, Institute for Cancer Research, Oslo University Hospital Radiumhospitalet, Oslo, Norway. <sup>8</sup>Department of Mathematics and Natural Science, University of Stavanger, Stavanger, Norway. <sup>9</sup>Department of Research, Stavanger University Hospital, Stavanger, Norway. <sup>10</sup>Department of Epidemiology, Rollins School of Public Health, Winship Cancer Institute, Emory University, Atlanta, USA. <sup>11</sup>Expert Center Pharmacogenetics, Department of Clinical Chemistry, Erasmus University Medical Center, Rotterdam, The Netherlands. <sup>12</sup>Department of Pathology, Stavanger University Hospital, Stavanger, Norway. <sup>13</sup>Department of Surgery, Section of Breast and Endocrine Surgery, Stavanger University Hospital, Stavanger, Norway.

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