The role of the androgen receptor and hydroxysteroid 17β dehydrogenase in breast cancer

Impact on tamoxifen treatment

Erik Hilborn
I find your lack of faith disturbing
- Darth Vader

Till min familj

III
Supervisor
Agneta Jansson, Associate Professor
Department of Clinical and Experimental Medicine, Linköping University, Sweden

Co-supervisor
Olle Stål, Professor
Department of Clinical and Experimental Medicine, Linköping University, Sweden

Faculty opponent
Yvonne Ceder, Associate Professor
Department of Translational Cancer Research, Lund University, Sweden

Board committee
Simin Mohseni, Associate Professor
Department of Clinical and Experimental Medicine, Linköping University, Sweden

Abdimajid Osman, Associate Professor
Department of Clinical and Experimental Medicine, Linköping University, Sweden

Helena Jernström, Associate Professor
Department of Clinical Sciences, Linköping University, Sweden
Abstract
The healthy breast is a tissue composed of centrally located milk producing glands connected to the nipple by ducts, surrounded by fat tissue and connective tissue. The growth of the breast is primarily mediated by the estrogens, while the androgens mediate tissue homeostasis and protect against growth signals. In breast cancer, the cells of the glands or ducts undergo malignant transformation, and start proliferating in an uncontrollable fashion. Breast cancer is the most common malignancy in women, and it is estimated that 10% of all women will be diagnosed with breast cancer during their lifetime. The primary classification of breast cancer is based mainly on the expression of the estrogen receptor, and 70-80% of breast cancers are estrogen receptor positive, and are classified as luminal. The remaining breast cancers are classified into HER2 positive or triple negative breast cancer. Out of all breast cancers, ~80% are androgen receptor positive. This varies in different subtypes, however, with the highest expression in luminal and lowest expression in triple negative breast cancers. The role of androgen receptor varies depending on subtype. It is considered tissue-protective in luminal breast cancer, while it’s role in HER2 positive and triple negative breast cancers is less defined, but is generally considered to be associated with worse outcome. The primary treatment for breast cancer is surgery, followed by chemotherapy and/or radiotherapy in order to reduce the risk of recurrence. Treatment is also subtype specific, and luminal breast cancers in premenopausal women are treated using the estrogen receptor blocker (antagonist) tamoxifen, which blocks estrogen signaling. In postmenopausal women, luminal breast cancers are treated using tamoxifen or aromatase inhibitors, which prevent the formation of estrogen. The knowledge of which patient will respond and who will develop treatment resistance is of great importance, and the development of markers which can be analyzed prior to treatment in order to reduce the risk of unwanted side effects or complications is the focus of a large body of research. One of the primary goals of this thesis was to establish biomarkers for prognosis and tamoxifen treatment in breast cancer, and paper I, paper II and paper III address this aim.

Steroid hormones, including estrogens and androgens, are normally synthesized from cholesterol in the adrenal gland, as well as in gender specific tissues such as ovaries in women or the testis or prostate in men. This synthesis takes place as a number of enzymatic conversions, mediated by several different enzymes, and the expression of these enzymes determines the final product of this conversion. In the adrenal gland, testis and prostate, androgens are the end-product, while the ovaries synthesize estrogens. These hormones are transported through the circulation, and upon reaching their target tissues, they mediate their effect. The impact of the steroids on their destination tissue is dependent on their relative concentration and exposure time, which in turn is dependent on the amount in the circulation, but also on the presence of local steroid converting enzymes, which are present in most tissues. The enzymes of the hydroxysteroid 17β dehydrogenase family are present in most tissues, primarily the oxidative member hydroxysteroid 17β dehydrogenase type 2, which facilitate the conversion of estrogens and androgens to the less active forms, thus protecting the tissues from their effect. In breast cancer, the reductive form, hydroxysteroid 17β dehydrogenase type 1 is often up-regulated, and mediates increased activation of estrogens, resulting in increased estrogen signaling, which results in increased proliferation and growth. The second goal of this
thesis was to further study the role of hydroxysteroid 17β dehydrogenase enzymes in breast cancer, and paper I and paper IV address different aspects of their role in breast cancer.

Following reduction of the expression of hydroxysteroid 17β dehydrogenase type 14, an oxidative member of the family, in breast cancer, the expression of C-X-C ligand 10 was found to be altered. In paper I, to determine the role of C-X-C ligand 10 and C-X-C receptor 3 in breast cancer, their expression was quantified using immunohistochemistry in breast cancer patients randomized to tamoxifen or no endocrine treatment irrespectively of estrogen receptor status. The expression of C-X-C ligand 10 and C-X-C receptor 3 was found to be associated with increased tamoxifen treatment benefit in the estrogen receptor positive group of patients, indicating that they could be useful markers for determining which patient would respond well to this treatment. Further, C-X-C receptor 3 expression was associated with worse outcome in patients who did not receive tamoxifen, and could be a potential target for inhibitors in order to improve patient outcome.

The role of the androgen receptor in breast cancer was evaluated. In paper II the expression was quantified using immunohistochemistry in the same cohort as in paper I. We show that in patients with estrogen receptor negative tumors, the androgen receptor is associated with worse outcome. In patients with high tumoral androgen receptor expression, tamoxifen signaling results in significant improvement in outcome, despite lack of the estrogen receptor. The opposite was observed in patients without tumoral androgen receptor expression, and tamoxifen treatment was associated with adverse outcome. Similar findings were made in the triple negative cases. In the luminal cases, the androgen receptor does not provide further information pertaining to outcome. In paper III we evaluated the role of mutations in the androgen receptor in the cohort of estrogen receptor negative and androgen receptor positive cases from paper II. The role of mutations in the androgen receptor appear to have a modest role in regard to patient outcome, but rs17302090 appear associated with tamoxifen treatment benefit.

The modulation of the members of the hydroxysteroid 17β dehydrogenase in breast cancer is associated with changes in the local steroid balance, and has been associated with worse outcome and changes in the response to tamoxifen. Further, the inhibition of hydroxysteroid 17β dehydrogenase type 1 has been proposed as an alternate treatment for breast cancer, but no inhibitors are currently used in the clinic. In paper IV, we evaluated several different mechanisms by which the expression of hydroxysteroid 17β dehydrogenase type 1 and type 2 are modulated in breast cancer. We show that the most potent estrogen estradiol, in an estrogen receptor dependent fashion, can result in decreased hydroxysteroid 17β dehydrogenase type 1 expression, and a short term reduction in type 2 expression or long term increased type 2 expression. We also show that the most potent androgen, dihydrotestosterone, can increase hydroxysteroid 17β dehydrogenase type 2 expression, but has limited impact on hydroxysteroid 17β dehydrogenase type 1. Further, we show that a number of genes involved in breast cancer, and microRNA are involved in modulating the expression of the hydroxysteroid 17β dehydrogenase type 1 and type 2 in breast cancer. These findings could potentially be used as an alternative to inhibitors, and help modulate the steroidal balance in target tissue.
Populärvetenskaplig sammanfattning

Ett vanligt friskt bröst består av mjölkproducerande körtlar, sammanlänkade till bröstvärtan via körtelgångar vars funktion är att transportera mjölk vid amning. Denna struktur ligger omvälvd av fettvävnad. Den huvudsakliga faktorn som styr bröstets utveckling och funktion är de kvinnliga könshormonen, östrogen. För att skapa balans i tillväxten finns även manliga könshormon, androgen, vars roll i bröstet är att motverka östrogenets effekt och skydda mot tillväxtsignaler.

I bröstcancer så genomgår cellerna i körtlar eller körtelgångarna en elakartad omvandling, och börjar växa okontrollerat. Bröstcancer delas in i undergrupper, och de olika undergrupperna har olika behandling och prognos. Den viktigaste faktorn för klassifikation är östrogenreceptorn, som uttrycks av 70-80% av alla bröstcancrar. En bröstcancer som uttrycker östrogenreceptorn är luminal. Resterande 20-30% delas upp i ungefär lika stora delar utifrån uttrycket av proteinet HER2, om tumören uttrycker HER2 klassas den HER2 positiv, annars är den trippelnegativ. Totalt är ca 80% av alla bröstcancrar positiva för androgenreceptorn. Detta uttryck varierar i olika undergrupperna, och det är högst i luminala, och lägst i trippelnegativa bröstcancrar. Androgenreceptorns roll varierar utifrån undergrupp och anses vara skyddande i luminal bröstcancer. Dess roll är mindre tydlig i HER2 positiv och trippelnegativa bröstcancrar, även om de generellt är associerad med sämre prognos.


Steroidhormon, vilket innefattar östrogen och androgen, syntetiseras normalt från kolesterol i binjuren, samt i ovari hos kvinnor. Denna syntes sker genom flera steg av enzymatisk transformation. I binjuren saknas enzymen för att katalysera omvandlingen av androgen till östrogen, vilket leder till att slutprodukten är androgener. I ovarierna finns de enzym som medierar omvandlingen av androgen till östrogen, aromatas, och därför syntetiseras östrogener. Dessa hormoner transporteras efter syntesen ut i blodet, och cirkulerar tills de når sin målvävnad, där de har sin effekt. Resultatet av hormonerna i vävnaden är helt beroende på mängden, men också verkningstiden. Detta i sin tur beror på mängden hormon i blodet, och på uttrycket av steroidomvandlande enzym i målvävnaden.

Enzymen i hydroxysteroid 17β dehydrogenas familjen finns i de flesta vävnader, speciellt den oxidativa medlemmen hydroxysteroid 17β dehydrogenas typ 2. Effekten av hydroxysteroid 17β dehydrogenas typ 2 anses skydda neger från vävnaderna och androgenernas verkan, genom att minska deras aktivitet. I bröstcancer så är hydroxysteroid 17β dehydrogenas typ 1 ofta uttryckt till högre grad än den skyddande typ 2, vilket resulterar i ökad aktivitet hos
östrogener, och ökad tillväxt. Det andra målet med denna avhandling var att vidare undersöka funktionen av hydroxysteroid 17β dehydrogenas enzymen i bröstcancer, och artikel I och artikel IV är kopplade till deras roll i bröstcancer.

Efter att en koppling mellan den vävnadskyddande hydroxysteroid 17β dehydrogenas typ 14 och C-X-C ligand 10 (som är kopplad till immunförsvar i bröstcancer) påvisades, valde vi i artikel I att undersöka funktionen hos C-X-C ligand 10 dess motpart C-X-C receptor 3 i bröstcancer. Detta gjordes genom att vi färgade tumörmaterial med markörer för C-X-C ligand 10 och C-X-C receptor 3. Patienterna i detta material hade behandlats med tamoxifen eller ingen tamoxifen, oberoende av undergrupp, vilket gör att vi kan studera effekten av tamoxifen i olika patientgrupper. Vi såg att högt uttryck av C-X-C ligand 10 och C-X-C receptor 3 var associerat med ett förbättrat utfall hos patienter som behandlats med tamoxifen, vilket antyder att de skulle kunna användas för att förutsöpa vilkapatienter som skulle svara väl på denna typ av behandling. Vidare var C-X-C receptor 3 uttrycket associerat med sämre prognos hos patienter som inte behandlats med tamoxifen, vilket kan vara till kliniskt nytta.

Androgenreceptorns roll i bröstcancer utvärderades i artikel II. Uttrycket av androgenreceptorn kvantifierades med samma metod i samma patienter som använts i artikel I. Närvaron av androgenreceptorn var kopplat till sämre prognos i patienter med östrogenerceptornegativa tumörer. Dock svarade patienter med androgenreceptorn bättre på tamoxifen, medan patienter vars tumörer saknar androgenreceptoruttryck klarade sig sämre när de fick tamoxifen. I de luminala tumörena gav androgenreceptorn ingen ytterligare information. I artikel III undersökte vi rollen av mutationer i androgenreceptorn i samma östrogenerceptornegativa och androgenreceptor positiva kohort som i artikel II visat god nytta av tamoxifen. Mutationernas roll var blygsam gällande utfall, men en av mutationerna (rs17302090) verkar associerad med god nytta av tamoxifen.

Förändringar i uttrycket av hydroxysteroid 17β dehydrogenas är förknippat med ändringar i balansen av steroidhormon i vävnaden, och det har kopplats till sämre prognos och förändring i svaret på behandlingen med tamoxifen. Blockad av hydroxysteroid 17β dehydrogenas typ 1 med inhibitorer har föreslagits som alternative behandling till patienter som utvecklar resistens mot traditionell behandling. Blockad skulle även kunna göras tillsammans med nuvarande primärbehandling för att ge ökad effekt. Idag finns dock inga sådana inhibitorer i kliniken. I artikel IV undersökte vi flera olika mekanismer för hur uttrycket av hydroxysteroid 17β dehydrogenas typ 1 och typ 2 regleras i bröstcancer och visar att östrogener och androgene kan förändra uttrycket av hydroxysteroid 17β dehydrogenas typ 1 och typ 2. Vi visar också att flera gener som är involverade i östrogensignalering i bröstcancer samt mikro-RNA kan påverka uttrycket av hydroxysteroid 17β dehydrogenas typ 1 och typ 2 i bröstcancer. Dessa fynd kan potentiellt leda till andra sätt att påverka hydroxysteroid 17β dehydrogenas balansen i patienter, och vara ett alternativ till inhibitorer för hydroxysteroid 17β dehydrogenas typ 1.
Foreword
Part of this thesis is based on a review, which was recently submitted, detailing the function and role of the enzymes of the hydroxysteroid 17β dehydrogenase family. As such, there may be some parts which share language similarities with this piece.
List of papers

Paper I
ERIK HILBORN, Tove Sivik, Tommy Fornander, Olle Stål, Bo Nordenskjöld, Agneta Jansson.
C–X–C ligand 10 and C–X–C receptor 3 status can predict tamoxifen treatment response in breast cancer patients.

Paper II
ERIK HILBORN, Jelena Gacic, Tommy Fornander, Bo Nordenskjöld, Olle Stål, Agneta Jansson
Androgen receptor expression predicts beneficial tamoxifen response in oestrogen receptor-α negative breast cancer
British Journal of Cancer, 2016, 114, 248-255

Paper III
ERIK HILBORN, Tommy Fornander, Bo Nordenskjöld, Olle Stål, Agneta Jansson
Androgen receptor Single Nucleotide Polymorphisms in androgen receptor positive and estrogen receptor negative breast cancer
Manuscript

Paper IV
ERIK HILBORN, Olle Stål, Agneta Jansson
The regulation of hydroxysteroid 17β-dehydrogenase 1 and 2 gene expression in breast cancer by estradiol, dihydrotestosterone, microRNAs and genes related to breast cancer
Manuscript
Table of Contents

The role of the androgen receptor and hydroxysteroid 17β dehydrogenase in breast cancer.... 1
Impact on tamoxifen treatment ................................................................................................. 1
Abstract ...................................................................................................................................... V
Populärvetenskaplig sammanfattning ...................................................................................... VII
Foreword ..................................................................................................................................... X
List of papers .......................................................................................................................... XI

Introduction .................................................................................................................................. 1
Normal breast ............................................................................................................................ 1
Breast cancer .............................................................................................................................. 2

MicroRNA ................................................................................................................................... 4
Estrogens ................................................................................................................................. 4
ER ................................................................................................................................................ 4
Androgens ............................................................................................................................... 5
AR ................................................................................................................................................ 5

The role of AR in tamoxifen treatment ....................................................................................... 6
Steroid hormone synthesis ......................................................................................................... 6
Hydroxysteroid dehydrogenase ................................................................................................. 9
HSD17B1 and HSD17B2.............................................................................................................. 9

Role of HSD17B1 and HSD17B2 in breast cancer ..................................................................... 9
The clinical relevance of HSD17B1 and HSD17B2 .................................................................. 10
Inhibitors of HSD17B1 and HSD17B2 ..................................................................................... 10
Control of expression and regulation of HSD17B1 and HSD17B2 ........................................... 10
Other HSD17B enzymes in breast cancer ................................................................................. 12
C-X-C ligand 10 and C-X-C ligand receptor 3 ......................................................................... 12

Methodical considerations ....................................................................................................... 15
Patients ....................................................................................................................................... 15
Immunohistochemistry ............................................................................................................ 15
Nucleic acid isolation ............................................................................................................... 16
PCR .......................................................................................................................................... 16
Reverse transcription-PCR ....................................................................................................... 17
Quantitative PCR ..................................................................................................................... 17

XII
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotyping</td>
<td>17</td>
</tr>
<tr>
<td>Diplotype construction</td>
<td>18</td>
</tr>
<tr>
<td>Statistics</td>
<td>19</td>
</tr>
<tr>
<td>Cell culture</td>
<td>19</td>
</tr>
<tr>
<td>Cell lines</td>
<td>19</td>
</tr>
<tr>
<td>Hormonal treatment</td>
<td>19</td>
</tr>
<tr>
<td>Silencing RNA treatment</td>
<td>20</td>
</tr>
<tr>
<td>Micro RNA treatment</td>
<td>20</td>
</tr>
<tr>
<td>Summary of papers</td>
<td>21</td>
</tr>
<tr>
<td>Paper I</td>
<td>21</td>
</tr>
<tr>
<td>Aim</td>
<td>21</td>
</tr>
<tr>
<td>Results</td>
<td>21</td>
</tr>
<tr>
<td>Discussion and conclusion</td>
<td>21</td>
</tr>
<tr>
<td>Paper II</td>
<td>22</td>
</tr>
<tr>
<td>Aim</td>
<td>22</td>
</tr>
<tr>
<td>Results</td>
<td>22</td>
</tr>
<tr>
<td>Discussion and conclusion</td>
<td>23</td>
</tr>
<tr>
<td>Paper III</td>
<td>23</td>
</tr>
<tr>
<td>Aim</td>
<td>23</td>
</tr>
<tr>
<td>Results</td>
<td>23</td>
</tr>
<tr>
<td>Discussion and conclusion</td>
<td>24</td>
</tr>
<tr>
<td>Paper IV</td>
<td>24</td>
</tr>
<tr>
<td>Aim</td>
<td>24</td>
</tr>
<tr>
<td>Results</td>
<td>24</td>
</tr>
<tr>
<td>Discussion and conclusion</td>
<td>25</td>
</tr>
<tr>
<td>Concluding remarks</td>
<td>27</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>29</td>
</tr>
<tr>
<td>References</td>
<td>31</td>
</tr>
</tbody>
</table>
Introduction
Normal breast
The breast normally develops during puberty in a number of stages as a result of signaling from primarily estrogens and progesterone. The final product is a tissue composed primarily of fat tissue, with centrally located glands connected to the nipple by ducts. The entire tissue is supported by connective tissue, Figure 1. These glands and ducts undergo growth and regression during the normal menstrual cycle and become active during lactation. The effect of sex steroids on breast tissue in genetic females is normally primarily mediated by estrogens. Estrogen signaling results in breast growth, and changes in estrogen exposure occur naturally in the different stages of life, such as puberty and pregnancy. The effect of androgens in breast tissue are in direct opposition to those of estrogens, mediating tissue homeostasis, and protection against proliferative signals and can lead to breast atrophy. Sufficient androgen concentrations prevents formation of breasts, even in genetic females, in certain disorders such as adrenal tumors. The balance of estrogens and androgens thus determine the future of the breast in any individual (1).

Figure 1. The anatomy of the breast. The centrally located lobule, supported by connective tissue and surrounded by fat deposits. Ductal structures emanating from the lobule and connect them to the nipple. The entire breast is supported by chest musculature attached to the chest wall.
Breast cancer
Breast cancer is the malignant growth of cells in the breast tissue, and 80% of breast cancers are derived from the ductal epithelial cells (Ductal carcinoma), and 5-15% are derived from the lobular epithelial cells of the glands (Lobular carcinoma). The remaining breast cancers include mucinous or apocrine breast cancers and are less common. Breast cancer is staged and graded based on the TNM and Nottingham Histological Grade (NHG) systems, respectively. In TNM tumor size (T), nodal status (N) and distant metastasis (M) are combined into a stage score. NHG combines a 1-3 score for tubule formation, nuclear irregularity and number of mitoses into a grade score, where score 3-5 is grade 1, 6-7 is grade 2 and 8-9 is grade 3. These systems are used to assist in determining the severity of the disease and to help determine the best treatment options for the patient. Breast cancer is further divided into subgroups based on the expression of certain proteins, as determined by immunohistochemistry (IHC). Luminal breast cancers are associated with the best prognosis, Luminal A breast cancers express estrogen receptor (ER)α, progesterone receptor (PgR), and have low Ki67 expression. Luminal B breast cancers are ERα-positive and either PgR or human epidermal growth factor (HER)2 positive, combined with moderate/high Ki67 expression. ERα-negative breast cancers are more heterogeneous in nature, and are often subdivided into HER2 amplified (ERα- HER2+) and triple negative breast cancer (TNBC), both of which have increased Ki67 expression.

Breast cancer is the most common malignancy in women, and the second most common malignancy overall (2). It is estimated that 10% of all women in the developed world will be diagnosed with breast cancer during their life-time. Hereditary breast cancer constitutes approximately 10-15% of breast cancer, and mutation to the DNA repair genes BRCA1 and BRCA2 is the primary risk hereditary risk factor, greatly increasing the risk of developing breast cancer. Being a woman, increased age, high breast density, height, exposure to harmful environmental factors, early menarche, late menopause, not bearing children, not breastfeeding, having hormone replacement therapy, having Caucasian descent, lack of physical exercise, certain high fat diets and alcohol consumption are all risk factors generally attributed to increased breast cancer risk (3, 4). The role of oral contraceptives in regard to breast cancer risk is unclear, a number of studies have shown an increase in relative risk which from oral contraceptives (5, 6) while, some studies show no increased risk for oral contraceptives (7, 8). The importance of oral contraceptives has been highlighted regard to the estrogen/androgen balance, since increased estrogen from oral contraceptives could reduce the de-novo synthesis of both estrogens and androgens, and since estrogens but not androgens are provided in many oral contraceptives, this would reduce in excessive estrogen signaling (1). Recently, increased focus has been put into trying to determine if different subtypes of breast cancer have different risk based of well-established risk factors. Table 1, details the findings of two recent meta-reviews on the topic (3, 4).

The primary treatment for all breast cancer is surgery, followed by chemotherapy and/or radiotherapy to reduce the risk of recurrence. Further treatment is based on the respective subtype. ERα is expressed in 70-80% of all breast cancers (9, 10). ERα-positive (luminal) patients are treated with endocrine treatment, either tamoxifen, a selective ER modulator (SERM) which binds the ER and prevents estrogen binding, or aromatase inhibitors, which inhibit the conversion of androgens into estrogens, thus limiting the available estrogen.
Tamoxifen has been the golden standard for treatment of ERα positive breast cancer, and is given to premenopausal women, and as a compliment to aromatase inhibitors in postmenopausal women. Fulvestrant, which marks ER for degradation is a second line treatment in ERα positive patients. Meanwhile, patients with HER2 positive disease are often treated using trastuzumab (11). While these treatments are efficient in reducing recurrence, progression and adverse outcome, a problem which frequently arise is resistance to treatment, either intrinsic or acquired. In order to know which patients will benefit from treatment, or who will develop resistance, so called biomarkers, are the focus of significant research.

| Table 1, overview of risk factors associated with subtypes of breast cancer |
|--------------------------------|------------------|------------------|------------------|------------------|
| Increased age of Menarche (4) | Probability of reduced risk | Possible reduced risk | Inconclusive | Reduced risk |
| Parity vs Nullparity (3, 4)   | Substantially reduced risk | No association | No association | Increased risk (4) |
| Young age of first birth (3, 4)| Reduced risk | No association | No association | No association |
| Increased time of breastfeeding (3, 4) | Reduced risk | Reduced risk | No association | Reduced risk |
| Increased age of menopause (4) | Increased risk | Inconclusive | Inconclusive | Probable increased risk |
| High BMI (prenopausal) (4)    | Reduced risk | Inconclusive | Inconclusive | Increased risk |
| High BMI (postmenopausal) (4) | Inconclusive | Inconclusive | Inconclusive | No association |
| Family history (4)            | Strongly increased risk | Strongly increased risk | Strongly increased risk | Strongly increased risk |
| Alcohol usage (4)             | Increased risk (7 drinks weekly) | No association | Possibly increased risk | Inconclusive |
| Oral Contraceptives (4)       | Reduced risk | Inconclusive | Inconclusive | Increased risk |
| Hormone replacement therapy (4) | Strongly increased risk | Increased risk | Inconclusive | Inconclusive |
MicroRNA
MicroRNAs (miRs) are ~21 nucleotides short inhibitory RNAs, involved in almost every part of carcinogenesis and have been estimated to regulate the majority of all genes (12). miRs bind their targets based on their SEED region, bases 2-7 of their 5’ region, however, the 8th base is also important in determining binding, and the 3’ portion of the miRNA has also been shown to influence binding specificity. Due to the relative shortness of the sequence, each miRNA can bind as many as hundreds of genes. Studies of miRNA function using overexpression or inhibition of miRNA have yielded much information into the role of miRNA. However, due to the broad spectrum of targets for each miRNA, the role of a specific miRNA is not always shared between different species, or even tissues. Normal miRNA modulation suppresses their targets by about 2-fold, a relatively modest change, and many seed regions are highly conserved across multiple miRNA. Further, knockdown studies of single miRNA often have little apparent detectible impact on the organism. These findings together have led to the idea that many miRNA form a net of fine tuning modulators of gene expression. Further, while single miRNA on their own may seem redundant based on limited phenotypical changes in knockdown animals, their role may be specific to specific stressors such as injury or stress. Another system in which miRNA may be important is as buffers, continuous suppressors of noise in transcriptional regulation, caused for instance by sudden changes in temperature, or hormonal fluctuations (13).

Estrogens
Per definition, estrogens are any of a group of hormones which promote the development and maintenance of female characteristics of the body. They include estrone (E1) and estradiol (E2) as well as a number of less common variants and any artificial compounds which act as oral contraceptives or for treatment of menopausal or menstrual disorders. The primary endogenous estrogens are E1 and E2, which bind to the ERα and ERβ. E2 is the most potent natural estrogen, and has an IC50 of approximately 10nM for both ERα and ERβ. E1 on the other hand, has an IC50 of roughly 1nM for ERα and 0.2nM for ERβ (14).

ER
Estrogen signaling by ERα in breast cancer cells results in proliferation and survival signals while suppressing the expression of antiproliferative and apoptotic targets (15). Additionally, there is a second form of ER, known as ERβ, which shares 56% sequence homology with ERα, which is more antiproliferative and mediates tissue homeostasis. The primary form of ER in healthy breast and most breast cancers is ERα, and as a result, most estrogen signaling is mediated through ERα signaling in breast cancer (16). ER actions can be divided into genomic and rapid response. The genomic response takes at least a few hours to occur. In this pathway ER undergoes dimerization and binds to ER binding sites. These binding sites are characterized by cofactors and by pioneering factors like AP-2, FoxA1 and GATA3. Pioneering factors are proteins which facilitate the binding of nuclear receptors to their targets by opening up the chromatin, without them the binding is greatly diminished (17, 18). Further cofactors are recruited following ER binding. ER binds to the estrogen response elements (ERE) located in promotors (18) or distal sites from where ER interacts with targets by chromatin looping (17). In the non-classical genomic pathway, phosphorylated ER can act independently of ligands, as
a response to p38 MAPK, JNK or PI3K/AKT signaling. Besides the genomic response, there is the rapid response, sometimes referred to as the non-genomic response. This can occur within seconds, and is mediated through phospholipase C (PLC), protein kinase C (PKCs) Ras/Raf/MAPK and cAMP protein kinase A (PKA). ERβ signaling can counteract ERα signaling somewhat, having growth inhibitory properties and by being able to form heterodimers with ERα, resulting in reduced activity compared to ERα dimers (15, 16, 19, 20).

**Androgens**
Per definition, androgens are sex hormones which promote male secondary characteristics. Androgens include DHEA, androstenediol, androstenedione, testosterone (T) and dihydrotestosterone (DHT). The different androgens differ mainly in binding affinity to different receptors. The greatest affinity for AR is DHT, which binds primarily to AR in order to mediate its effect. Slightly less potent AR binding results from T binding, with 2-10 fold reduced effect compared to DHT binding. T can also bind directly to the ER. Further, DHEA has some affinity for AR but less than DHT and T, with androstenediol and androstenedione having the least AR affinity compared to the other androgens (21, 22). Androstenediol also has some affinity for the ER (23, 24).

**AR**
The androgen receptor (AR) is frequently expressed in normal breast epithelium and in malignant breast tumors (up to 80%) (25, 26), its expression differs in breast cancer subtypes, with 84-95% in luminal, 50-63% in HER2 amplified and 10-53% in TNBC (27). The great variation in percentages stems from varying cutoff values, antibodies and different selection criteria for the cohorts.

AR modulates the expression of more than a thousand genes, many of them highly tissue specific. The AR can mediate its effect by several different pathways. Following ligand binding to the AR it will dimerize. The AR-AR dimer binds to distal AR-binding-sites, and will subsequently recruit cofactors which can reach across the folded chromosome to interact with the promotors of androgen regulated genes. Classic binding of the AR to target sites such as the androgen response element (ARE) and glucocorticoid response element (GRE) is another important mechanism of its action. It is believed that the sequence of the binding site in itself is only part of the requirement for specific binding, since cofactors and pioneering factors such as FoxA1 and GATA2 are pivotal in facilitating AR binding. The primary AR response occurs within seconds and peaks around a few hours (18).

The role of AR in breast cancer is greatly dependent on the subtype studied. Several studies have reported an improved patient outcome associated with increased AR expression in ERα-positive breast cancer, possibly associated with an AR mediated inhibition of proliferation (28-33). The role of AR in the ERα-negative subgroup is more controversial, being associated with either improved or worsened patient outcome. This discrepancy likely stems from variation in the patient selection criteria of the cohort studied as well as differing AR cut-off values (33, 34). TNBC is a diverse group which is difficult to treat with high risk of recurrence and poor prognosis compared to other subtypes. Several reports on TNBC indicate a positive correlation between AR expression and better clinical outcome (35-38). Further, the TNBC AR positive
group has been shown to respond to AR antagonists, in addition a portion of these patients express the luminal AR (LAR) gene expression profile, which resembles that of ER positive breast cancer and is associated with improved outcome (27, 39).

The role of AR in tamoxifen treatment

While patients with ERα-negative breast cancer generally do not respond to tamoxifen therapy, studies in both ERα-positive and negative breast cancer have shown a connection between AR and tamoxifen response. Park et al., showed that AR status is a positive factor in determining response to tamoxifen in patients with ERα-positive breast cancer (31). On the other hand, using an in vitro model, De Amicis and colleagues showed that increased AR to ERα ratio was an indicator of tamoxifen resistance (40). In paper II we show that patients with ERα-negative and AR-positive tumors benefit from tamoxifen treatment, while patients with ERα-negative and AR-negative tumors did worse when treated with tamoxifen. Recently, using a cohort with both ERα-positive and negative patients, Lundin et al., showed that diplotypes based on six AR single nucleotide polymorphism (SNP)s (rs17302090, rs6152, rs7061037, rs1337080, rs5031002 and rs5964607) could predicted patient outcome and tamoxifen response (41). Furthermore, rs17302090, rs6152 and 7061037 were associated with an increased risk of developing prostate cancer (42) and rs17302090 was borderline associated with worse outcome (43). In paper III we show that WT-variant rs17302090 had a somewhat improved tamoxifen response compared to the entire cohort. Further, patients grouped based on the outcome of their diplotype, revealed a group of patients with adverse outcome, which benefitted from tamoxifen treatment.

Steroid hormone synthesis

Steroid hormones such as estrogens and androgens are synthesized from cholesterol through a series of pathways which begin in the mitochondria of steroidogenic cells in the cortex of the adrenal gland. Most available cholesterol is derived from low density lipoproteins (LDL), which transports dietary cholesterol to the cells. However, cholesterol can also be synthesized de novo from acetate, which is sufficient for normal steroid synthesis in statin treated patients (44, 45). The synthesis of steroid hormones takes places in three distinct layers of the adrenal cortex; the zona glomerulosa where aldosterone is produced, the zona fasciculata synthesizes cortisol and cortisone and the zona reticularis where the synthesis of androgens DHEA and androstendione takes place. The biotransformation of cholesterol to the respective steroids is detailed in figure 2a. All products of the adrenal gland are exported by a centrally located vein. Of primary interest in breast development are the adrenal androgens. They are exported into the circulation from where they make their way to peripheral tissues and either mediate their action or become converted into androstenediol, E1, E2 or DHT by steroid converting enzymes, Figure 2a (44, 45). In men, additional testosterone synthesis occurs in the testes Leydig cells, Figure 2b. In women, the theca and granulosa cells in the ovaries mediate the conversion of cholesterol to estrone or estradiol. Further, in the corpus luteum, progesterone is synthesized, Figure 2c (44).
Figure 2. Schematic representation of the synthesis of steroids. 
Steroids continuously assert their influence on the tissues, and the resulting effect of this stimulation is dependent on duration and intensity of the steroid stimulation. This in turn is dependent on the circulating concentrations of the respective steroid. However, the effect and affinity of steroids is modulated by steroid converting enzymes in the peripheral tissue. Hence duration and intensity of steroid stimulation in any tissue is dependent in part of circulating steroids, but also on the relative local concentration of steroid converting enzymes, since this will determine the final availability of steroids in the target tissue. The primary site of estrogen production in premenopausal women is the ovaries, while most androgens are synthesized in the adrenal glands. In postmenopausal women, the ovarian production of estrogens is greatly diminished, and adrenal androgens and biologically inactive sulfated estrogens become the primary circulating steroids. This results in a shift of the primary estrogen production site from the ovary to the peripheral tissues. In the breast tissue this conversion is primarily mediated by a number of enzymes, including aromatase, steroid sulfatase (STS) and hydroxysteroid 17β hydrogenase (HSD17B) 1, 2, 4, 5 and 7, Figure 3 (46-53). The focus of this thesis is on the enzymes of the HSD17B family.

Figure 3. Schematic representation of the enzymatic conversion of sex steroids in breast tissue.
Hydroxysteroid dehydrogenase
The HSD17B family was first reported in the 50’s when enzymes mediating conversion of 17β-hydroxysteroids (androgens and estrogens) in the placenta were discovered (54). In the 90’s the first members of the HSD17B family were cloned, sequenced and their function documented (46-50). The enzymes of the HSD17B family are numbered in the order in which they were discovered. To date, 14 members have been identified, and with the exception of HSD17B5, which is an aldo-keto reductase (AKR), they are all part of the short-chain dehydrogenase/reductase (SDR) family. The HSD17Bs share a relatively low sequence homogeneity, approximately 20-30%. Despite this, there is a substantial overlap in enzymatic activity between family members, with HSD17B1, 3, 5, 7 and 12 catalyzing reduction and 2, 4 and 14 the oxidation of 17β-hydroxysteroids. The primary differences between the different reductive and oxidative members are the preferred substrate and their pattern of expression. The reduced forms of both androgens and estrogens, T and E2 respectively, have higher binding affinity to their respective receptors than their oxidized counterparts androstenedione and E1 respectively. The oxidizing reaction is considered protective against the effects of sex hormones, and the enzymes which catalyze the oxidizing reactions are more widely expressed than the reductive counterparts, and the oxidative enzymes are sometimes lost or down-regulated in cancer.

HSD17B1 and 2
HSD17B1 is the first member of the HSD17B family discovered. The gene HSD17B1 is localized to 17q11-q21 and encodes a 6 exon protein composed of 328 amino acids with a molecular mass of 34.95 kDa. The enzyme is expressed in the cytoplasm (49). HSD17B1 is active as a homodimer composed of two subunits. HSD17B1 is a reductive enzyme, catalyzing the reduction of E1 to E2, DHEA to androstenediol and DHT into 3β-diol and 3α-diol (55), both of which have much lower affinity for AR and increased affinity for ERβ and to some degree ERα compared to DHT (56-58). HSD17B1 has been shown to be the most active enzyme in regards to E2 production (59). Maintenance of low DHT concentration in the breast tissue is important for ERα-positive breast cancer progression, since increased DHT concentrations will result in inhibition of proliferation (60, 61). In healthy tissue, HSD17B1 is primarily located to the placenta and ovary (49), but it is also expressed at low levels in breast epithelium (62, 63).

The gene HSD17B2 is localized to 16q24.1-q24.2 and encodes a 6 exon protein composed of 387 amino acids with a molecular mass of 42.785 kDa. HSD17B2 is likely located to the endoplasmic reticulum, as indicated by its endoplasmic retention motif (48). HSD17B2 catalyzes the oxidation of E2 to E1, testosterone to androstenedione and androstenediol to DHEA (64). HSD17B2 is expressed in placenta, lung, liver, pancreas, kidney, prostate, colon, small intestine, endometrium (49) and breast epithelial cells (62).

Role of HSD17B1 and HSD17B2 in breast cancer
In the healthy breast, the oxidative reaction of estradiol catalyzed by HSD17B2 is preferred over the reductive reaction (62, 63). In breast cancer, HSD17B1 expression is often increased, and the preferential reaction is reductive (53, 65-67). In postmenopausal patients, the ratio of
E2 to E1 in the breast tissue is increased due to decreased circulating E1. This is accompanied by increased \textit{HSD17B1} mRNA expression levels, but no change in aromatase or sulfatase levels (68). \textit{In vitro} and \textit{in vivo} experiments highlight the role of HSD17B1 in mediating the growth response of E1 in breast cancer, with a significant reduction in E1 mediated proliferation when HSD17B1 is down-regulated. The loss of proliferation was accompanied by reduced E2 but increased DHT levels (55, 59, 69). Recently, a steroid independent function of \textit{HSD17B1} was demonstrated, and an increase in apoptotic gene profile accompanied its expression (70).

The tissue protective HSD17B2 is frequently down-regulated or lost in breast cancer compared to benign tumors (53, 71). Further, in breast cancer, the expression of HSD17B2 has been inversely correlated to E2 levels and numerous adverse clinical factors (66, 72-74). In ERα-negative breast cancer the relevance of HSD17B2 is not as defined, and it has been reported to be overexpressed in this group of patients (65). In invasive lobular carcinoma (ILC) the HSD17B2 expression was significantly higher than in invasive ductal carcinoma (IDC), and it was accompanied by reduced tumor size when expressed (75).

**The clinical relevance of HSD17B1 and HSD17B2**

Increased expression of HSD17B1 has been shown to be related to significantly adverse outcome, including shorter disease-free survival in postmenopausal patients as well as worse prognosis in postmenopausal patients with ERα-positive tumors (74, 76, 77). In patients with ERα-positive tumors, a high HSD17B2 expression was associated with improved prognosis and reduced risk of recurrence. The ratio of HSD17B1 to HSD17B2 is also prognostic, and high HSD17B1 to HSD17B2 is associated with worse outcome, while high HSD17B2 to HSD17B1 is associated with improved outcome (74, 77). Further increased copy number of the \textit{HSD17B1} gene was associated with reduced breast cancer survival (78). In postmenopausal patients with tumors expressing a high HSD17B1/HSD17B2 protein ratio, patients had less benefit from tamoxifen treatment (79). Further, in ERα-positive premenopausal breast cancer patients who received tamoxifen treatment, low HSD17B1 expression was associated with reduced risk of recurrence (80).

**Inhibitors of HSD17B1 and HSD17B2**

Several authors have proposed the use of HSD17B1 inhibitors for breast cancer, either as a single treatment, conceivably once resistance to aromatase inhibitors has arisen, or in combination with other treatments (55, 81, 82). The benefit of HSD17B1 inhibition would be that the reduction of E2 levels and increase in DHT levels would be limited to tissues expressing HSD17B1 (primarily ovary, placenta and many breast cancer). As a result, side effects should be more limited than current anti-hormonal treatments (49, 62, 63). There are two primary forms of inhibitors available, steroidal and nonsteroidal (83, 84). However, despite a plethora of tested inhibitors, there is currently no clinically used HSD17B1 inhibitors, and more testing is needed to find suitable candidates.

**Control of expression and regulation of HSD17B1 and HSD17B2**

HSD17B1 has been shown to be situated with a promotor in the 5’ flanking region from -78 to +9, and a silencer element located -113 to -78. The binding sites of transcription factors specificity protein (SP)1 and SP3 are present at -52 to -43, and regulate 30-60% of promotor
activity. Additionally, activating protein (AP)2 binds at -62 to -53 and counteracts the actions of SP1 and SP3. Further, GATA 3 has also been shown to reduce the HSD17B1 promotor activity (85). Both SP1 and GATA3 are targets of ERα, which indicates that there may be a connection between ERα and HSD17B1 expression. A recent meta-analysis on the impact of HSD17B1 polymorphism rs605059 show that it might confer genetic cancer susceptibility in Caucasians, but authors propose more studies are needed (86). On the other hand, the SNP rs4445895_T was shown to be associated with lower intratumoral HSD17B2 mRNA levels and inversely correlated with E2 levels (87), indicating that HSD17B2 polymorphisms may have clinical relevance.

Besides genomic factors, it has been reported that progestins, used as treatment for endometriosis or in combination with hormone replacement therapy, can influence the oxidative and reductive capacity of tissues (63, 67, 88). In PgR positive breast cancer cell line T-47D and MCF7, progesterone, levonorgestrel, and medroxyprogesterone acetate were shown to modulate HSD17B1, HSD17B2 and HSD17B5 expression (89). The expression of HSD17B1 is also known to be regulated by growth factors like insulin-like growth factors Types I and II and retinoic acid and immunological factors like interleukin 1 (IL-1), IL6 and tumor necrosis factor a (TNFα). In light of this, it is possible that HSD17B1 is a target of the immune system in breast tumors (65). Following aromatase inhibitor treatment in postmenopausal ERα-positive breast cancer patients, the HSD17B1 expression was shown to be increased. It was hypothesized that this upregulation of HSD17B1 could be a response to estrogen depletion where the tissue attempts to restore estrogen signaling by alternative pathways (90). Supporting findings were made using lung cancer cell lines A549 and LK87, in which aromatase inhibitor treatment resulted in increased HSD17B1 expression (91). In ERα- and AR-positive breast cancer cell line T-47D, aromatase inhibitor treatment resulted in increased HSD17B2 and DHT expression. The change in HSD17B2 was shown to be AR dependent, suggesting that DHT can directly upregulate HSD17B2 expression. They also show that treatment with E2 counteracts this DHT mediated effect (92). DHT depletion in prostate cancer cell lines using Salpha-reductase type I and type II resulted in increased HSD17B1 (93). While very little work on the role of microRNA (miRNA) regulation of HSD17B1 has been conducted, a study in placental cells show that miRNA-210 and 518c modulate HSD17B1 expression (94) and microRNAs-10b, 145, 342, 17, 26a and 106b have been predicted to interact with HSD17B1 and HSD17B2 in breast cancer (95). In paper IV, using cell-lines, we show that E2 signaling acts as a negative modulator of HSD17B1 expression, while having a time-dependent effect on HSD17B2. Furthermore, DHT upregulates HSD17B2 but has a limited effect on HSD17B1 in tested breast cancer cell-lines. We also identify mir-17, mir-210, mir-7-5p and mir-1304-3p as modulators of HSD17B1 expression, while mir-498, mir-579-3p, mir-204-5p and mir-205-3p are modulators of HSD17B2 expression. Finally, downregulation of CX3CL1, EPHB6, and TP63 were shown to increase HSD17B1 and HSD17B2, while GREB1 downregulation suppressed HSD17B1 and promoted HSD17B2 expression.
Other HSD17B enzymes in breast cancer
While this thesis work has focused on HSD17B1 and HSD17B2, this section briefly describes the role of other HSD17B enzymes relevant in breast cancer.

HSD17B4 is an oxidative enzyme, and similarly to HSD17B2, it is expressed in virtually all human tissues. The primary reaction is the conversion of E2 to E1 and androstenediol to DHEA (46), its activity is reported to be much lower than HSD17B2 (96).

HSD17B5 is a reductive enzyme, and it is expressed in the ovary and breast ductal epithelial cells. It catalyzes the conversion of androstenedione to testosterone, but it has also been reported to transform DHT and progesterone to less active forms (47, 76). It is overexpressed in breast cancer (76, 97), prostate cancer (98) and ovarian cancer (99). In breast cancer, HSD17B5 expression has been shown to be correlated to worse prognosis (76) and increased risk relapse (100).

HSD17B7, a reductive enzyme, is expressed in the ovary, placenta, breast tissue, testis, liver and brain (101). It catalyzes the conversation of E1 to E2 (50). Experimental studies in breast cancer show that reduction of HSD17B7 expression resulted in reduced proliferation, and as such it could be a potential target for inhibition in breast cancer (59).

HSD17B14, a weakly oxidative enzyme, was shown to be expressed in the endometrium, ovaries, breast, testis, GI, kidney and retina (102). It catalyzes the conversion of E2 to E1 and androstenediol to DHEA (103, 104), albeit at very low levels compared to HSD17B2 (102). Breast cancer patients with high HSD17B14 mRNA expression have improved recurrence-free survival and breast cancer-specific survival (100). HSD17B14 expression can also be used to predict tamoxifen treatment outcome with recurrence-free survival as outcome in ERα-positive lymph node negative breast cancer patients (105). Experimental data using downregulation of HSD17B14 in breast cancer cell-lines, resulted in altered gene expression a number of genes, including of C-X-C motif Ligand 10 (CXCL10), also known as γ-Interferon-induced protein of 10 kDa (IP 10) (unpublished data).

C-X-C ligand 10 and C-X-C ligand receptor 3
The relevance of the microenvironment has been reported in regard to survival, proliferation, differentiation and migration of breast cancer cells. One important component of the microenvironment is the immune system and C-X-C ligand (CXCL)10 is a potent chemoattractant for T-cells (106-115). CXCL10 was reported to prevent tumor formation in vivo, and could mediate the regression of pre-existing tumors (116). The primary receptor for CXCL10 is C-X-C motif Receptor 3 (CXCR3). CXCR3 has two reported isoforms, CXCR3-A, which is expressed on immune and some tumor cells, and results in migration and proliferation. It has also been correlated to angiogenesis (117-119). CXCR3-B is reported to have opposing function (117-120). Increase of total CXCR3 has been observed in breast cancer, but analysis of CXCR3-A expression on its own has been difficult due to lack of specific antibodies and a high degree of similarity between CXCR3-A and CXCR3-B (113, 117, 118).
In paper I, we show that in ERα-positive breast cancer, high expression of CXCL10 and CXCR3 expression were associated with tamoxifen treatment benefit, and that CXCR3
expression is associated with adverse outcome in patients who did not receive endocrine treatment. Finally, there was a clinical connection between HSD17B14 and CXCL10 or CXCR3 in the studied cohort (unpublished data).
Overall aim of the thesis

The aim of this thesis was to identify biomarkers for the prognosis of breast cancer and for the prediction of benefit following tamoxifen treatment. It was also to study the roles of hydroxysteroid 17β dehydrogenase in breast cancer, in regard to related proteins and modulating of the expression of hydroxysteroid 17β dehydrogenase type 1 and type 2 in breast cancer cell-lines.
Methodical considerations

Patients

Papers I, II and III used immunohistochemistry or DNA from the ‘Stockholm Trial’. Tumors from patients participating in a randomized tamoxifen trial conducted 1976-1990 in Stockholm, Sweden were used. Results and details of the ‘Stockholm Trial’ were previously described (121). All patients were postmenopausal with tumors ≤30 mm and negative for axillary lymph node involvement (N0). The patients received either breast-conserving surgery followed by radiation treatment with a dose of 50 Gy with 2 Gy per fraction 5 days weekly or modified radical mastectomy. After surgery, patients were randomized to tamoxifen 40 mg daily or to no endocrine treatment. After two years of tamoxifen treatment, most disease-free patients were randomized to tamoxifen for an additional three years or no further therapy. Retrospective studies of biomarkers were approved by the Research Ethics Committee at the Karolinska Institute (dnr 97–451, with amendments). In paper I ERα protein cut-off levels at 10% of positively stained tumor cell nuclei were used. In paper II and III 1% of positively stained tumor cell nuclei was used as cut-off. The original cytosol measurements were used in the case of missing immunohistochemical data, with a cut-off of 0.05 fmol/µg DNA (121). HER2 protein expression scored 0-3+ was previously described (122), and for all analysis in the present study, the clinically used 3+ expression was considered HER2-positive. Grade was scored previously according to the NHG system (123).

Immunohistochemistry

Immunohistochemistry (IHC) is a method where tissue sections are stained with antibodies in order to visualize the amount, intensity or distribution of a specific protein in a tissue section slide. The method is widely used in diagnosis, as well as to stain for biomarkers to help make informed decisions for disease treatment and prognosis. In our papers I and II IHC was used to determine the intensity and distribution of CXCL10, CXCR3 or AR in tumor tissues from the patients previously described. In short, sample slides are deparaffinized, rehydrated and treated for antigen retrieval. In paper I this was done by heating, treatment with tissue clear (HistoLab, Göteborg, Sweden), rehydrated by washing followed by boiling in DIVA-buffer (BioCare, Concord, CA) in a decloaking chamber (BioCare). In paper II, this was done using Pre-Treatment Module for Tissue Specimens (DAKO, Glostrup, Denmark) with Buffer Envision™ FLEX (Target Retrieval Solution; DAKO) for high pH, and treated according to the manufacturer’s instructions followed by endogenous peroxidases were blocked with 3% H₂O₂ + MeOH. In both paper I and II the slides were subsequently washed and incubated with Protein Block (Spring Bioscience, Pleasanton, CA) which reduces unspecific binding. The primary antibody was added overnight, followed by a secondary EnVision secondary anti-mouse antibody conjugated to HRP (DAKO). The HRP coupled antibodies were visualized using 3,3’-diaminobenzidine tetrahydrochloride (DAB) with hydrogen peroxide. This was followed by counterstaining of the tissue with hematoxylin (BioRad, Hercules, CA). All IHC staining was evaluated by two separate evaluators, without evaluators’ knowledge of clinical or pathological data for patients. In paper I samples were scored using a 0-3 scaling system for tumor cell intensity, - indicating no staining, + indicating weak expression, ++ indicating moderate expression and +++ indicating a strong expression. In paper II tumor cell nuclei
were scored and the occurrence of positive nuclei was divided into three groups, 0% (−); 1-
10% (+) and >10% (++). Table 2 details the primary antibodies used.

<table>
<thead>
<tr>
<th>Target</th>
<th>Type</th>
<th>Concentration</th>
<th>Clone</th>
<th>Distributor</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL10</td>
<td>Rabbit, polyclonal</td>
<td>111 ng/mL</td>
<td>Ab9807</td>
<td>Abcam, Cambridge,</td>
<td>I</td>
</tr>
<tr>
<td>CXCR3</td>
<td>Mouse, monoclonal</td>
<td>500 ng/mL</td>
<td>2Ar1</td>
<td>Abcam, Cambridge,</td>
<td>I</td>
</tr>
<tr>
<td>AR</td>
<td>Mouse, monoclonal</td>
<td>1:400</td>
<td>AR441</td>
<td>DAKO, Glostrup,</td>
<td>II</td>
</tr>
</tbody>
</table>

Nucleic acid isolation

The purpose of nucleic acid isolation is to separate DNA or RNA from the remaining contents of the cell. This is done by lysis of the cellular and nuclear membrane, separation of the nucleic acids from proteins and general cell debris. Meanwhile, one wishes to minimize the loss of integrity of the nucleic acid in question, while seeking to achieve a high purity in terms of lack of salt contaminants, and of other nucleic acids. When purifying RNA, which is highly unstable, DNA contamination and RNA degradation are the two primary problems which can arise. DNA isolation primarily suffers from RNA contamination, but can also suffer from degradation.

Isolation of DNA from formalin fixed paraffin embedded (FFPE) tumor tissue has inherent problems, since the FFPE treatment often results in fragmentation of the DNA before the extraction has started. In paper III, genomic DNA was extracted from FFPE tumor tissues using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer’s protocol, with the exception of paraffin removal. The paraffin was removed with Histolab Clear (Histolab, Gothenburg, Sweden). Up to five sections of 10 µm FFPE tissue per sample were used. The DNA concentration was measured with QuantiFluor® ONE dsDNA Dye kit (Promega, Madison, WI, USA) on a Quantus™ Fluorometer (Promega).

Isolation of RNA in paper IV was done using fenol based TRIzol (Thermo Fisher Scientific) according to manufacturer’s instructions. Sample quality was verified using bioanalyzer (Agilent, Santa Clara, CA) to determine RNA Integrity Number (RIN), and NanoDrop (NanoDrop, Wilmington, DE) measurement was used to determine purity in terms of contaminants, both salts and other nucleic acids.

PCR

In order to study specific changes in the genome, a large amount of genomic template is needed. In order to be able to study samples where smaller amounts of template are available, the genomic template must be amplified. Using polymerase chain reaction (PCR), it is possible to amplify a specified region of the genome, containing the area of interest. In order to perform PCR, four general components are needed. A Taq Polymerase for DNA synthesis. This enzyme has been modified to survive the temperatures of the PCR reaction. The primers, two complementary strands of nucleic acids designed to bind at specific locations in the genome. Nucleic acids, deoxynucleotide triphosphates (dNTPs). And finally, the template DNA. The
PCR works by repeat cycles of heating, which results in disassociation of the DNA and DNA-primer complexes, followed by an annealing step, where the primers and Taq Polymerase bind their targets, and finally a transcription stage, where the area of interest is replicated. Following each cycle of the PCR reaction, the amount of replicated DNA is doubled, leading to an exponential growth of DNA fragments from the area of interest. The PCR reaction is the basis for a number of methods used in this thesis.

**Reverse transcription-PCR**
In order to facilitate analysis of mRNA, it is often synthesized into complimentary DNA (cDNA). This has several reasons, besides making it more stable, which facilitates storage and practical handling, it allows more leeway in choice of assay for future analysis. cDNA generation is a reverse transcription PCR reaction where primers designed to amplify the entire genome are mixed with dNTPs and a reverse transcriptase and allowed to work over a single long PCR reaction cycle. This results in a transcription of the RNA into cDNA (124). In **paper IV** reverse transcription-PCR was performed using the High Capacity cDNA kit (Thermo Fisher Scientific).

**Quantitative PCR**
In order to quantify the expression of a single gene, researchers have designed several approaches, including visualization of PCR product on electrophoresis gels. A need for more sensitive detection resulted in the design of quantitative PCR (qPCR). This can be performed through two primary methods, by use of an intercalated dye, such as SYBR Green, which binds all double stranded DNA. It can also be performed by TaqMan assay, in which the primers are complimented by a nonextendable fluorescent probe complementary to a region located between the two primers. These probes contain both a florescent reported and a quencher. TaqMan chemistry utilizes the 5’ to 3’ exonuclease activity of the Taq polymerase, which degrades the probe following hybridization. The cleavage of the quencher and reporter allows them to disassociate, resulting in fluorescent emission. As a result, if no template is present, or if the amount is too low, no emission will be detected. As the PCR cycles are repeated, and the amount of template (if any) increases, the emission will increase. The emission in modern qPCR is measured after each cycle, which allows for “real time” quantification of the reporter in each sample. This allows researchers to detect the relative emission between samples over the course of the run, which gives the researcher a relative concentration of the reported gene in each sample which greatly increases sensitivitiy compared to end-point PCR quantification (125). For **paper IV** TaqMan® Fast Universal PCR Master Mix without AmpErase UNG (Thermo Fisher Scientific) was used. In lieu with the MIQE guidelines for qPCR experiments, two reporters Peptidylprolyl Isomerase A (Cyclophilin A) and beta-actin were used as endogenous controls for all experiments. Data analyses were performed according to the ΔΔCt method, and relative concentrations were calculated against the appropriate control.

**Genotyping**
Genotyping is the process of determining the genetic variant of an individual, the genotype. Examination of the genotype in individuals is performed through a variety of methods, but often involves PCR for the amplification of the DNA. For genotyping using PCR, a convenient approach utilizes two probes similar to those in qPCR, but which overlap the area where the
variation is located. One probe will bind the normal genotype, while the other will bind to the variant analyzed. Both probes are synthesized with a quencher, but will have different fluorescent reporters. As a result, the color(s) of the emission will reveal the presence of the respective genotype in the sample. In Paper III we used droplet digital PCR in order to genotype our samples. Droplet digital PCR (ddPCR) takes the standard PCR reaction, but performs it in ~20 000 monodispersed droplets. Following the PCR reaction, the droplets are transferred into an automated reader which measures the emission the droplets from each sample. Poisson statistics combined with manual thresholds are used to determine the sample genotype, figure 4 (126). The advantage of ddPCR over conventional genotyping include increased sensitivity and robustness, albeit at lower throughput than conventional methods. In paper III, droplet digital PCR (Bio-Rad, CA, USA) was used according to manufacturer’s protocol for SNP analysis. Genotypes were determined by EH who was blinded to patient outcome and characteristics at the time of genotyping, using the Quantasoft 1.7 software (Bio-Rad).

Diplotype construction
In order to study the impact of several genotypes which are inherited together from a single parent, genotypes are formed into haplotypes. Each individual has a pair of haplotypes, which constitutes a diplotype. These diplotypes were used to analyze outcome for the full set of genotypes for each patient. In paper III, haplotypes were constructed using Haploview 4.2 (Broad Institute, Cambridge, Massachusetts), which were formed into diplotypes manually.

Figure 4. example of droplet clusters which were subsequently grouped for genotyping using Quantasoft 1.7.
Statistics
For all studies where patient materials were used, a common approach was used to the statistics. The relationships between grouped variables were analyzed using Spearman's rank order correlation. The significance level varied based on study, but was set at either $p=0.05$ or $p=0.01$. The survival curves were produced according to the lifetable method described by Kaplan and Meier and differences between groups were evaluated with log-rank tests. Patients with missing data were excluded. Univariate and multivariate analysis were conducted using Cox proportional hazards regression and $P < 0.05$ was considered significant. For paper I, end points used were breast cancer specific mortality, defined as when patients had a local or distant recurrence at time of death or when breast cancer was registered as cause of death by the Swedish cause of death registry. Local recurrence defined as a relapse on either chest wall or in a regional lymph node or distant recurrence, defined as the remaining metastatic events. The statistical package Statistica 10.0 (StatSoft Scandinavia, Uppsala, Sweden) was used for all calculations. For paper II, the chosen endpoint was recurrence, defined as regional relapse or distant metastasis. Breast cancer-specific survival was chosen as a secondary endpoint. The statistical package Statistica 12.0 (StatSoft Scandinavia, Uppsala, Sweden) was used for all calculations with the exception of the comparison of the TMA and the original cohort, where STATA 13.1 (StataCorp, Stockholm, Sweden) was used. For paper III, the chosen endpoint was recurrence, defined as regional relapse or distant metastasis. Breast cancer-specific survival was chosen as a secondary endpoint. The statistical package SPSS (IBM corporation, NY, USA) was used for all calculations. In paper IV, student’s t-test was used in order to compare the means of the groups of data derived from in vitro experiments. GraphPad Prism 7 (GraphPad Software, CA, USA) was used for all calculations and constructing of graphs.

Cell culture
The following section exclusively details work conducted in paper IV.

Cell lines
Cell culture is often used to study the effects of stimuli in controlled conditions, which allows the researcher to study the outcome using a variety of downstream applications. The primary drawback of cell culture is the simplicity of the system compared to live tissue inside patients, where other types of cells and circulatory factors constitute a microenvironment.

The breast cancer epithelial cell lines ZR-75-1, MCF7 and T-47D as well as the immortalized epithelial cell line MCF10A were used. ZR-75-1, MCF7 and T-47D cells are ER- and AR-positive and MCF10A cells are ERα- and AR-negative. All cell lines used were positive for HSD17B1 and HSD17B2. All cell culture experiments were conducted in technical triplicates, and repeated 2 or 3 times.

Hormonal treatment
In order to test the effect of a steroid hormone on cultured cells, the medium was supplemented with charcoal treated (steroid free) serum for growth. In order to minimize changes in cell behavior, cells were seeded in normal medium, allowed to grow for 8 hours to adjust to the new flask, after which the media was changed to charcoal treated medium for the duration of the experiment. 24 hours after seeding, the first treatment was added. Cells were treated with...
E2 or DHT (Merck, Darmstadt, Germany) for 6, 24, 48, 72 hours or 7 days, during which the media was replaced every 24 hours.

**Silencing RNA treatment**

Silencing RNA (siRNA) are short RNA sequences designed to mediate complimentary binding and degradation of specific mRNA sequences. This is mediated by RISC which facilitates degradation of mRNA complementary to the siRNA guiding strand, leading to a reduction of target mRNA and frequently protein expression.

In order to determine if E2 signaling was ERα dependent, we performed knockdown of ERα using Dharmafect 1 (GE Healthcare, Little Chalfont, United Kingdom) and silence select siRNA 4392420 (Thermo Fisher Scientific, MA, USA) targeting ERα. Following <80% reduction in ERα expression after 30 hours, cells were treated with E2 for 48 hours, as per the protocol for hormonal treatment. ERα levels remained low for the duration of the experiment.

For the final siRNA experiments, genes were selected based on their co-expression in ERα patients with HSD17B1 and HSD17B2 using published transcriptomics datasets (Cancer Genome Atlas Network, 2012). The top 80 genes in all the transcriptomics datasets together were subjected to a literature search of possible connections of these genes to estrogen or androgen signaling in breast cancer. Finally, the 13 genes most promising genes were selected for siRNA knock-downs.

**Micro RNA treatment**

Computer algorithms based on these 7 nucleotides of the 5’ region are frequently used to predict binding. Due to the shortness of the sequence, each miRNA can bind as many as hundreds of genes, and different algorithms predict somewhat different targets. As a result, several tools are often used together to predict miR binding sites.

The miRNAs were chosen based on a literature study and bioinformatics predictions using miRSearch v3.0 (exiqon), microT v4 (127) and TargetScan (128). ZR-75-1, MCF7, T-47D and MCF10A cells were seeded 24 hours prior to transfection. The microRNA mimic was added at a final concentration of 40nM combined with Dharmafect 1 (GE Healthcare) with a final concentration of 0.5%. The cells were harvested at 48 hours after transfection.
Summary of papers

Paper I
Aim
The purpose of paper I was to investigate the expression levels of CXCL10 and CXCR3 in tumors from breast cancer patients randomized to adjuvant tamoxifen treatment or no endocrine treatment, to determine if they could be used to determine the outcome in regard to prognosis (defined as outcome irrespective of treatment status) and prediction of tamoxifen treatment benefit (defined as outcome influenced by treatment). This was done using a retrospective cohort of lymph node negative postmenopausal breast cancer patients with a long follow-up period that were randomized to no endocrine treatment or tamoxifen treatment, independently of ER expression.

Results
Out of 912 patients, data for CXCL10 expression was acquired from 793 cases and for CXCR3 data was acquired for 735 cases. There was no correlation between CXCL10 and CXCR3. Further, neither CXCL10 nor CXCR3 were correlated to ERα expression. All survival analysis was conducted in ERα positive patients. Patients with strong tumoral CXCL10 expression had reduced local recurrence following tamoxifen treatment (RR=0.46; C.I. 95% 0.25-0.85, p=0.01). There was no impact of CXCL10 on distant recurrence or breast cancer specific survival. Tamoxifen treatment did not yield any change in outcome in the group of patients with weak tumoral CXCL10 expression. Patients with strong CXCR3 expression had a risk reduction in regard to risk of local (RR=0.35; C.I. 95% 0.22-0.58 p<0.001) and distant recurrence (RR 0.44; C.I. 95% 0.24-0.81, p=0.009) as well as breast cancer specific survival (RR 0.34; C.I. 95% 0.19-0.62, p<0.001) following tamoxifen treatment. There was no change in outcome following tamoxifen treatment in patients with weak CXCR3 expression. CXCR3 was also a prognostic factor in this cohort, with strong expression being associated with adverse outcome in regards to distant recurrence (RR 1.48 (C.I. 95% 1.2-1.9, p=0.05) and breast cancer specific survival (RR 1.40 (C.I. 95% 1.02-1.92, p=0.036).

Discussion and conclusion
Within paper I we show that high CXCL10 and CXCR3 can predict tamoxifen benefit in the ERα positive cohort of patients. The improvement in local recurrence free survival for CXCL10 expression could be related to the ability of CXCL10 to recruit immune cells and protect the tissue from recurrence of tumor cells (106, 107). Further, CXCL10 has been shown to prevent estrogen dependent tumor formation (129). This anti-estrogenic effect could synergize with tamoxifen, and could explain the improved response we see in our cohort.

CXCR3 is an oncogene driving proliferation and metastasis when expressed (115, 117-119, 130-132), as supported by the adverse outcome seen in patients with high CXCR3 expression in this cohort. The prognostic impact previously observed in breast cancer by Ma et al. was in a cohort like ours, with early disease, and no nodal involvement, which could indicate that CXCR3 is an early marker for prognosis (118). A mechanism of action for the relevance of CXCR3 in tamoxifen response could be that since CXCR3 expression is dependent on the cell-cycle, being primarily expressed in G2/M phase, upon tamoxifen treatment, the CXCR3
expressing cells, would suffer dual inhibition, expression of CXCR3 would be lost and the cells would suffer normal inhibition by tamoxifen (115, 117-120, 130-135).

In conclusion, CXCR3 and CXCL10, are biomarkers for tamoxifen treatment benefit in the ERα positive patients. CXCR3 on its own has prognostic impact in this cohort of early breast cancer, and could be considered relevant for further testing both as a prognostic and treatment predictive marker, with possible future clinical application.

After the publication of paper I, a few studies have been published on the topic. CXCR3 expression was shown to be adversely correlated to metastasis and survival in 1800 patients. Further, knockdown or inhibition of CXCR3 was associated with reduced metastasis and improved host tumor response in a model of metastatic disease in mice (136). CXCL10 expression was found to be increased in breast cancer compared to healthy control, this increase was subsequently increased with higher grade (137). Further, CXCL10 was shown to be involved in progression and metastatic disease (138). A recent study used qPCR to quantify CXCR3-A and CXCR3-B, and their findings suggest CXCR3-A is the primary isoform in breast cancer, but that CXCR3-B may be associated with lung colonization and are elevated in mammosphere forming cells. However, they used primers which could quantify both CXCR3-A and CXCR3-B when analyzing CXCR3-A, and used SYBR green assay over probes which could have enhanced specificity. As a result, their findings are difficult to interpret (139).

**Paper II**

**Aim**

The purpose of paper II was to investigate the prognostic and tamoxifen predictive relevance of AR protein expression in breast cancer and its subgroups. This was done using a retrospective cohort of lymph node negative postmenopausal breast cancer patients with a long follow-up period that were randomized to no endocrine treatment or tamoxifen treatment, independently of ER expression.

**Results**

AR status was determined in 769 of patients. AR status was correlated to ERα, PgR and inversely correlation to grade, mitotic index and tumor size. For survival analysis, AR ≥1% was considered positive. Patients with ERα-negative tumors had worse outcome when AR was expressed (HR = 2.64 95% C.I. 1.04-6.66; p=0.040). When analyzing the benefit of tamoxifen treatment, AR positive cases had a significant benefit of tamoxifen treatment (HR = 0.34 95% C.I. 0.14-0.81; p=0.015) with the opposite observed in patients with AR negative tumors (HR = 2.92 95% C.I. 1.16-7.31; p=0.022). In the TNBC cases, high AR was associated with adverse prognosis (HR = 3.80; 95% C.I. 1.11-12.99; p=0.033). In regard to tamoxifen treatment benefit, patients with high tumoral AR expression had reduced risk (HR = 0.12; 95% C.I. 0.014-0.95 p=0.044), while those with tumors without AR expression had had increased risk (HR = 3.98; 95% C.I. 1.32-12.03; p=0.014). Patients with ERα-positive tumors did not show any difference in outcome based on AR status, and benefitted from tamoxifen treatment irrespectively of AR status. When analyzing breast cancer specific survival, patients with low AR expression did significantly worse when given tamoxifen.
Discussion and conclusion
We show that in patients with ERα-negative or TNBC disease, AR status determined tamoxifen treatment benefit. We also show that AR status on its own can predict patient outcome in ERα-negative or TNBC disease. Previous studies into the role of AR in breast cancer show conflicting results in the ERα-negative or TNBC cohorts, with AR being associated with worse (34, 140-143) or improved (32, 33, 35-38, 144) outcome. With others showing no effect of AR expression (28, 145, 146). There is no clear indicator as to why these studies have opposing results, the number of patients with high grade, metastatic and nodal involvement varied somewhat between studies, as did AR cut-off value, treatment and the fraction of AR positive patients. The mechanism of action for this tamoxifen benefit in ERα-negative and TNBC patients could be related to direct binding of tamoxifen to AR, or for tamoxifen to inhibit AR signalling as shown in prostate cancer (147-149). While we show no added benefit of AR in ERα-positive patients, several discrepancies between this and other cohorts could explain the differing results, as many previous cohorts were more heterogeneous in terms of stage, age and endocrine treatment status (9, 28, 30, 34, 150).

With the large degree of heterogeneity in the AR field, further validation of these findings are needed. However, it is possible that ERα-negative or TNBC patients with AR expression could be potential candidates for tamoxifen treatment, which could be a potential treatment option in these difficult to treat cohorts.

Following the publication of paper II, a large number of papers have been published regarding the androgen receptor and breast cancer, and only some of them will be discussed here. The role of AR as a defining factor in the proposed quadruple negative breast cancer (QNBC) has been highlighted recently, since patients with TNBC AR-positive disease are potential targets of anti-AR treatment (151). A study in ERα-positive breast cancer show that tamoxifen can mediate AR dependent EGFR activation and subsequent ERα signaling, which could be a mechanism of resistance in this cohort (152). Further, a number of studies further detailing the prognostic and predictive role of AR in different subtypes have been published, however the final definition of a role for AR outside ERα-positive disease still needs further clarification.

Paper III
Aim
The aim of paper III was to determine the frequency of the AR SNPs rs17302090, rs6152, rs7061037, rs1337080, rs5031002 and rs5964607 in ERα-negative AR-positive breast cancer, and to determine if these SNPs could be used to predict patient outcome in terms of prognosis and tamoxifen benefit.

Results
Of 58 patients, a complete genotype profile was successfully determined for 39 patients. Rs1337080 was inversely correlated to size, with similar trends for rs7061037, rs5031002 and rs5964607. No SNP genotype could predict outcome (data not shown). Patients with the wild type variant GG of rs17302090 had a non-significant improvement in benefit from tamoxifen treatment compared to the whole cohort (HR=0.08. 95% confidence interval (C.I.) 0.01-0.65; p=0.02; HR=0.16. 95% C.I. 0.03-0.72; p=0.02, respectively). Patients were grouped based on
the outcome of their diplotype. The first group of diplotypes had no clinical events (A) (n=11), and the other 1 or more clinical events (B) (n=47). Group B showed a significant improvement in recurrence-free survival with tamoxifen treatment (log-rank p=0.036) Figure 2, (HR=0.37, 95% C.I. 0.14-0.98; p=0.045). When accounting for size and grade, the estimated benefit from tamoxifen was somewhat improved (HR=0.3; 95% C.I. 0.10-0.83; p=0.02).

Discussion and conclusion
These findings suggest that SNP analysis in this subset of patients does not provide prognostic information, although the SNPs may indicate the size of the tumor. Patients with ERα-negative disease are currently not given tamoxifen, but results based on paper II show that the subset of patients with AR positive disease may benefit from such treatment. In this paper we demonstrate that patients with the variant GG of rs17302090 in their tumors tended to have a reduced risk of recurrence following tamoxifen treatment. This SNP is located in the AR promoter, and the mechanism of action for this change in tamoxifen response is likely dependent on changes in AR expression. Interestingly, variant A of rs17302090 was associated with resistance to androgen deprivation therapy in prostate cancer (43). While the role of these SNPs appear limited, the data presented here suggest some overlap between the role of rs17302090 in breast and prostate cancer suggesting that their role may be conserved between the two forms of cancer.

Diplotypes, the combination of two sets of haplotypes (a combination of genotypes inherited together) of a patient, were created based on the available SNP data. These diplotypes were grouped based on their outcome. Group A diplotypes had no regional recurrences or distant metastasis, while patients in group B had one or more events. Patients in group B have a considerable benefit from tamoxifen treatment. Lundin et al., showed that in ERα-positive patients, AR diplotype groups identify patients with different benefit from tamoxifen treatment. While the study cohorts differed in ERα status, the groups that benefitted from tamoxifen treatment were similar in terms of increased size. The diplotype analysis could, given further study in additional validation cohorts, be a tool in determining treatment options for patients with AR positive tumors. In addition to the limitations discussed in the paper, the number of patients available was a limiting factor, increasing the risk of false negative findings.

In conclusion, variant GG of rs17302090 may have increased benefit of tamoxifen treatment. Further, patients in diplotype groups in which recurrences were observed tended to benefit from tamoxifen treatment, and may be candidates for adjuvant tamoxifen treatment.

Paper IV
Aim
We aimed to investigate if estrogen and androgen-mediated signaling can influence the expression of HSD17B1 and HSD17B2 in breast cancer cell lines. Furthermore, to identify microRNA responsible for the regulation of HSD17B1 and HSD17B2 and to identify genes which control the expression of HSD17B1 and HSD17B2 in breast cancer cell lines.

Results
We detected a distinct decrease in HSD17B1 expression following E2 treatment for 24 hours, 48 hours and 7 days in ZR-75-1, MCF7 and T47D. In terms of HSD17B2 expression, there
was a reduction in ZR-75-1 and T-47D at 48 hours, which returned to baseline at 7 days. For MCF7, there was no effect at 24 or 48 hours, but an up-regulation of HSD17B2 at 7 days. To determine the role of ERα, cells treated with siRNA against ERα which had greatly reduced ERα were treated with E2. These cells did not respond to E2, and no detectable change to E2 stimulation was measured. In cells with DHT the change in HSD17B2 was most apparent after 7 days, but was visible at 24 and 48 hours. The change in HSD17B1 was more modest, with small changes at 24 and 48 hours or at 7 days for the different cell-lines, but no consistent change was detected.

Based on literature and bioinformatics predictions, we identified 50 miRNA predicted to target HSD17B1 or HSD17B2. After initial moderate throughput screening by transient transfection into 4 different cell-lines where interesting miRNA were selected, we performed repeated experiments to validate the effect on HSD17B1 and HSD17B2 gene expression. We identified miR-17, miR-210, miR-7-5p and miR-1304-3p as modulators of HSD17B1 expression, while miR-498, miR-579-3p, miR-204-5p and miR-205-3p modulated HSD17B2 expression.

Since ER signaling was shown to interact with HSD17B1 and HSD7B2, we used bioinformatics prediction to identify genes which were correlated to HSD17B1 or HSD17B2 in breast cancer. We then performed a literature search, determining which had previously known involvement in breast cancer. We performed transient downregulation of 13 different genes, and chose 9 genes for validation. Out of these, EPHB6 and KLK5 suppressed HSD17B1 in two cell-lines. Further, GREB1 promoted HSD17B1 expression in T-47D. GREB1, EPHB6 and TP63 were similarly shown to suppress HSD17B2 in ZR-75-1 and T-47D.

**Discussion and conclusion**

In this paper, we present evidence that E2 alters the expression of HSD17B1 and HSD17B2, two of the enzymes responsible for mediating the activity of E2 in breast cancer cell lines. The downregulation of HSD17B1 in response to E2 stimulation is relatively rapid, occurring after 24 hours and becoming more prominent after prolonged exposure. In breast cancer patients, E2 depletion was accompanied by an increase in HSD17B1 expression (90), and similar observations were made in lung cancer (91). In regard to HSD17B2, there appears to be a time-sensitive response in response to E2. It is possible that the transient reduction of HSD17B2 expression at earlier time points is a means to reduce conversion of E2 back to E1, which would allow for existing E2 to mediate its effect, with the increased HSD17B2 expression after 7 days constituting a mechanism to reduce the E2 effects after prolonged exposure. The loss of any detectible effect of E2 stimulation on HSD17B1 or HSD17B2 following ERα downregulation suggests that this effect is ERα-dependent in ZR-75-1, MCF7 and T-47D cells.

Based on our findings for HSD17B1, DHT likely has relatively modest effect on HSD17B1 expression in breast cancer. It has previously been shown that DHT can mediate HSD17B2 upregulation in the ERα and AR-positive cell line T-47D and that this effect was AR-dependent (92). We wanted to determine if this held true in additional cell-lines, and show similar effects in MCF7 and ZR-75-1 in regard to HSD17B2. Increased HSD17B2 expression represent a return to a more normal-like phenotype of the tissue, where the ratio of HSD17B1 to HSD17B2 is decreased (62, 63).
The role of miRNA in the regulation of the enzymes of the HSD17B family is poorly understood. The primary work in the field of miRNA and HSD17B1 or HSD17B2 to date is a publication showing that miR-210 and miR-518c control the expression of HSD17B1 in placental tissue (94). We were unable to see any HSD17B1 modulating ability from miR-518c (data not shown), however, we were able to verify a role of miR-210. To our knowledge, the findings that miR-17, miR-210, miR-7-5p and miR-1304-3p modulates HSD17B1 and miR-498, miR-579-3p, miR-204-5p and miR-205-3p modulates HSD17B2 are the first to characterize miRNAs controlling HSD17B1 and HSD17B2 in breast cancer cell lines. Further knowledge of the expression patterns of these miRNAs in breast cancer, and of their other targets is still needed.

Following modulation of genes predicted to target HSD17B1 or HSD17B2, we found that EPHB6 suppresses both HSD17B1 and HSD17B2. There was also some suppressive effect of CX3CL1 and TP63 on both HSD17B1 and HSD17B2. These findings could indicate a switch to a more steroid independent phenotype as the tumor progresses, and steroid activity is less pivotal in determining the future of the tumor. Meanwhile, the estrogen response gene GREB1 seemed to promote HSD17B1 and suppress HSD17B2 in T47D cells, which would promote increased steroid activation, further reinforcing its role in the literature as an ERα response gene.

In conclusion, we describe several mechanisms of HSD17B1 and HSD17B2 regulation, which may be involved in the normal control of expression and ratio of these two steroid converting enzymes. We show that estradiol is a potent suppressor of HSD17B1 and has a time dependent effect on HSD17B2. This effect appears to be reliant on ERα. DHT has some ability to modulate HSD17B1, but the primary impact is on HSD17B2 expression. We found eight miRNAs which modulate HSD17B1 or HSD17B2 expression, and five genes which appear able to control HSD17B1 or HSD17B2 expression in breast cancer, including GREB1, a well-known ERα response gene. These findings help chart the control of two primary steroid converting HSD17 enzymes in breast cancer. Our hope is that further knowledge of HSD17B1 and HSD17B2 regulation could lead to therapeutic interventions.
Concluding remarks

Breast cancer is a complex disease with many factors influencing prevalence and prognosis. In this thesis, we have focused on the role of the androgen receptor in breast cancer, both in regard to prognosis and tamoxifen treatment prediction. Further we analyzed sex steroid converting enzymes of the HSD17B family. This included the regulation of members HSD17B1 and HSD17B2, as well as an indirect HSD17B14 effect in how CXCL10, and its receptor CXCR3, can predict breast cancer outcome and tamoxifen treatment benefit. Table 3 summarizes the papers of this thesis.

<table>
<thead>
<tr>
<th>Table 3, summary of the primary findings in papers I-IV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Method</strong></td>
</tr>
<tr>
<td>Paper I</td>
</tr>
<tr>
<td>Paper II</td>
</tr>
<tr>
<td>Paper III</td>
</tr>
<tr>
<td>Paper IV</td>
</tr>
</tbody>
</table>

CXCL10 and CXCR3 are an important part of the microenvironment, in cell survival and migration and are involved in the recruitment of T-cells. CXCL10 and CXCR3 are associated with changes in survival and metastasis in breast cancer. We show that both CXCL10 and CXCR3 are prognostic, and are able to predict tamoxifen treatment benefit. Recent studies have shown that CXCR3 expression is increased in breast cancer, and further definition of the roles of CXCR3 isoforms, CXCR3-A and CXCR3-B has been published. The analysis of CXCR3 isoforms is confounded by the lack of specific assays for CXCR3-A, and hopefully future studies will address this. The connection of CXCR3 and CXCL10 to the response to tamoxifen as shown in paper I, is to our knowledge the only published such association, and further validation would be an important step in applying these findings in the clinic. Since the publication, the prognostic role of CXCR3 and CXCL10 both have been verified in multiple studies, and further development of direct inhibitors could be an alternate approach to treating these cases.
The role of the androgen receptor has come under new focus in recent years, and it has been shown to be able to predict outcome in all studied types of breast cancer, with differing roles in ERα-positive cases compared to ERα-negative cases. Many recent studies highlight the importance of AR in ERα-negative breast cancer, and its role in TNBC may be even more interesting due to lack of targets for treatment in this subgroup. Recently the term quadruple negative breast cancer was proposed to compliment TNBC, in order to discern TNBC AR+ from TNBC AR- breast cancer, and treatments directly targeting AR in TNBC are currently undergoing clinical trials, with early results indicating that treatments are well tolerated and yield clinical benefit. In paper II we found that AR positive patients have adverse outcome in the ERα-negative groups, we also found that AR status could be associated with response to tamoxifen treatment, and that AR positive patients could benefit from such treatment, despite lacking the ERα. In paper III we show relatively modest impact of mutations in the AR, however in ERα-negative AR positive breast cancer the wild type of rs17302090 may have improved benefit compared to non-wild type patients. In order to continue examining the role of AR in the response to tamoxifen, further studies in tamoxifen treated patients would be required, and validation in further tamoxifen treated cohorts needed. A prospective study of patients who are ERα-negative with mixed AR status would be the most efficient way, although this approach has ethical limitations, and limiting the study to ERα-negative AR negative who would not receive tamoxifen and ERα-negative and AR positive who would receive tamoxifen would be a more ethical approach.

The enzymes of the HSD17B family are involved in modulating the activity of estrogens and androgens both in pre- and postmenopausal women, although at different sites. The inhibition of HSD17B1 has been proposed as an alternative second-line treatment for breast cancer or as a first-line treatment in combination with existing treatments like tamoxifen or aromatase inhibitors. However, problems in designing effective inhibitors for HSD17B1 have limited this approach, and as a result we started considering alternate paths to achieving a similar effect, for instance modulation of HSD17B1 or HSD17B2 in breast cancer. In paper IV we detailed several mechanisms by which HSD17B1 or HSD17B2 are regulated, and it is possible that any of these mechanisms of regulation could be exploited to change the steroid balance in the tumor in favor of the patient. Further research into each of these pathways would be needed, followed by in vivo and in situ studies.
Acknowledgements

My main supervisor, Agneta Jansson. Thank you for all time we spent working through research questions and ideas, and trying to overcome obstacles. Thank you also for believing in me and letting me develop as a researcher and showing me I could overcome many obstacles on my own. Thank you also for being a fun person to talk to on everything not related to research, and for the conference discussions we had, especially in Seattle!

Olle Stål, my co-supervisor. Thank you for all your help through the years with discussions about research, holding us together for group meeting to share and develop ideas and for all the knowledge and wisdom you have shared throughout the years!

Charlotta Dabrosin, my Subject Representative, thank you for allowing me this opportunity to do my PhD here at Linköping University.

Emina Vorkapic, from our first discussion when you first came here, to discussions about the future, life, dreams and ambitions and everything else. Without you these years would have been so much less entertaining. Thank you for all the fun times we’ve had doing boxing, dancing and running! Oh, and why are there so many police cars here?

Cynthia Veenstra, for always being there to talk to, sharing an office with you has made this time much more enjoyable. Thanks for all the fun times we’ve had during after works, going boxing and or away on conferences!

Olof Sandberg, my nerd and running buddy. Thank you for the great times we’ve had talking about life questions, science or games. Your thoughtfulness and kindness are always a highlight of any day, thank you.


Sebastian Gnosa, for every time I was feeling bad about something, you were there to make it worse, thank you! Sharing an office with you was fun, thank you for all the good discussions and activities we’ve had!

Thank you DOMFIL, especially the board of 2013/2014, Anna Södergren, Malin Silverå Ejneby and Kjersti Claesson, for all the fun times, after works, board game nights and other social events.

For the people in the group, past and present; Elin Karlsson, Birgitta Holmlund, Gizeh Perez Torino, Linda Bojmar, Tove Sivik, Phia-Lotta Jerevall, Josefine Bostner and Birgit Olsson.

Jag vill även tacka er från Norrköping för alla tokigheter, allt figurspelande, målande, pizzaätande och alla roliga fester.

To all the friends, colleagues, co-workers and relatives who have been there throughout the years, thank you!
Min familj. **Sandra**, min underbara fru som alltid stöttar mig och står ut med min jobbiga humor och dåliga skämt. Tack för att du alltid är där och är bäst! Älskar dig massor, och mest! I love you like old people love chicken, its perfect! **Emil** och **Vincent**, busfröet och tänkaren. Ni är så underbart roliga att spendera tid med, och det ska bli så roligt att se er växa upp!

Mina föräldrar. **Krister** och **Ingrid**, tack för att ni alltid ställer upp, med barnen, flytta, handla, få idéer till vilken dator eller bil man ska köpa eller bara prata. Tack för allt ni har gjort under min uppväxt! **Amelia**, min lillasyster som jag alltid lekte med när vi växte upp, lycka till med allt som händer!

**Örjan, Marie**, tack för allt ni gjort för mig och för att ni fått mig att känna mig välkommen in i ert hem. Tack även **Bill, Madde, Cissi**, och **Peo**. Tack allihopa för alla trevliga middagar, filmstunder, kräftskivor och utflykter med hästar och traktorer!
References


Papers

The articles associated with this thesis have been removed for copyright reasons. For more details about these see:

http://urn.kb.se/resolve?urn=urn:nbn:se:liu:diva-132453