Testosterone and the postmenopausal breast
-aspects on cell proliferation and mammographic density

Marie Hofling
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Marie Hofling
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Gårdsvägen 4, 169 70 Solna
To my beloved family Mikael, Erika, Filippa
Gåvan

En dag så full av lycka.
Arbetade i trädgården, dimman lyfte tidigt.
Kolibrierna stod stilla över kaprifolens blom.
Det fanns på jorden inte en sak jag ville äga.
Jag visste ingen värld att avundas.
Vad ont som hänt hade jag glömt.
Jag visste ingen värld att avundas.
Vad ont som hänt hade jag glömt.
Skämdes inte för tanken att vara den jag alltid varit.
Kände i kroppen ingen smärta.
När jag rätade på ryggen såg jag blå hav och segel.

Czeslaw Milosz
Testosterone and the postmenopausal breast
-aspects on cell proliferation and mammographic density
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ABSTRACT
The breast is a target organ for sex steroids, and hormonal treatments have been associated with a risk of breast cancer. There is increasing interest in androgen treatment for postmenopausal women. Testosterone has been shown to improve bone density, body composition, mood, psychosexual function and general well-being. Little is known about the effects of testosterone on the breast.

The aims of this thesis were: to study the effects of testosterone addition to combined estrogen/progestogen treatment on breast cell proliferation and mammographic breast density; to assess possible relations to breast symptoms; to compare the effect of tibolone and combined hormone therapy on circulating sex steroids, binding proteins and their relationship to mammographic density; to explore the expression of androgen receptor (AR) and Syndecan-1 in primate breast tissue after long-term hormonal treatments. Postmenopausal, healthy women were recruited for prospective, randomized, placebo-controlled trials.

Tibolone and combined estrogen/progestogen treatment caused distinct differences in estrogen/androgen status and blood levels of possible breast mitogens. Treatment with tibolone resulted in elevated free testosterone levels. There was a negative association between free testosterone and mammographic density. This can be one mechanism to explain why tibolone has less influence on the breast than combined HT.

The fine needle aspiration biopsy technique is a useful tool to evaluate the proliferative response to hormonal treatments. During combined estrogen/progestogen treatment there was on average a four to five fold increase in breast cell proliferation. In contrast, when testosterone was added, no such increase was seen.

Mammographic density, a strong and independent risk factor for breast cancer, showed no significant difference between the treatment groups. Thus testosterone addition had a seemingly neutral effect on breast density. Breast symptoms of soreness and pain were found to increase during treatment, with a peak at 2 months. There was a correlation between symptoms and increase in mammographic density.

In a monkey model, long-term treatment with estrogen/progestogen resulted in a suppression of AR expression and a concomitant increase in Syndecan-1. After treatment with tibolone AR levels were markedly increased and around ten-fold higher than after estrogen/progestogen. The effects on Syndecan-1 expression were quite similar. After treatment with estrogen alone, values for both AR and Syndecan-1 expression did not differ from those in untreated monkeys.

In conclusion, testosterone and other androgens may have a protective influence on the breast.

Key words: Menopause, testosterone, breast cell proliferation, fine needle aspiration biopsy, mammographic density, Syndecan-1, androgen receptor, cynomolgus macaques.
LIST OF PUBLICATIONS

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


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**LIST OF ABBREVIATIONS**

AR androgen receptor  
BMD bone mineral density  
BMI body mass index  
BP blood pressure  
BRCA breast cancer susceptibility gene  
CC cranio caudal  
CEE conjugated equine estrogen  
CNS central nervous system  
DHEA dehydroepiandrosterone  
DHEAS dehydroepiandrosterone sulfate  
DHT dihydrotestosterone  
DNA deoxyribo nucleic acid  
E1 estrone  
E2 estradiol  
EPT estrogen progestogen therapy  
FAI female androgen insufficiency  
FNA fine needle aspiration  
FSH follicle stimulating hormone  
\( fT \) free testosterone  
GPRD general practice research database  
HCG human chorionic gonadotropin  
HERS heart and estrogen /progestin replacement study  
HT hormone therapy  
IGFBP-3 insulin like growth factor binding protein-3  
IGF-I insulin like growth factor –I  
ITT intention to treat  
LH luteinizing hormone  
MLO mediolateral oblique  
MPA medroxyprogesterone acetate  
NETA norethisterone acetate  
PCOS polycystic ovary syndrome  
RCT randomized controlled trial  
SHBG sex hormone binding globulin  
T testosterone  
\( tE1 \) total estrone  
WHI womens health initiative
INTRODUCTION

Breast cancer is the most common malignancy among women in the western world. It can be estimated that one out of nine women will develop breast cancer during her life span. Reproductive events and endogenous sex steroid hormones have been established as important risk factors in the etiology of breast cancer. Premature menopause, or oophorectomy, is well known to exert strong protective effects (Clemons & Goss 2001; Veronesi et al., 2005).

The expected postmenopausal lifetime for women in the western world is about 30 years. Hormonal treatment with estrogen/progestogen is known to relieve menopausal symptoms, prevent bone loss, and to improve quality of life. The effects of hormonal treatments on the normal breast in healthy women are incompletely understood and currently the subject of much discussion. Clearly, any adverse effects and even a small increase in risk of breast cancer from such treatments are of great importance for women’s health. During the last years, serious concerns have been raised about the long-term safety of combined estrogen/progestogen therapy and, in particular, about the effects on the breast (Chlebowski et al., 2003).

There is a need to define treatment regimens for postmenopausal women that have a minimum of effects on the breast but still maintain the many advantages of such treatment. While there has been a substantial decline in the use of traditional estrogen/progestogen treatments there is also an increasing interest in androgen treatment for women (Gelfand 2004; Somboonporn & Davis 2004). Very little is known about the effects of androgens on the breast.

Human studies on the effects of testosterone on the breast have been hampered by the lack of preparations suitable for women. With preparations for men, you very easily get an overtreatment and risk of androgenic side effects. Relevant information can be obtained from clinical experience of the use of compounds with some androgenic properties such as danazol and tibolone. Danazol, previously frequently used for the treatment of endometriosis, was also shown to relieve breast symptoms of tenderness and pain (Pye et al., 1985). DHEA, a weak androgen, was suggested to inhibit breast cancer development and growth in an animal model (LaBrie et al., 2003). In previous studies tibolone was found to have less influence on the breast and a stronger influence on libido when compared to continuous combined estrogen/progestogen HT (Hammar et al. 1998; Lundström et al., 2002).

Recently, a transdermal patch releasing 300 µg of testosterone per day, a dosage suitable for women, has been introduced after extensive clinical trials (Shifren et al., 2000; Mazer & Schifren 2003).

![Figure 1 The testosterone molecule](image_url)
ETIOLOGY AND RISK FACTORS FOR BREAST CANCER

The identification of effective strategies and interventions to prevent breast cancer remains an unsolved challenge. Epidemiological observations have identified a number of hereditary and lifestyle related associations (Clemons & Goss 2001; Veronesi et al., 2005).

Table I. Risk factors for breast cancer and estimated relative risks among women

<table>
<thead>
<tr>
<th>Variable</th>
<th>Relative risk</th>
<th>High risk group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>150</td>
<td>Women</td>
</tr>
<tr>
<td>Age</td>
<td>&gt;10</td>
<td>Elderly individuals</td>
</tr>
<tr>
<td>Age at menarche</td>
<td>1.5</td>
<td>Before 12 years</td>
</tr>
<tr>
<td>Age at menopause</td>
<td>2.0</td>
<td>After 54 years</td>
</tr>
<tr>
<td>Age at first delivery</td>
<td>3.0</td>
<td>After 40 years</td>
</tr>
<tr>
<td>Breast feeding and parity</td>
<td>* )</td>
<td>Women who do not breastfeed</td>
</tr>
<tr>
<td>Family history</td>
<td>2.6</td>
<td>BC in first degree relative</td>
</tr>
<tr>
<td>Benign breast disease</td>
<td>4.5</td>
<td>As typical hyperplasia</td>
</tr>
<tr>
<td>Socio-economic group</td>
<td>2</td>
<td>High groups</td>
</tr>
<tr>
<td>Alcohol</td>
<td>1.4</td>
<td>Current use</td>
</tr>
<tr>
<td>Body Mass Index</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre menopause</td>
<td>0.7</td>
<td>High BMI</td>
</tr>
<tr>
<td>Post menopause</td>
<td>2</td>
<td>High BMI</td>
</tr>
<tr>
<td>Bone mineral density</td>
<td>3</td>
<td>High BMD</td>
</tr>
<tr>
<td>Mammographic density</td>
<td>&gt;5</td>
<td>Extensive dense breast tissue</td>
</tr>
<tr>
<td>Oral contraception</td>
<td>1.2</td>
<td>Current use</td>
</tr>
<tr>
<td>Menopausal hormone therapy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estrogen only</td>
<td>1.0 - 1.4</td>
<td>Current use</td>
</tr>
<tr>
<td>Estrogen + progestogen</td>
<td>1.3 - 1.7</td>
<td>Current use</td>
</tr>
</tbody>
</table>

*) 4 % reduction for every 12 month of breast feeding
7 % reduction for every child birth

Age. As for most other tumours, breast cancer incidence increases with age. Before the age of thirty the incidence is very low, less than 25 cases per 100 000, while at the age of 80 there are 500 out of 100 000 women diagnosed with the disease.

Family history. About 5-10 % of breast cancers are attributed to known hereditary factors, such as the two breast cancer susceptibility genes BRCA-1 and BRCA-2. For the woman who carries a mutation in these genes, the lifetime risk to develop breast cancer is around 60-70 %. In addition, apart from breast cancer with defined genetic linkage, it is estimated that some 15 % of cases have a familiar disposition where a putative genetic background is still unknown. The size of risk seems to vary as a function of age and the type (first, second degree) and number of relatives affected. In clinical practice a genetic investigation is indicated in women with either a) three first degree relatives with breast cancer, one diagnosed before the age of fifty, b) two first degree relatives with breast cancer, one diagnosed before the age of forty or c) one first degree relative with breast cancer diagnosed before the age of thirty (Olsson et al., 1996). However, eight out of nine women with breast cancer do not have a family history for the disease.
Benign breast disease. Benign breast disease includes a wide range of different conditions and the histologic classification is important. Lesions with no over-growth of cells are called non-proliferative and do not increase breast cancer risk. About one out of twenty breast lumps shows atypical hyperplasia increasing the breast cancer risk between 2 and 5 times the average.

Reproductive and hormonal factors.
The connection between breast cancer and sex steroid hormones has been recognised for more than hundred years when bilateral oophorectomy was found to induce remission of breast cancer in premenopausal women. Subsequent evidence has implicated both endogenous and exogenous estrogen/progestogen in the pathogenesis of breast cancer. Many of the observed associations between breast cancer risk and reproductive factors, body composition including bone mass and socio-economic status may reflect the cumulative influence and sensitivity to endogenous estrogen during a woman’s life span.
The pathophysiological mechanisms for estrogen/progestogen associated carcinogenesis are unclear. As regards estrogen, different mechanisms for its possible carcinogenicity have been proposed: receptor-mediated hormonal activity, which stimulates cellular proliferation, resulting in more opportunities for genetic damage; a cytochrom P450-mediated metabolic activation, which elicits direct genotoxic effects by increasing mutation rates; and induction of aneuploidy by estrogen (Veronesi et al., 2005).
Large epidemiological studies have clearly indicated an early menarche and a late menopause to be risk factors for breast cancer. The theory of breast cancer as connected with ovarian function and the number of menstrual cycles is also supported by the observation that women where both ovaries were removed before the age of forty, showed a 45 % reduction in breast cancer risk as compared to women with a natural menopause.
Age at first full-term pregnancy is also important, there is a significant risk reduction at young age which is followed by a gradual increase in risk after the age of 30. There seems to be no protective effect from an early short-term pregnancy ending as a spontaneous miscarriage or as an induced abortion. The cell differentiation in the breast during the last part of a woman’s first full-term pregnancy and subsequent lactation may be protective against carcinogenesis in epithelial stem cells.
Breast feeding and parity. Long term breast feeding has shown a protective effect. The short lifetime duration of breast feeding that is typical for women in developed countries may contribute to the high incidence of breast cancer in these areas. In a large re-analysis (Collaborative Group, 2002), women with longer lifetime duration of breast feeding were less likely to develop breast cancer with a risk reduction rate of 4.3 % for every 12 months of breast feeding. Multi-parous women were less likely to develop breast cancer than women with few children. For every delivery there was a decline in risk by 7 %.
Body composition and lifestyle. Many of the associations with breast cancer risk as reported for antropometric and socio-economic factors could indirectly reflect levles of circulating estrogen and the relative amount of breast tissue. In postmenopausal women, a high BMI is a risk factor for breast cancer, probably since adipose tissue is an extra gonadal source of bio-available estrogen. On the other hand, in premenopausal women, a high BMI is rather protective probably because of its connection with anovulation. In pre- and postmenopausal women, accumulation of body fat is usually abdominal being strongly associated with hyperinsulinimia which by itself is a risk factor for breast cancer.
Height and socio-economic status may both reflect nutrition and dietary habits. Tall height has consistently been associated with increased breast cancer risk among both pre- and postmenopausal women. An increase of 5 cm in length implies a 5-15 % increased risk. Tall women may also have
more breast tissue parenchyma. Breast cancer is most common in the left breast which on average contains somewhat more parenchyma than the right one. Daughters and grand-daughters to women who migrate from countries with a low breast cancer incidence to countries with high incidence take on the higher rates of the new host country. Therefore, nutrition has been proposed as an important determinant of breast cancer. However, despite large efforts any clear cut associations between e.g. dietary fat intake and breast cancer have been hard to demonstrate. Furthermore, no significant differences were found in breast cancer mortality between vegetarians and non-vegetarians with an otherwise similar lifestyle. Alcohol intake is the best established specific dietary risk factor and even moderate consumption may increase endogenous estrogen levels. Relative risks for one, two and three drinks per day were 1.1, 1.2 and 1.4 respectively. Smoking has not been associated with increased risk and may rather have a slight protective effect among older women due to “anti-estrogenic effects” e.g. earlier menopause, increased osteoporosis and a reduced risk of endometrial carcinoma. Physical activity may be protective for both pre- and postmenopausal breast cancer, but it is also associated with many other lifestyle factors and the interpretation of current data is complex. Physical activity in postmenopausal women may reduce BMI which is an established risk factor.

Mammographic breast density.
The mammographic pattern of the breast varies depending on the relative amount of fat, connective and glandular tissue. Fat is radiologically lucent and appears dark on the image whereas stroma and epithelial tissue are more dense and appear white. High amounts of connective and epithelial tissue cause an increased density on the mammogram. Mammographic density has been identified as a strong and independent risk factor for the development of breast cancer (Warren 2004; Boyd et al., 2005). Odds ratios for risk in women with high density ranging from 2 to 6 are higher than for many other risk factors.

BREAST CELL HOMEOSTASIS
Development of the breast
The development of the breast starts already during foetal life but the organ is not fully developed at birth. Few other organs undergo such profound changes in size, shape and function during puberty, pregnancy and lactation. In young, premenopausal women approximately 15% of the breast consists of epithelial cells. Gradually, later in life, epithelium is replaced by fat tissue and in the postmenopausal women less than 5% consists of epithelium (Reid et al., 1996). In adult women, fat and stroma constitute more than 80% of the breast volume. The tissue components are inseparable and present in continuity with each other. The mature female breast parenchyma contains thousands of hormone sensitive potentially milk producing lobules. Each lobule is drained by a terminal duct attached to the main duct system, the determinal ductal lobular unit, which naturally regresses at menopause. Most breast diseases arise in the ductal lobular units.

Four different lobular structures, type 1-4, have been identified in the breast of postpubertal women, each representing different developmental stages and phases in the life cycle (Russo et al., 2005(a); Russo et al., 2005(b)). Type 1 lobules are the most undifferentiated ones with only a few ductuli per lobule. They are present in the immature breast before menarche. Lobules type 2 evolve from type 1 and are more complex containing more ducts per lobule. In lobules type 1 and 2, the epithelial cells comprise stem cells with a high proliferative capacity and susceptibility of malignant transformation. Lobules type 3 with an average of 80 ductulus or alveoli per lobule are seen in the breast during pregnancy or other hormonal stimulation. Lobule type 4 is considered to
represent maximal parenchymal differentiation and is only found during lactation. Following the differentiation acquired during pregnancy, stem cells become more refractory to carcinogenesis. In a rodent model this protective effect of pregnancy can be mimicked by treatment with the pregnancy hormone human chorionic gonadotrophin (HCG). It appears that pregnancy and/or HCG induces differentiation and a change in the genomic signature of the stem cell making it more refractory to carcinogenesis (Russo et al., 2005(b)). Studies on human breast cancer pathogenesis indicate that lobules typ 1 are the origin of invasive ductal carcinoma whereas lobular carcinoma may emerge from lobules type 2. Lobules type 3 and 4 do not seem to undergo malignant transformation but may be the source of benign conditions e.g. cysts and fibroadenoma.

**Proliferation and epithelial/mesenchymal interaction**

The basis of risk associated with hormonal therapies may lie in the regulation of cell proliferation. Within populations of cells *in vitro* and *in vivo* a higher rate of cell proliferation may increase the risk of transformation into the neoplastic phenotype. During normal cellular division, approximately one in a million breast cells undergoes spontaneous mutation. Therefore, the risk of developing cancer is highly influenced by the rate of mitosis (Preston-Martin et al., 1990). In many organs, e.g. the endometrium, progesterone and progestogens are known to counteract the proliferative effects of estrogen. In contrast, in the breast addition of progesterone will enhance cell proliferation. During the normal menstrual cycle, there is only little cell proliferation from estrogen alone in the follicular phase whereas after ovulation, under the combined influence of estradiol and progesterone, proliferation is markedly increased (Söderqvist et al., 1998; Conner 2007). Likewise, in experimental models for hormonal treatment, administration of estrogen alone implies only little effect and proliferative activity is generally much higher during the combined influence of estrogen plus progestogen (Conner 2007).

*Figure 2* Schematic illustration of the female breast anatomy.
Epithelial mesenchymal interactions play an important role both in normal mammary gland development and during neoplastic transformation. Part of steroid action in target tissues is mediated via binding to specific intranuclear receptors. Apart from estrogen and progesterone receptors the androgen receptor is a third member of the nuclear receptor super family. Estrogen receptors are found in both epithelial and stromal cells within the mammary gland. However, most cells that proliferate in response to estrogens do not contain estrogen receptors. Experimental data suggest that the estrogenic stimulation and epithelial growth in the mammary gland is a paracrine event and mediated via stromal cells (Cunha et al., 1997; Guo et al., 2001; Imagawa et al., 2002; Shekhar et al., 2003). Proteoglycans in the stroma seem to have an important role in stromal-epithelial interaction and paracrine signaling. Proteoglycans may be regarded as multireceptor molecules which promote the integration of cellular signals (Delehedde et al., 2001; Alowami et al., 2003). Syndecan-1 is a cell surface heparansulfate proteoglycan which participates in cell proliferation, cell migration and cell matrix interaction (Roskelley & Bisell et al., 1995; Barbareschi et al., 2003; Beauvais et al., 2004). The biological effects of syndecans are thought to be mediated through the binding of various growth factors. Using a cDNA micro array technique it was demonstrated that the gene expression of Syndecan-1 in the rat uterus is clearly estrogen dependent (Wu et al., 2003). An increased expression of Syndecan-1 was demonstrated in the stroma of invasive breast cancer (Stanley et al., 1999; Leivonen et al., 2004; Maeda et al., 2004). Syndecan-1 has also been found to promote proliferation of human breast cancer cells in vitro (Maeda et al., 2004). Expression of Syndecan-1 may be a predictor of a poor prognosis in breast cancer (Leivonen et al., 2004).

Apoptosis. Normal homeostatic functioning in breast tissue is dependent on epithelial cell turnover and the balance between the proliferation and apoptosis. Apoptosis, or programmed cell death, is an active and physiological mode of cell death where the cell designs and executes the process of its own destruction. Apoptosis is involved in tissue remodelling during e.g. embryogenesis and is vitally important to remove potentially malignant cells exposed to mutagens before tumours can develop (Wyllie 1992; Cohen 1993). Not only the change in the rate of cell proliferation but also the ability of the cell to respond to programmed cell death is associated with tumour development and progression. Growth factors and trophic hormones seem to regulate apoptosis and proliferation in a reciprocal manner. During the normal menstrual cycle in young women, apoptotic activity generally peaks 2 to 3 days after the maximum proliferative activity in the luteal phase (Reid et al., 1996). During the high estrogen levels before ovulation, apoptotic activity has been shown to be low. In women on hormonal treatment, the progestogen exposure is important for the balance between proliferation and apoptosis. There are indications that the discontinuation of a progestogen is in fact a trigger to initiate the apoptotic pathway (Foidart et al., 1998). In a relevant monkey model, continuous progestogen administration was found to suppress apoptosis (Conner et al., 2005).

**BREAST RESPONSE TO HORMONAL THERAPY**

It is well known that different principles for hormonal therapy e.g. estrogen alone and estrogen in cyclic or continuous combination with progestogen have quite different effects in many target organs such as the endometrium. During the last years, it has become clear that there are significant differences between various hormonal treatments with regard to their effects on the breast. The differential effects on surrogate markers, such as breast cell proliferation and mammographic density, largely seem to parallel differences in breast cancer risks.
**Breast cell proliferation**

Carefully conducted *in vitro* studies of both normal and transformed breast cells in culture have produced a wealth of information about the hormonal regulation of breast epithelium. However, cultured breast epithelial cells lack the normal complement of blood vessels, fat tissues, stroma and myoepithelial cells. These components are known to exert considerable paracrine and hormonal influence *in vivo*. In fact, the results from many proliferation analysis in cell cultures on the effects of progestogen are quite opposite to the findings obtained under *in vivo* conditions (*Andersson et al.*, 1982; *Gompel et al.*, 1986; *Longacre et al.*, 1986; *Isaksson et al.*, 2001). While many cell culture experiments indicate an inhibitory effect of progestogen on estrogen induced proliferation, the majority of *in vivo* studies show an enhanced proliferative activity.

For obvious reasons, studies on long-term hormonal treatment can be difficult to perform in women and there is a need for relevant animal models. The Cynomolgus Macaque (*Macaque fascicularis*) is a non-human primate. Macaques have well-documented similarities to women in terms of reproductive physiology and anatomy, mammary gland development, peripheral steroid hormone metabolism and sex steroid receptor expression. In contrast to e.g. rodents, the breast epithelial cells also share a characteristic cytokeratin immun-phenotype which reflects the close phylogenetic relation with the human species. In general, experimental findings in macaques have been predictive of outcome in human reproductive studies (*Cline et al.*, 2001). Data from this experimental *in vivo* model have repeatedly shown that in surgically postmenopausal macaques the proliferative breast response after combined estrogen/progestogen treatment is much more pronounced than for treatment with estrogen alone (*Cline et al.*, 1996; *Cline et al.*, 1998). In addition, the apoptotic rate, as reflected by Caspase-3 expression in breast tissue was much lower for the combined regimen (*Conner et al.*, 2005). An increased proliferation in combination with decreased apoptosis could tentatively explain the excess risk reported for combined therapy in clinical and epidemiological studies.

![The Cynomolgus Macaque](image)

**Figure 3** The Cynomolgus Macaque
The fine needle aspiration (FNA) biopsy technique was developed at the Karolinska Hospital and has become an established tool for the pre-operative diagnosis of palpable tumours in the breasts. Over the years, numerous studies have shown a high concordance between cytological findings obtained by FNA and the histopathological assessment after surgery (Söderqvist 1998; Löfgren et al., 2003; Conner 2007). There is an increased proliferative activity in normal breast epithelium from young women during the luteal phase of the menstruation cycle and also a significant correlation with blood levels of progesterone (Isaksson et al., 2001). Proliferation during oral contraceptive use showed a positive correlation with circulating levels of progesterone and also a negative correlation with levels of free testosterone. During continuous combined estrogen/progestogen treatment with standard doses, there is on average a 4 to 5-fold increase in proliferating cells as recorded by staining for the marker Ki-67/MIB-1. The results have been quite similar for different combinations of estrogen and progestogen (Conner et al., 2003).

![Fine needle aspiration biopsy performed from the upper lateral quadrant of the left breast.](image)

Tibolone is a synthetic compound which after oral intake is rapidly converted into 3α- and 3β-hydroxy tibolone both having estrogentic properties, and the Δ4 isomer, which is known to possess progestogenic as well as androgenic activity (Moore 1999). Treatment with tibolone has been repeatedly shown to have less influence on breast cell proliferation than estrogen/progestogen treatment (Cline et al., 2002; Conner et al., 2004; Valdivia et al., 2004). The effects of endogenous testosterone levels as well as from testosterone treatment on breast cell proliferation are unknown.

Mammographic density.

Breast density is associated with sex steroid hormones and ovarian function. It varies with age, parity, height and body weight as well as menopausal status (Boyd et al., 1998). Breast density is also related to reproductive status, circulating levels of endogenous sex steroids, peptide hormones, growth factors and their binding proteins (Boyd et al., 2002). In fact, density seems to reflect the net influence of hormonal and reproductive factors and its background genetics on the breast (Warren 2004). Density has been suggested as an intermediate phenotype for breast cancer and may account for as much as 30% of all breast cancer cases (Boyd et al., 2005). While breast density is associated with breast epithelial cell proliferation, its major characteristic seems to be tissue remodelling and

22
an increase of stromal proteoglycans (Warren & Lakhani 2003; Boyd et al., 2005; Lundström et al., 2006; Harvey et al., 2008).

Figure 5. Mammogram of a 53 year old woman before (to the left) and after (to the right) six months of treatment with continuous combined estrogen/progestogen therapy demonstrating an increase in mammographic density.

Mammographic density may be of particular importance since it is the only known risk factor which is present in the very organ that will eventually develop the disease. Numerous reports have shown that mammographic breast density is increased in a significant proportion of women using hormone therapy. Breast cancer screening may be hampered by increased density making small occult tumours more difficult to diagnose. It is clear that regimens for hormone therapy have different impacts on the mammographic breast density pattern (Greendale et al., 1999; Lundström et al., 1999; Valdivia et al., 2004). Overall, data show that an increase in breast density is much more frequent and pronounced among women on combined estrogen/progestogen treatment than in those receiving estrogen alone. The increase in density, when it occurs, is an early event and is fully developed within the first few months of treatment. Thereafter, in women who continue on the same regimens, there is little change in mammographic status during long-term follow-up (Lundström et al., 1999; Conner 2007). There are also data to show that breast density during hormone therapy is dynamic, increasing with initiation and decreasing with discontinuation and change of therapy (Rutter et al., 2001). While there may be a small effect of the estrogen doses, the marked differences between treatments are associated with the addition of progestogen. So far, there seems to be little differences between the effects of e.g. 19 nor-steroids like norethisterone and 17-hydroxyprogesterone compounds like medroxyprogesterone-acetate (MPA) in this respect (Conner et al., 2004). However, tibolone has been demonstrated to have less influence on mammographic density than combined estrogen/progestogen treatment. The impact of this compound with somewhat androgenic properties is more similar to treatment with estrogen alone (Lundström et al., 2002; Valdivia et al., 2004). Whether treatment with testosterone alone or in combination with estrogen/progestogen will affect mammographic breast density is unknown.
ANDROGENS IN WOMEN

Androgens represent natural anabolic steroids and have a great variety of important effects not only as sex steroids but also in several extragenital organs. In women androgens are important for e.g. energy, bone mass, body composition, mood and libido (Rosenberg et al., 1997; Scherwin 1998; Davison & Davis 2003; Labrie et al., 2003). Testosterone is the most important circulating androgen in both men and women. Normal serum levels in premenopausal women range 0.7-3.0 nmol/L. Some 0.5-1.5 nmol/L of circulating testosterone is of adrenal origin and 0.5-1.0 nmol/L is produced by the ovary (Figure 6). LH has an important role to stimulate hormone synthesis in the ovarian stroma.

Testosterone effects can be mediated directly via androgen receptor binding or after peripheral aromatization to estradiol via the estrogen receptor system. Androgen receptors are found in several organs and tissues including the CNS, cardiovascular system, bone, liver, muscle, fat and reproductive organs including the breast.

Furthermore, after 5-α reduction in e.g. hair follicles and sweat and sebaceus glands, it can exert its action in the form of dihydrotestosterone, DHT, the most potent androgen in the body.

The changes in circulating testosterone levels with age have not been clearly documented and there is a lack of longitudinal studies to elucidate this issue (Burger et al., 2000; Couzin et al., 2001). While levels of estrogens dramatically decline after menopause, levels of testosterone are generally thought to be largely maintained (Speroff & Fritz 2005). Several studies in postmenopausal women have shown two- to tenfold higher testosterone levels in ovarian veins than in the peripheral circulation. Bilateral oophorectomy has been shown to cause a 50% reduction in in serum levels of testosterone in both pre- and postmenopausal women (Schifren et al., 2000; Lobo 2001). Much of the uncertainty relates to the fact that most testosterone assays are unreliable to quantify low concentrations (Miller et al., 2004; Somboonporn & Davis 2004). Validation of routine immunochemical testosterone assays against an established standard has been scarce. When analyzing samples from women, methods without extraction steps may be compromised by a
high cross reactivity from androgen conjugates. A comparison between ten commercial methods versus one including extraction and isotope dilution gas chromatography mass-spectrometry showed that many assays in the female concentration range were not acceptable. In addition, available commercial assays for free testosterone are inconsistent. Hardly any assays include an evaluation of the amount bound to albumin. Calculations on the basis of the law of mass action and equilibrium dialysis are considered to be the most accurate methods to assess levels of free testosterone (Miller et al., 2004).

In women 75% of circulating testosterone is bound to sex hormone binding globulin, SHBG, and some 25% to albumin (Figure 7). Only the few percent of unbound testosterone will pass the cell-membrane in target organs, bind to the androgen receptor and thus exert a biological effect (Vermeulen et al., 1998).

![Figure 7 Amount of free and protein bound circulating testosterone.](image)

**SHBG and the estrogen/androgen balance**

Variations in SHBG levels may change the amounts of bioavailable testosterone and thus the estrogen/androgen balance in individual women. It is important to notice that oral administration of estrogens in oral contraceptives or postmenopausal treatment will enhance SHBG production in the liver. Many of the clinical effects of hormonal treatment are due to the change in levels of circulating steroid and peptide hormones and their binding proteins and as a consequence an altered response in various target tissues. The increase in SHBG has been suggested to reflect the estrogenicity of the treatment at the hepatic level (Rosner 1991; von Schoultz 1998; Tchernoff et al., 2000). In young women using oral contraceptives the relative increase in SHBG has even been suggested as a surrogate maker for the estrogen dependent risk of thromboembolic disease (Odlind et al., 2002). At the hepatic level, thyroid hormone stimulates the SHBG synthesis. High insulin levels inhibit production, and may be one of the underlying causes of hyperandrogenism in the hyperinsulinemic metabolic syndrome.

**Androgen treatment in women**

During the last years, there has been an increasing interest in androgen treatment in women. The term Female Androgen Insufficiency (FAI) has been proposed as a pattern of clinical symptoms in the presence of decreased bioavailable testosterone and normal estrogen status (Bachmann et al., 2002). Since clinical symptoms can be vague and are quite unspecific, correct measurement of free testosterone is of great importance. The addition of testosterone to hormone therapy has shown benefits for psycho-sexual function, mood, energy, psychological well being, bone mineral density, muscle mass and strenght and adipose tissue distribution. (Davis et al., 1995; Sherwin 1998; Schifren et al., 2000; Flöter et al., 2002).
Although studies have shown a positive effect of testosterone treatment on psycho-sexual function, no clear correlation between levels of free testosterone and sexual dysfunction have been demonstrated.

Little is known about the long-term safety of androgen treatment in women (Basaria & Dobbs 2006). Large doses of testosterone and supraphysiological blood levels may result in androgenic side effects such as hirsutism, acne and virilization. There are also concerns about adverse effects on carbohydrate and lipid metabolism (Bachmann et al., 2002). However, in the WHILA (Women’s Health in Lund Area) study women with cardiovascular disease were found to have low serum androgen levels and there was a positive association between androgens and HDL-cholesterol (Khatibi et al., 2007). Conditions with androgen excess such as the polycystic ovary syndrome (PCOS) have been linked to an increased risk of type II diabetes and cardiovascular disease (Catrall & Healey 2004; Alberti et al., 2006). There is also uncertainty on effects on the endometrium. Recently, short term treatment with testosterone was demonstrated not to increase endometrial proliferation but rather to counteract estrogen/progestogen induced stimulation (Zang et al., 2007).

**Androgens and the breast**

Currently there is a lack in our basic understanding as to how testosterone and other androgens influence the normal breast (Birell et al., 1995). Data from in vitro studies suggest that testosterone and other androgens may have two distinct primary effects. Under estrogen deprived conditions, androgens after aromatase conversion may stimulate growth via the estrogen receptor α, and this effect can be blocked by antiestrogens. On the other hand, in the presence of estrogens, androgens will inhibit the growth stimulatory effect of estrogen. This antagonistic effect is mediated via the androgen receptor and can be blocked by antiandrogens (Burak et al., 1997; Conde et al., 2004). Thus, when considering testosterone treatment in both pre-and postmenopausal women, the estrogen status of the woman is of strong importance.

There are conflicting data on endogenous testosterone levels and breast cancer risk. In case-control studies, high endogenous total testosterone levels have been associated with increased breast cancer risk in postmenopausal women (Endogenous Hormone and Breast Cancer Coll Group, 2002; Key et al., 2002) but not in premenopausal women. The causal relationship can be difficult to establish since increased aromatase activity in the setting of estrogen depletion after menopause, and increased capacity to convert testosterone to estradiol may be a major factor. After adjustment for estradiol levels this association does not remain (Cauley et al., 1999). Furthermore, interpretation of available data is complicated by methodological difficulties and the low sensitivity of most available assays (Miller et al., 2004).

Tibolone is a synthetic compound which after oral intake is rapidly converted into $3\alpha$- and $3\beta$-hydroxy tibolone both having estrogenic properties, and the $\Delta 4$ isomer, which is known to possess progestogenic as well as androgenic activity.

Tibolone has been suggested as an alternative to the common estrogen/progestogen therapy, and been found to exert estrogenic, progestogenic and also androgenic effects in target tissues (Moore et al., 1999; de Gooyer et al., 2003). In previous studies tibolone was found to have only little influence on mammographic breast density and breast epithelial cell proliferation (Lundström et al., 2002; Conner et al., 2004; Valdivia et al., 2004). Also a stronger influence on libido from tibolone was reported when compared to continuous combined estrogen/progestogen HT (Hammar et al., 1998).
After extensive clinical trials a transdermal testosterone patch, Intrinsa®, has recently been approved by the Swedish Medical Products Agency (MPA) and will soon be available on the market (www.lakemedelsverket.se). The indication for testosterone treatment according to the MPA is hypoactive sexual desire disorder (HSDD) after surgical menopause during concomitant estrogen treatment. Previous studies have shown that oophorectomy results in impaired sexual desire to a greater extent than natural menopause, and these surgically menopausal women to have lower levels of free testosterone compared to women with normal libido (Shifren 2000; Mazer & Schifren 2003). In previous clinical trials, women treated with the patch in combination with transdermal estrogen showed a larger improvement on sexual function compared to women treated with the patch and oral estrogen. The patch is a twice a week application to the abdomen. (Figure 9). Testosterone levels are increased to the upper normal range for premenopausal women (Mazer & Schifren 2003).

Figure 8 Tibolone and its metabolites

Figure 9 The transdermal testosterone patch.
Testosterone is absorbed through the skin by passive diffusion and the patch delivers 300 µg of testosterone per day. Maximal serum concentrations of testosterone are achieved within 24-36 hours with a large interindividual variation. Since testosterone has a short half life of two hours, serum levels return to baseline within 12 hours after removal of the patch, and there is no risk of accumulation of testosterone. Safety data on 882 patients exposed for 20 weeks and 348 patients exposed for 48 weeks showed no difference in adverse events between treatment with the testosterone patch and placebo (Mazer & Shifren2003). There were no differences between groups in blood lipids, glucose metabolism or liver function. Long term data for safety is required.

**Breast cancer risk from hormonal therapies**

Valid evidence from randomized controlled trials (Table II) indicate that breast cancer risk is increased with combined estrogen/progestogen use and that such treatment implies a risk beyond that of estrogen alone (Chlebowski et al., 2003; Hulley et al., 2003; Stefanick et al., 2006).

### Table II  Breast cancer risk in Randomized Controlled Trials

<table>
<thead>
<tr>
<th></th>
<th>HERS II</th>
<th>WHI</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>E + P</td>
<td>E + P</td>
</tr>
<tr>
<td>Follow-up</td>
<td>6-8 years</td>
<td>6.2 years</td>
</tr>
<tr>
<td>RR of BC (ITT)</td>
<td>1.27</td>
<td>1.26</td>
</tr>
<tr>
<td>95% CI</td>
<td>0.8-1.9</td>
<td>1.0-1.6</td>
</tr>
<tr>
<td>RR of BC (adherent)</td>
<td>1.49</td>
<td>0.67</td>
</tr>
<tr>
<td>95% CI</td>
<td>1.13-1.96</td>
<td>0.47-0.97</td>
</tr>
</tbody>
</table>

Available evidence from observational studies, including more than 2 million women, broadly agrees with trial findings (Collaborative Group 1997; Beral et al., 2003; Santen 2003; Collins et al., 2005; Chen et al., 2006; Fournier et al., 2008; Opartny et al., 2008). Overall, risk estimates from observational studies (Table III) are somewhat higher than in RCTs but remain modest as compared to other risk factors, even after long-term treatment.

For combined estrogen/progestogen therapy, risk is gradually increasing to become significant after 4 to 5 years. As regards estrogen-only treatment, the large WHI trial indicated a non-significant decrease in risk, whereas epidemiological evidence rather suggest a small risk increase after 10 to 15 years of treatment. After cessation of therapy, risk estimates decline to reach baseline levels after around 5 years.

The majority of available data, and in particular the large Million women study, suggest that neither the type of estrogen nor the type of progestogen will effect breast cancer risk. Although the adverse effects of progestogen addition may be a class effect, there are reports from the French E3N cohort to suggest that natural micronized progesterone, and eventually dydrogesterone, may carry less risk (Fournier et al., 2008).

There are conflicting data on the association between androgen levels and breast cancer risk. In cell cultures and animal experiments, non aromatizable androgens have been shown to exert anti-proliferative effects, whereas in some reports aromatizable androgens displayed proliferative action (Birell et al., 1995).

In the Million Women Study, the risk estimate for breast cancer from treatment with tibolone was clearly lower than for conventional estrogen/progestogen combinations, but still significant at
RR 1.45; 95% CI 1.25-1.68 (Beral et al., 2003). However this finding was recently contradicted (RR 0.86; 95% CI 0.65-1.13) in a large case control study of a cohort of postmenopausal women from the UK’s General Practice Research Database (Opatrny et al., 2008). In the GPRD material, as in many other studies, there was little risk association for treatment with estrogen only and also a suggestion that combined treatment with the estrogen patch would carry a lower risk than oral administration.

There are only few studies on exogenous testosterone therapy and breast cancer risk. Dimitrakakis et al found no increase of breast cancer in a group of postmenopausal women when testosterone was added to HT, during a mean follow up of 5.8 years (Dimitrakakis et al., 2004). In contrast, recent data from the Nurses’ Health Study suggested combined estrogen/testosterone to be associated with an increased risk (Tamimi et al., 2006). The need for prospective, randomized trials on this subject is apparent.

**Table III** Risk estimates (RR/OR/HR) for breast cancer in some observational studies published after the Collaborative report 1997.

<table>
<thead>
<tr>
<th>Study</th>
<th>E only</th>
<th>E + P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Collins et al 2005</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average risk estimate from</td>
<td>current use</td>
<td>1.18 (1.01-1.38)</td>
</tr>
<tr>
<td>studies published after the</td>
<td></td>
<td>1.70 (1.36-2.13)</td>
</tr>
<tr>
<td>Collaborative Study.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estrogen only 2 862 cases; E</td>
<td></td>
<td></td>
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<tr>
<td>+ P 3 455 cases; overall</td>
<td></td>
<td></td>
</tr>
<tr>
<td>weight of the Million</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Women Study 65% and 84%.</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Chen et al 2006</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>934 cases from the Nurses</td>
<td>5-10 years use</td>
<td>0.90 (0.73-1.12)</td>
</tr>
<tr>
<td>Health Study Cohort.</td>
<td>&gt; 20 years use</td>
<td>1.42 (1.13-1.7)</td>
</tr>
<tr>
<td><strong>Fournier et al 2008</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 354 cases from the French</td>
<td>E + natural</td>
<td>1.29 (1.02-1.65)</td>
</tr>
<tr>
<td>E3N cohort mean follow-up 8.1</td>
<td>progesterone</td>
<td>1.0 (0.83-1.22)</td>
</tr>
<tr>
<td>years.</td>
<td>E + synthetic</td>
<td>1.69 (1.50-1.91)</td>
</tr>
<tr>
<td></td>
<td>progestogen</td>
<td></td>
</tr>
<tr>
<td><strong>Opatrny et al 2008</strong></td>
<td>oral estrogen + P</td>
<td>0.97 (0.86-1.09)</td>
</tr>
<tr>
<td>6 341 cases and 31 516 controls from the UK General Practice Research database.</td>
<td></td>
<td>1.38 (1.27-1.49)</td>
</tr>
<tr>
<td></td>
<td>estrogen patch + P</td>
<td>1.08 (0.81-1.43)</td>
</tr>
</tbody>
</table>
AIMS

- To compare the effect of tibolone and estrogen/progestogen treatment on serum levels of sex steroids and binding proteins and their relationship to mammographic density.

- To explore the effect of testosterone addition on breast cell proliferation during postmenopausal estrogen/progestogen treatment.

- To evaluate the effect of testosterone addition on mammographic density during estrogen/progestogen treatment.

- To evaluate breast symptoms during HT and their possible correlation to mammographic density.

- To analyze the expression of Androgen receptor(AR) and Syndecan-I in breast tissue during different long-term hormonal treatments in cynomolgus monkeys.
MATERIAL AND METHODS

SUBJECTS (I-III)

Postmenopausal apparently healthy women aged 50-70 (I) and 45-65 (II, III) years without any previous history of breast disorder were recruited. All women were postmenopausal for at least twelve months or had FSH levels > 40 IU/L. None of the women had taken any sex steroid hormones during the last three months preceding the study. Exclusion criteria were: any previous history of cancer, any history of previous breast disease or abnormal mammogram, hypertension (systolic blood pressure > 170 mm Hg or diastolic > 105 mm Hg), hyperlipidemia (total cholesterol > 8.0 mmol/L, triglycerides > 3.0 mmol/L), diabetes mellitus, history of thromboembolic disease, undiagnosed vaginal bleeding, any sign of hepatic dysfunction or concomitant treatment known to influence the study medication (warfarin, rifampicin, carbamazepine, griseofulvin, hydantoins, primidone, barbiturates and broad-spectrum antibiotics).

In study I a total of 166 women were randomized to receive either estradiol 2 mg/norethisterone acetate 1 mg (E2/NETA Kliogest®), tibolone 2.5mg (Livial®) or placebo once daily for 6 months. The study medication used was individually numbered for each subject. Random assignment of subject code numbers over treatment groups was consecutively performed. Blinding was maintained until completion of the study. Compliance was checked after 3 and 6 months from diaries where the women registered their daily medication intake. The study was approved by the independent Ethics Committee and all women gave their written informed consent prior to inclusion.

In study II and III a total of 99 postmenopausal women were given continuous combined estradiol 2 mg/norethisterone acetate 1 mg and were equally randomized to receive additional treatment with either a testosterone patch releasing 300 µg/24h or a placebo patch. Blinding was maintained until completion of the study. Compliance was checked at clinical visits after 2, 4 and 6 months. Mammography was performed and breast cells were collected at baseline and after 6 months.

Both studies were approved by the independent Ethics Committee at the Karolinska University Hospital and by the Swedish Medical Products Agency. All women gave their written informed consent before inclusion.

Animals

The subjects in study IV were were 60 feral adult female cynomolgus macaques (Macaca fascicularis) imported from Indonesia to the United States by Wake Forest University. They were a subset from a larger study on breast proliferation (Cline et al 2002). They were 6-8 years old at entry into the study. Animals were housed in social groups of 4-6 monkeys each in a facility accredited by the Association for the Advancement and Accreditation of Laboratory Animal Care. Experimental protocols were approved by the Institutional Animal Care and Use Committee. Bilateral oophorectomy were done on all animals 3 months prior to start of hormonal treatment. The 60 macaques were randomized into four treatment groups with either tibolone at 0.2mg/kg, conjugated equine estrogens (CEE) 0.042mg/kg or CEE + medroxyprogesterone acetate (MPA) at 0.167mg/kg. Controls were given no hormonal treatment (n=15 in all groups). Treatments were given continuously in the diet for 2 years; doses were scaled on a caloric intake basis. The doses were designed to correspond to 0.625 mg CEE, 2.5mg MPA.
and 3mg of tibolone when given in women (Adams et al., 1997; Cline et al., 2002). Animals were fed twice daily, with drug treatments split between the two feedings.

**FINE NEEDLE ASPIRATION BIOPSY (II)**

Before and after six months of treatment percutaneous FNA biopsies from the upper outer quadrant of the left breast were performed using a needle with an outer diameter of 0.6 millimeter (Franzen & Zajicek, 1968; Skoog et al., 1990). In order to produce several identical slides, the aspirated cells were mixed with 0.5-1.0 ml 4% buffered (pH 7.4) formalin in the syringe used to procure cells. The cells were concentrated by centrifugation at 700 rpm for 3 minutes in a Shandon cytoSpin centrifuge and after resuspension in 200 μl buffer, volumes of 110 μL were sedimented on to pretreated glass slides.

**IMMUNOCYTOCHEMICAL ANALYSIS (II)**

Immunostained cells were quantified using cell counting. Slides were blinded for identity, type of treatment and sequence of biopsy and stained for the nuclear antigen Ki-67. The Ki-67/MIB-1 monoclonal antibody reacts with a human nuclear antigen which is present in proliferating cells but absent in quiescent cells. Cell cycle analysis shows that the antigen is expressed in the phases of G1, S, G2 and mitosis (Gerdes et al., 1991). MIB-1 analyses were performed using reagents supplied by Immunotech, Marseilles, France. The staining procedure uses an avidin-biotin peroxidase system, modified for the cytoSpin technique. We considered samples obtained by FNA to be assessable only if they contained intact cells and no free lying nuclei. All slides were examined by an experienced cytopathologist (LS), and around 80% of cells were judged to be of epithelial origin. Stromal cells were identified morphologically by size (twice that of epithelial cells), an oval shape and initially also by a positive staining for Vimentin using a commercial kit (Monoclonal Mouse anti-Vimentin, M7020, DAKO A/S, Glostrup, Denmark). Vimentin is a skeletal protein present only in cells of mesenchymal origin (Herrmann & Aebi, 2000). On average 150-200 cells were counted per slide and only samples with a minimum of 40 cells were included in the analysis.

**MAMMOGRAPHIC BREAST DENSITY (I, III)**

Mammography examinations were performed in accordance with the Quality Control Regulations as stipulated by the Swedish National Board of Health and Welfare and the Swedish National Radiation Protection Institute.

Mammograms were obtained at baseline and at six months to determine breast density and to evaluate any abnormalities. Mammography examinations comprised mediolateral oblique (MLO) and craniocaudal (CC) views of both breasts. Only the MLO view of the left breast was used for the visual classifications of breast density. Previous studies have shown very little difference between the left and right breast in the response to hormonal treatment (Lundström et al., 1999; Lundström et al., 2001). For technical reasons e.g. to avoid the pectoral muscle when the assessable area was defined, the CC view was used for the digitized assessment. All mammograms were assessed by two independent radiologists (GS, EA) who were blinded to treatments. Any differences of opinion in the classification of a mammogram were discussed and resolved with a consensus result.
Visual classification (I, III)

Mammographic density of all coded films was classified according to the Wolfe classification (Wolfe, 1976) in four categories: N1, essentially normal breast with a parenchyma composed primarily of fat and with, at most, a few fibrous connective tissue strands; P1, prominent ductal pattern in up to one fourth of the breast volume; P2, prominent ductal pattern in more than one fourth of the breast volume; and DY, extremely dense parenchyma, which usually denotes connective tissue hyperplasia. In addition to the Wolfe classification, for each individual woman, all coded films were classified according to a percentage scale (Lundström et al., 2002; Conner et al., 2004) with five categories of the amount of dense breast parenchyma in relation to the whole breast volume. The five categories were: 0-20%, 21-40%, 41-60%, 61-80% and 81-100%.

Digitized breast density (III)

In addition to visual judgment and classification a computer based quantitative assessment was also performed (Figure 10). The identifying data was removed from the films and the operators (EL, MH) were unaware of the patients identity and duration of treatment.

Figure 10. Digitized mammographic density in a healthy postmenopausal woman after HT demonstrating an area of dense tissue.

All films were digitized and the dense area of the left cranio-caudal (CC) view image was measured by using a computer-assisted program (Cumulus, Sierra plus, Vidar Systems Corporation, Medical Imaging, Herndon, VA, USA) (Byng et al., 1994; Byng et al., 1996; Lundström et al., 2006). In this procedure the operator establishes “thresholds” for the edge of the breast and the edge of dense tissue. A computer then records the number of pixels in the digitized image that fall within the defined areas. This method of measurement has been shown to give highly reproducible results and details have been given elsewhere (Byng et al., 1994; Byng et al., 1996). In the present study the intra-assay variation was 7%, as calculated from five repeated measurements in five different mammograms, i.e. a total of 25 mammograms. Each value for density was calculated as the mean of three measurements.
BREAST SYMPTOM QUESTIONNAIRE (III)

All women were asked to complete a breast symptom questionnaire at baseline and after two, four and six months. The questionnaire comprised 5 items on the presence of subjective symptoms during the last days before the visit. The symptoms were sensations of stinging, pain, and soreness and whether the women thought that the breasts had become swollen or enlarged. Symptoms were graded from 0 (no symptom at all) to 10 (a maximum of symptom). Symptoms grades for all 5 items were thereafter summarized and expressed as a total symptom score with values ranging from 0 to 50.

TISSUE COLLECTION (IV)

Breast tissues were collected at the end of the treatment period when all monkeys were euthanized and necropsied. Serum levels of estradiol, estrone sulfate, MPA and tibolone metabolites were measured as previously described (Cline et al., 2002).

Tissues were fixed in 4% paraformaldehyde for 24h and stored at 4°C in 70% ethanol. Thereafter, tissues were trimmed to 3mm thickness, embedded in paraffin and sectioned at 5 μmeters for immunostaining.

IMMUNOHISTOCHEMISTRY (IV)

A standard immunohistochemical technique (avidin-biotin-peroxidase) was used to visualize the distribution of AR and Syndecan-1.

A monoclonal mouse anti-human antibody was used for detection of AR (M3562, DakoCytomation, Glostrup, Denmark). The tissue sections were dewaxed and rehydrated in descending concentrations of ethanol. Sections were then pretreated in 0.01M sodium citrate buffer (pH 6.0), in a microwave oven at high power for 10 to 20 min, and allowed to cool for a further 20 min. Following washing in buffer; 0.1M phosphate-buffered saline (PBS) pH 7.4, non-specific endogenous peroxidase activity was blocked by treatment with 3% H2O2 (Merck Darmstadt, Germany) in methanol for 10 minutes. Sections were then incubated in a solution consisting of non-immune horse serum (Vector Laboratories Inc, Burlingame, CA, USA) to block non-specific binding sites. The tissue sections were thereafter incubated with the primary antibody. The AR-antibody was diluted 1:100 in PBS and incubated on sections at 4°C overnight. Negative controls were obtained by replacing the primary antibody with mouse IgG of the equivalent concentration. Following primary antibody binding, the sections were incubated for 30 min at room temperature (RT) with a biotinylated secondary horse anti-mouse IgG antibody (Vector Laboratories Inc).

The tissue sections were then incubated for 30 minutes at RT with horseradish peroxidase-avidin biotin complex (Vectastain Elite, Vector, CA). The site of the bound enzyme was visualized by the application of 3,3’-diaminobenzidine (DAB kit, Dako Cytomation), a chromogen which produces a brown, insoluble precipitate when incubated with enzyme. Thereafter, sections were counterstained with haematoxylin and dehydrated before mounting.

A polyclonal rabbit anti-human antibody was used to detect Syndecan-1 (CD 138, M7788, DakoCytomation, Glostrup, Denmark). The antibody was diluted 1:50 in Tris/HCl and the immunohistochemistry was performed in an automatic system using the “Dako REAL EnVision Detection System, peroxidase/DAB+, rabbit/mouse” kit, according to the manual provided by the manufacturer (Dako Denmark A/S, Glostrup, Denmark).
**Image analysis**

A Leica microscope connected to a computer using Colorvision software (Leica Imaging System Ltd. Cambridge, England) was used to assess AR immunostaining quantitatively by a computer image analysis system. Quantification of immunostaining was performed on the digitized images of systematic randomly selected mammary glands, from which stromal elements were interactively removed. All glands, or 10 fields, were measured separately in each tissue section. By using color discrimination software, the total area of positively stained nuclei (brown reaction product) was measured, and expressed as a ratio of the total area of cell nuclei (brown reaction product + blue haematoxylin).

**Manual scoring**

Manual scoring of Syndecan-1 expression in epithelium and stroma/myoepithelial cells was performed. Two observers (MH, SN), blinded to the identity of the slides, performed all the assessments. The Syndecan-1 staining was evaluated using a grading system. The staining intensity was graded on a scale of (0) = no staining, (1) faint staining and (2) moderate/ strong staining.

**SERUM ANALYSES (I-III)**

Venous blood samples were drawn at baseline and after six months of treatment. Serum concentrations of testosterone (T) and estradiol-17β (E2) were determined by radioimmunoassay using commercial kits from Diagnostic Products Corp., Los Angeles, CA (“Coat-a-Count® Testosterone) and from CIS Bio International, Gif-sur-Yvette, France (“ESTR-US-CT®”, E2). Serum levels of dihydrotestosterone (DHT) were determined with radioimmunoassay after removal of cross-reacting T by oxidative cleavage of the 4-ene double bond with potassium permanganate, using a commercial kit (‘DHT®’, Diagnostic Systems Laboratories Inc, Webster, Tx, USA).

Sex hormone-binding globulin (SHBG), dehydroepiandrosterone sulfate (DHEAS) and insulin-like-growth factor I (IGF-I) were determined by chemiluminiscence enzyme immunoassay using commercial kits obtained from Diagnostic Products Corp., Los Angeles CA (“Immulite® SHBG” and “Immulite® DHEA-SO4”) and from Nichols Products Corp., San Juan Capistrano, CA (“Advantage® IGF-I”).

Insulin-like growth factor binding protein 3 (IGFBP-3) was analysed by enzyme linked immunosorbent assay using a commercial kit obtained from Diagnostics Systems Laboratories, Webster, TEX.

Dehydroepiandrosterone (DHEA), 4-androstene-3,17-dione (A-4), unconjugated estrone (E1) and total estrone (tE1; ≥ 85% estrone sulfate) were determined by radioimmunoassay after extraction with diethyl ether (Carlström & Sköldefors 1997). For tE1 estrone sulfate was hydrolysed by Helix pomatia sulfataser plus glucuronidase preparation prior to extraction.

Apparent concentrations of free testosterone (fT) were calculated from values for total T, SHBG and a fixed albumin concentration of 40 g/L by successive approximation using a computer program based upon an equation system derived from the law of mass action(Södergård et al., 1982).

The detection limits and within and between assay coefficients of variation were for T: 0.1 nmol/L, 6% and 10%; f-T: 6 pmol/L, 7% and 10%; DHT: 14 pmol/L, 4% and 8%; E2: 5 pmol/L, 13% and 18%; E1: 30 pmol/L, 7% and 10%; tE1 0.3 nmol/L, 7.0% and 8.9%; SHBG: 0.2 nmol/L, 6.5 % and 8.7%; A-4: 0.6 nmol/L, 6% and 10%; DHEA: 1.6 nmol /L, 5% and 7%; DHEAS: 0.002 µmol/L; 8.2% and 12%; IGF-I: 6 µg/L, 5% and 7 %; and for IGFBP-3: 0.04 µg/L, 9% and 10 % respectively.
STATISTICAL ANALYSES

Differences between groups were tested by Kruskal-Wallis test followed by post hoc analysis with t-test for unpaired observations or Mann-Whitney U-test according to distribution and in paper IV Dunn’s test. Changes from pre-treatment values were tested by t-test for paired observations or by Wilcoxon signed rank test according to distribution. Correlations were assessed by Spearman’s rank correlation test and by multiple regression. The significance level was set at p<0.05.
RESULTS AND DISCUSSION

During the last years it has been well established from a number of clinical and epidemiological studies that hormonal treatment for the relief of menopausal symptoms is associated with an increased risk of breast cancer. Different principles for hormonal therapy e.g. estrogen alone and estrogen in cyclic or continuous combination with progestogen clearly differ with regard to their effects on the breast (Table II and Table III). The differential effects on surrogate markers, such as breast cell proliferation and mammographic density, largely seem to parallel differences in breast cancer risk. While extensive studies have been performed on the effect of estrogen/progestogen treatment in various preparations and routes of administration, data on effects of testosterone on the breast is scarce and interpretation is complicated by the incoherent testosterone measurements (Dimitrakakis et al., 2004; Somboonporn & Davis (a) 2004; Tamimi et al., 2006). There is an ongoing discussion on the possible general benefit of such treatment and a great need of prospective randomized trials on the effects of testosterone treatment, both in pre- and postmenopausal women.

VARIATIONS IN ANDROGEN STATUS BY DIFFERENT THERAPEUTIC PRINCIPLES

In paper I, two alternative treatments for postmenopausal symptoms were compared. In clinical practice both E2/NETA and tibolone are frequently used to relieve vasomotor symptoms in postmenopausal women (Hammar et al.,1998; Dören et al.,2001). In this comparative study, we found distinct differences between these two regimens as regards their impact on blood levels of hormones, growth factors and binding proteins.

Treatment with E2/NETA resulted in increased estrogen and decreased androgen levels, in the latter case in total and free T and DHEAS. These changes are characteristic for conventional oral estrogen/progestogen HRT. It is well known that estrogens, when given orally markedly influence liver metabolism and protein synthesis. After the administration of estrogen, dose dependent changes are recorded in the circulating levels of a number of liver derived proteins such as SHBG; renin substrate, various coagulation factors and anti thrombin (Mattsson et al.,1983; von Schoultz,1998). There is also evidence that the type and dose of progestogen, when added to the estrogen, is important for liver metabolism and protein synthesis. Androgens and progestogens with androgenic properties are known to counteract some of the effects of estrogen on liver metabolism (Lobo 1992; Kuhl 1996; Bernardi et al.,2003). The dramatic decrease in fT is mainly a result of the increased SHBG levels and the decrease in DHEAS is likely to reflect the liver impact of oral estrogens i.e. decreased circulating levels of its main binding protein albumin (Kluft & Lansink,1997).

The picture following treatment with tibolone was quite different. There was only a minor influence on circulating estrogens and SHBG levels were reduced by 50%. Androgens are known to suppress SHBG production at the hepatic level (Rosner et al.,1991; Lobo 1992; Tchernof & Deprés,2000; Bernardi et al.,2003). After oral intake, tibolone is rapidly converted into 3α- and 3β-hydroxy tibolone both having estrogeic properties, and the Δ4 isomer, which is known to possess progestogenic as well as androgenic activity. In fact, the receptor affinity for this isomer is about
40% of that of the potent androgen dihydrotestosterone (Chetrite et al., 1999; Moore et al., 1999; de Gooyer et al., 2003). The marked reduction in SHBG levels and as a consequence increased concentrations of free testosterone implies an enhanced circulating androgenic activity. This may be important as regards some clinical effects of tibolone.

After six months of treatment women on E2/NETA had lower IGF-I and IGFBP-3 values than those on tibolone. In premenopausal women, high concentrations of IGF-I have been reported as a risk factor for breast cancer (Campagnoli et al., 1994; Hankinson et al., 1998; Pollak et al., 1998) and mammographic density has previously been correlated to IGF-I (Byrne et al., 2000). It has previously been demonstrated (Lundström et al., 1999) that there was an increase in mammographic density in about fifty percent of women after treatment with E2/NETA whereas only few women (2-6%) had a similar response during treatment with tibolone (Lundström et al., 2002). Here, in the same material we could demonstrate an inverse relationship between mammographic density and free testosterone levels. Mammographic status at baseline displayed an inverse association with BMI (r_s=-0.29; p<0.001) and a positive association with SHBG (r_s=0.34; p<0.001). After six months of treatment, these associations remained, and there was also a negative correlation to fT (r_s=-0.27; p<0.001). Multiple regression after six months revealed SHBG as the most important factor (p<0.01). Apart from a direct effect of the Δ4 isomer on the androgen receptor, also the increase in endogenous free testosterone could be one possible mechanism to explain why tibolone has less influence on the breast than continuous combined HT.

**EFFECTS OF TESTOSTERONE ADDITION TO E2/NETA**

It is well established that combined estrogen/progestogen treatment can result in both increased breast cell proliferation and increased mammographic density. These two effects should be regarded as adverse events during therapy and might even reflect the increase in breast cancer risk.

There are a number of observations to suggest that androgens may counteract the proliferative effects of estrogen and progesterone in the mammary gland. In a monkey model, treatment with flutamide, an androgen receptor antagonist was shown to enhance breast epithelial proliferation (Dimitrakakis et al., 2003). Furthermore, in castrated animals testosterone was shown to inhibit breast cell proliferation as induced by estrogen and progesterone. Women with polycystic ovaries tend to have raised endogenous androgen levels and may also carry a lower breast cancer risk (Gammon et al., 1991). Androgen receptor mutations have been reported in some men with breast cancer (Lobaccaro et al., 1993) and a genetic linkage has been suggested between androgen receptor dysfunction and BRCA-1 mutations (Haiman et al., 2002). Breast atrophy has been observed among women athletes taking androgenic drugs to improve performance.

Figure 11 illustrates a reanalysis of baseline data from a material of healthy untreated postmenopausal women. All women were participants in previous clinical trials performed at our department (Conner et al., 2003, Conner et al., 2004). There was an inverse correlation between endogenous levels of fT and breast cell proliferation, indicating a possible protective role of testosterone on the breast. However, no RCTs on the effect of testosterone treatment on the breast have previously been performed.

In papers II and III, the objective was to study the effects of testosterone addition during conventional, postmenopausal EPT on two surrogate markers for breast cancer risk, i.e. breast cell proliferation and mammographic density.
As illustrated in Figure 12, healthy postmenopausal women with a mean age of 54.9 years and a mean BMI of 24.7 kg/m² thus representing a population suitable for HT were recruited to the study. In the study, all women were treated with E2 2mg/NETA 1 mg. Effects on both breast cell proliferation and mammographic density by this relatively high dosage preparation have been well documented in previous studies (Lundström et al., 2002; Conner et al., 2004). In addition to oral estrogen/progestogen the transdermal testosterone patch in a dosage of 300 μg/day or a placebo patch was added. The effect on testosterone levels after treatment with the patch was quite moderate. After six months of treatment mean values (± SEM) for total testosterone were 1.75 ± 0.2 nmol/l compared to 0.43 ± 0.02 in the placebo group. The normal upper range reference value was 3.0 nmol/l for premenopausal women according to our local laboratory so there was no overtreatment. The patch was well tolerated and no clinical androgenic side effects were recorded.

Effects on breast cell proliferation

Among the 88 women who completed the study (Figure 12), 50 (57%) had evaluable aspirates both before and after six months of treatment. Of these 50 women, 27 received E2/NETA plus the testosterone patch and 23 received E2/NETA plus placebo. The percentages of total MIB-1 positive breast cells before and after treatment in women with two evaluable samples are given in Table IV.

During treatment with E2/NETA there was a more than fivefold increase in total cell proliferation from a median value of 1.1% at baseline to 6.2% after 6 months (p < 0.001). This was expected and is in good agreement with previous data from women with the same treatment.

Figure 11. Inverse correlation between free testosterone levels and breast cell proliferation expressed as percentage of Ki-67/MIB-1 positive cells in untreated postmenopausal women. (Conner et al., 2003; Conner et al., 2004).
In contrast, when the testosterone patch was added in women receiving the same estrogen/progestogen treatment, no significant increase in breast cell proliferation was recorded. Median values were 1.6% and 2.0% respectively at baseline and after six months.

Breast stroma accounts for more than 80% of the resting breast volume (Reid et al., 1996; Shekhar et al., 2003). This supportive platform for the epithelial cells is composed of collagen, fibroblasts, endothelial cells, adipocytes and a macromolecular network of proteoglycans. In the present study, for the first time, we assessed stromal and epithelial cell proliferation separately (Figure 13). We found these two distinct cell types to respond in a quite similar manner to treatment with estrogen/progestogen alone or in combination with testosterone. In fact, the proliferative activity was seemingly even more pronounced in stromal than in epithelial cells (Table IV).

Estrogen receptors are found in both epithelial and stromal cells within the mammary gland (Haslam et al., 1992; Couse et al., 1997; Beato & Klug, 2000). However, most cells that proliferate in response to estrogens do not contain estrogen receptors (Cunha et al., 1997; Zeps et al., 1998; Imagawa et al., 2002). Experimental data suggest that the estrogenic stimulation of epithelial growth in the mammary gland is a paracrine event and mediated via estrogen receptor positive

Figure 12. Flow chart for the randomized clinical trial in papers II and III.
stromal cells. Information about androgen receptor content in breast epithelial and stromal cells is limited (Beato & Klug, 2000). It could be that the apparent inhibitory effect of testosterone on breast epithelium as demonstrated in the present study is mediated in a similar way i.e. via a change in stromal cell signaling. These results support the concept that androgens may counteract the proliferative effect of estrogen and progestogen in the mammary gland.

Table IV. Mean values, median and range for percentage of MIB-I positive breast cells in postmenopausal women with assessable fine needle aspiration samples both before and after six months of treatment with E2/NETA either in combination with placebo or testosterone patch (* =p<0.05; ***=p<0.001).

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Testosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>6 months</td>
</tr>
<tr>
<td>Total cells (n=23)</td>
<td>(n=27)</td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>1.7</td>
<td>5.1</td>
</tr>
<tr>
<td>median</td>
<td>1.1</td>
<td>6.2***</td>
</tr>
<tr>
<td>range</td>
<td>0.0 – 8.5</td>
<td>0.0 – 9.7</td>
</tr>
<tr>
<td>Epithelial cells (n=22)</td>
<td>(n=21)</td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>1.1</td>
<td>3.4</td>
</tr>
<tr>
<td>median</td>
<td>0.0</td>
<td>2.8***</td>
</tr>
<tr>
<td>range</td>
<td>0.0 – 6.1</td>
<td>0.0 – 8.3</td>
</tr>
<tr>
<td>Stromal cells (n=9)</td>
<td>(n=4)</td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>4.5</td>
<td>13.0</td>
</tr>
<tr>
<td>median</td>
<td>2.4</td>
<td>12.7*</td>
</tr>
<tr>
<td>range</td>
<td>0.0 – 16.7</td>
<td>1.8 – 25.0</td>
</tr>
</tbody>
</table>

Figure 13. Proliferating epithelial (left) and stromal (right) breast cells obtained by fine needle aspiration from a healthy 54 year old postmenopausal woman.
Effects on mammographic density

While testosterone addition to E2/NETA caused a significant inhibition of breast cell proliferation, no such inhibitory effect could be demonstrated on mammographic density. After six months there was an increase in mammographic density according to Wolfe in 18% of women receiving E2/NETA, and in 29% according to the percentage scale. The corresponding figures for women receiving E2/NETA and the testosterone patch were 22% and 30% respectively. All these changes were highly significant, but there were no differences between the treatment groups.

Also values for the percentage area of dense breast according to the digitized assessment were quite similar for both treatment groups. The mean increase during treatment was 7.4% in the placebo group and 5.4% in the testosterone group. Although a difference in numerical value, it did not reach statistical significance.

What is the effect of testosterone addition on density? One important observation in this study regards the magnitude of increase in mammographic density after treatment with E2/NETA. Compared to previous studies performed (Lundström et al., 2002; Conner et al., 2004), the increase in density according to visual classification scales was lower than what was previously observed from the same treatment. In paper III only 18% and 29% were upgraded within the Wolfe and percentage scale classifications. In comparison (Table V) among the somewhat older women from previous RCTs the corresponding figures ranged 45-68%. As illustrated in the table women in paper III were slightly younger, with fewer years since menopause compared to women in the studies performed by Lundström et al and Conner et al.

Breast density is well known to decline after menopause. Overall older women seem to be more sensitive to hormonal treatments and to react with a more pronounced increase in density than recently menopausal women (Conner 2007). The differences between these clinical materials are also obvious from Figure 14. Clearly in study III more women had a baseline breast density fulfilling the criteria for the P1 and P2 categories of the Wolfe classification. In fact these women comprised over 90% of the total patient material. In comparison many more women had a low

Table V. Mean values for some baseline patient characteristics in study III compared to previous RCTs with a similar design. All women were treated with E2 2 mg/NETA 1 mg and increase in mammographic breast density was recorded after 6 months.

<table>
<thead>
<tr>
<th></th>
<th>Age years</th>
<th>Years since menopause</th>
<th>BMI kg/m²</th>
<th>E2 pmol/l</th>
<th>T nmol/l</th>
<th>fT pmol/l</th>
<th>Density increase Wolfe</th>
<th>Density increase percentage scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hofling III</td>
<td>54.7</td>
<td>4.9</td>
<td>25.1</td>
<td>74.5</td>
<td>0.5</td>
<td>8.2</td>
<td>18%</td>
<td>29%</td>
</tr>
<tr>
<td>Lundström et al 2002</td>
<td>57.1</td>
<td>8.2</td>
<td>24.8</td>
<td>24.8</td>
<td>0.8</td>
<td>15.1</td>
<td>46%</td>
<td>50%</td>
</tr>
<tr>
<td>Conner et al 2004</td>
<td>56.2</td>
<td>5.6</td>
<td>25.6</td>
<td>61.8</td>
<td>0.9</td>
<td>16.0</td>
<td>45%</td>
<td>68%</td>
</tr>
</tbody>
</table>
baseline density and were classified as N1 in the studies of Lundström and Conner. This could be of considerable importance and may have reduced the ability to demonstrate a possible inhibition of density by testosterone addition.

Study III was based on the assumption that 40-60% of the women would respond with an upgrading of at least one class. For this assumption a total number of 80 patients was judged to be sufficient to demonstrate a significant difference between treatments at an α-error of 0.05 and a β-error of 0.1. In reality only 20-30% of the women in Study III showed an upgrading of class and thus the power of the study could have been insufficient.

Still in paper III, density both at baseline ($r_s -0.35; p<0.01$) and change during treatment ($r_s -0.28; p<0.01$) showed a negative association with free testosterone levels. Testosterone addition resulting in only moderate serum levels displayed a neutral effect on density. Thus the possibility remains that in women with a stronger response to HT or when given higher doses of testosterone an inhibitory effect of testosterone could be demonstrated.

**Percentage of women**

![Graph showing percentage of women across different density categories](image)

**Figure 14.** Comparative data on mammographic density according to Wolfe at baseline in three clinical studies.

**Effects on breast symptoms**

Breast symptoms of soreness and pain are well known to occur in some women during estrogen/progestogen treatment. These symptoms are probably related to a remodelling of breast tissue that is also associated with a change in mammographic density. As illustrated in Table VI, breast symptoms were significantly increased during treatment (paper III). The highest symptom scores were reported already at two months. Breast symptoms showed positive associations with both absolute values for digitized mammography at six months ($r_s 0.29; p<0.01$) and for the increase in density during treatment ($r_s 0.34; p<0.01$). Thereafter symptoms showed a gradual decline. The reduction of symptoms after four and six months was highly significant, but still symptoms were increased as compared to baseline. Previously new-onset breast symptoms were found to be associated with a stronger increase in digitized density during 12 months of HT (Crandall et al., 2006).
Although numerical values for the breast symptoms score were somewhat lower in the testosterone group there were no significant differences between treatments. The findings are in good accordance with clinical observations. After initiation of hormonal therapy, patients frequently report symptoms during the first two or three months of treatment. Symptoms then tend to decrease or at least be tolerated by the patient.

Table VI. Breast symptoms score before and after treatment with E2/NETA in combination with the testosterone patch or a placebo patch.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>2 months</th>
<th>4 months</th>
<th>6 months</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E2/NETA+Testosterone</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean ± SD</td>
<td>0.4±1.2</td>
<td>8.1±9.7</td>
<td>4.5±6.1</td>
<td>3.6±6.2</td>
</tr>
<tr>
<td>median</td>
<td>0</td>
<td>5</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>range</td>
<td>0-6</td>
<td>0-37</td>
<td>0-22</td>
<td>0-30</td>
</tr>
<tr>
<td><strong>E2/NETA+Placebo</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean ± SD</td>
<td>0.3±1.3</td>
<td>9.4±13</td>
<td>8.6±12</td>
<td>4.9±9.1</td>
</tr>
<tr>
<td>median</td>
<td>0</td>
<td>5</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>range</td>
<td>0-8</td>
<td>0-50</td>
<td>0-50</td>
<td>0-50</td>
</tr>
</tbody>
</table>

ANDROGEN RECEPTOR AND SYNDECAN-1 DURING DIFFERENT LONG-TERM TREATMENTS

Information on androgen receptor (AR) content in normal and malignant breast tissue is limited. Divergent effects concerning androgens and AR in the regulation of proliferation in breast epithelial cells and breast cancer have been reported from both \textit{in vitro} and \textit{in vivo} studies (Birell et al., 1995; Kollara et al., 2001).

Proteoglycans may be regarded as multireceptor molecules which promote the integration of cellular signals (Delehedde et al., 2001; Alowami et al., 2003). Syndecan-1 is a cell surface heparansulfate proteoglycan which participates in cell proliferation, cell migration and cell matrix interaction (Roskelley et al., 1995; Barbareschi et al., 2003; Beauvais et al., 2004). An increased expression of Syndecan-1 was found in the stroma of invasive breast cancer (Stanley et al., 1999, Levionen et al., 2004; Maeda et al., 2004).

In paper IV, we analysed the expression of the AR and of Syndecan-1 in breast tissue from surgically postmenopausal macaques after long term treatment with either estrogen alone, estrogen in combination with progestogen or tibolone. After two years of treatment we found apparent differences between treatments and in particular between CEE/MPA and tibolone, and between these two treatments and estrogen alone (Figure 15).

In the CEE/MPA group there was a suppression of AR expression and a concomittant increase in Syndecan-1. There are reports that low or absent levels of AR are characteristic of malignant breast tumor tissue (Isola et al., 1993; Shan et al., 2000). Mammographic density is an established risk factor for breast cancer and was found to be more pronounced among women with AR dysfunction and multiple CAG repeats (Lillie et al., 2004). Also AR dysfunction has been reported in some men with breast cancer (Lobaccaro et al., 1993). Recently a genetic linkage was suggested...
between AR dysfunction and BRCA-1 mutations (Haiman et al., 2002). It is tempting to speculate that AR suppression from CEE/MPA treatment could be associated with an increase in breast cell proliferation and eventually cancer risk.

Likewise, the increase in stromal content of Syndecan-1 might be regarded as an adverse effect from CEE/MPA. Previously an increase of Syndecan-1 and a redistribution from epithelium to stroma was suggested to be a characteristic feature of mammographically dense breast tissue (Lundström et al., 2006). Treatment with CEE/MPA is well known to increase breast density which in itself is a risk factor for breast cancer. In malignant breast stroma the amounts of Syndecan-1 were more than ten-fold higher than in normal tissue from the same breast and patient (Löfgren et al., 2007). Whereas epithelial staining for Syndecan-1 was quite similar between groups, the amounts of this proteoglycan were apparently increased in stromal tissue after treatment with both tibolone and CEE/MPA.

Figure 15. Immunohistochemical staining for AR and Syndecan-1 in control group (C) and after treatment with CEE, CEE/MPA (=COMB) and tibolone.
A reactive stromal response seems to be a characteristic of infiltrating carcinomas. There is evidence that stromal fibroblasts promote tumor development and growth. Syndecan-1 is believed to act as a co-receptor for growth factors and extra cellular matrix interactions. Previously an increase of Syndecan-1 expression has been demonstrated in reactive stromal cells (Maeda et al., 2004). Alterations in Syndecan expression may have a dramatic effect on breast epithelial cells. Accumulation of Syndecan-1 within the stroma has been suggested to enhance angiogenesis and stimulate epithelial proliferation (Stanley et al., 1999; Leivonen et al., 2004; Maeda et al., 2004).

After treatment with tibolone AR levels were markedly increased and around ten-fold higher than after CEE/MPA. On the other hand, the effects on Syndecan-1 expression were quite similar. Currently the association between tibolone treatment and breast cancer risk is uncertain and controversial. Data from surrogate markers like breast cell proliferation and mammographic density clearly suggest that tibolone implies less stimulation of the breast than traditional estrogen/progestogen therapy. The effect on these markers are more similar to those of treatment with estrogen-alone (Conner 2007). In the Million Women Study, the risk estimate for breast cancer from tibolone was clearly lower than for estrogen/progestogen combinations, but still significant and numerically higher than for estrogen alone (Beral et al., 2003). On the other hand in a recent large case control study of a cohort of postmenpausal women from the UKs General Practice Research Database there was no increase in breast cancer risk from tibolone (Opatrny et al., 2008).

Also in contrast to the Million Women Study the GPRD material in agreement with many other reports showed that there was little risk association for treatment with estrogen-only. Here in paper IV, unlike the findings for CEE/MPA and tibolone, treatment with CEE alone displayed a seemingly neutral effect. Values for both AR and Syndecan-1 expression did not differ from those in untreated monkeys. This is in good accordance with observations that estrogen alone excerts only little stimulation on breast cell proliferation and mammographic density. Apart from its many beneficial health effects, the breast safety data for estrogen-alone are quite reassuring (Table II, Table III). The WHI not only confirmed observational data of a different risk between unopposed and combined estrogen, but also reported a significant 30 % risk reduction among women adherent to estrogen-only treatment (Stefanick et al., 2006). Certainly, the finding to suggest that long-term treatment with estrogen should not imply an increase in risk, but rather a protective influence must be interpreted with caution. Even in the large WHI trial, there were only 94 exposed cases. Also, in a report from the Nurses Health Study cohort it was suggested that very long-term treatment might in fact imply an increased risk also from estrogen-only to become significant after some 15 years or more of treatment (Chen et al., 2006). Still, it seems clear that the breast cancer risk from estrogen-alone, if any, is much less of a clinical problem than for the combined treatment.

In paper IV, we found alternative regimens for hormonal therapy to differ in their influence on two markers of possible importance for the development of breast cancer. These results may be relevant for the ongoing clinical discussion on the long-term safety of different hormonal treatments.
A CRITICAL ASSESSMENT AND FUTURE PROSPECTS

The present results concerning some hormonal effects on the breast were based on a limited human and experimental animal material and should be interpreted with caution. The regulation of the normal breast is complex and dependent on a variety of hormones, proteins and growth factors that interact with the epithelium and neighbouring stroma. During normal reproductive life events such as puberty, growth, pregnancy and postmenopausal involution there is a marked individual variation in breast tissue differentiation and sensitivity to exogenous hormonal treatment (Reid et al., 1996; Russo et al., 2005(a)). This is further influenced by genetic predisposition, body constitution, lifestyle factors and different pharmaceutical treatments. Thus, to draw general conclusions on the effect of hormonal treatments on the breast is difficult.

The fine needle aspiration (FNA) biopsy technique is currently a routine method for evaluating suspicious lumps in the breast as well as in other locations in the body. Here the method was used for the assessment of breast cells from the normal postmenopausal breast which in general has a very low cellularity due to involution. Although most FNA samples contained several hundreds of cells to be counted, a cut-off level of 40 cells per slide for representative samples was used. Assessable samples were achieved in 75% of the biopsies. This limited the number of women with two assessable samples for comparison of treatment effects to 57% of the subjects who concluded the study, hereby reducing the statistical power. There was also a considerable intra and inter individual variation in response. However the results in paper II for the proliferative breast response in women on E2/NETA correspond well with previous findings in women using the same treatment (Conner et al., 2003, Conner et al., 2004).

Despite its limitations the FNA biopsy is well tolerated by the volunteer women in studies and they can be subject to repeated biopsies. Multiple and repeated samples from the same woman would most certainly increase the number of assessable samples which would have increased the power of the study. The use of a larger core needle biopsy would possibly have given a better yield of cells, but the risk of vessel injury and development of hematomas could also have limited the recruitment of women for repeated biopsies.

All biopsies were performed from the upper outer quadrant of the left breast. This area was chosen due to an on average somewhat higher amount of breast epithelium and also a higher cancer incidence in this location (Donegan, 1995). Clearly, there may be regional differences in the breast concerning the proliferative response to treatment. However, in a previous study, proliferation and receptor content was assessed in ten different locations of the macaque breast in order to ascertain any regional differences in response to treatment. No significant differences were seen (Cline et al., 1997).

In this thesis our conclusions are based on changes in cell proliferation. An increase in proliferation increases the risk of a transformed cell to develop into a malignancy, and also for spontaneous new mutations to occur (Hesch & Kenemans 1999). In addition to assessment of proliferation, it would have been appropriate to include markers for apoptosis in order to settle a relationship, or a net effect between proliferation and apoptosis.

Quantification of mammographic breast density was performed using the Wolfe and a percentage visual classification scale. Both of these methods require a 20-25% increase in density in order to fulfil the criteria for an upgrading of one class. Visual classification scales are subjective methods. In order to objectivize the measurements, all mammograms were coded and read by two experienced, independent radiologists blinded for treatment. In addition to visual judgment and classification, digitized assessment of mammographic breast density was performed. This method of measurement has been shown to give highly reproducible results, and is more sensitive to small changes in density than the rather crude visual classification scales.
The use of an animal model can always be questioned concerning its relevance in a human situation. Here we used the well established macaque model with documented similarities to humans in terms of reproductive physiology. The macaque model has previously been used extensively in cardiovascular research. Experimental findings in macaques have also been confirmed in human studies (Cline et al., 1996; Cline et al., 1998; Cline et al., 2002). It is noteworthy that the monkeys were oophorectomized i.e. surgically menopausal which results in a different androgen/estrogen balance than natural menopause. This may well have affected the results of the expression of androgen receptor in paper IV and must be taken in consideration when interpreting the data. Also, previous findings on the effect of tibolone in women were from naturally menopausal subjects.

**Immunohistochemistry** was performed for AR and Syndecan-1 expression in breast tissue. The scoring of staining intensity is subjective and semiquantitative so findings may vary between investigators. Therefore all readings were done by two investigators and strict blinding was maintained. There was good concordance between the two investigators readings. For the few slides with different scorings, a second reading was performed by the two investigators and a consensus score was settled. The visual scoring for the androgen receptor was supported by a quantitative digitized image analysis.

**Future prospects**

From a clinical perspective increased breast cell proliferation and increased mammographic density during hormonal treatment should be regarded as unwanted and potentially hazardous adverse events. The effects of different doses, routes of administration and types of estrogen and progestogen as well as principles to counteract the stimulatory effect of HT should be further explored.

There is a strong interindividual variation in sensitivity for treatment and different treatments may cause markedly different effects in the same individual. In the future, efforts should be made to identify the responders, i.e. those women who respond strongly to hormonal treatments. The breast is a functional unit of many cell types and breast tissue homeostasis should be further explored. During hormonal treatment the given doses and resulting serum levels may not reflect tissue concentrations and receptor expression in the breast. There is a substantial conversion of hormones at the tissue level as well as paracrine action of hormones in the breast. For evaluation of the estrogen/androgen balance in breast tissue, measurement of testosterone metabolites may give a more correct picture of treatment effects (Labrie et al., 2006).

Androgen receptor polymorphism may be an underlying cause to variation in response and sensitivity for treatment and should be further explored in particular for the comparison of responders and non-responders.

In the case of testosterone, studies on the effect of testosterone alone or in combination with estrogen alone in hysterectomized women can further reveal whether androgens may serve as modulators of estrogen action on the breast. Possibly hormonal treatment in the future will comprise not only substitution of estrogen but also of testosterone for those women who could benefit from such treatment. This could be of great importance, increase quality of life and result in a treatment with an adequate estrogen/androgen balance resembling the premenopausal status of the woman. Adequate hormonal substitution is of importance especially for younger women after oophorectomy and women who suffer from premature ovarian failure. Not only type of treatment and doses, but also the importance of timing of intervention as well as duration of treatment must be further explored in prospective, randomized studies. The aim should be to reveal underlying mechanisms at the tissue level, and to evaluate not only effects on breast cancer risk but also possible beneficial health effects of hormonal treatments.
GENERAL CONCLUSIONS

This clinical and experimental study has shown that:

- Tibolone and E2/NETA represent two alternative regimens for menopausal hormone therapy. These two treatments cause distinct differences in estrogen/androgen status and blood levels of possible breast mitogens. Estrogens are markedly increased and androgens decreased by E2/NETA. In contrast, tibolone has only little influence on circulating estrogens while levels of androgens are increased. These findings may be relevant for the reported differences in breast cell proliferation and cancer risk between tibolone and E2/NETA.

- Mammographic breast density has emerged as a strong and independent risk factor for breast cancer. In the individual woman density is negatively correlated to age and BMI and positively to SHBG. During treatment with tibolone there is an increase in free testosterone. After treatment there is a negative association between levels of free testosterone and mammographic density. This could be one mechanism to explain why tibolone has less influence on the breast than continuous combined HT.

- There is increasing interest in testosterone treatment in postmenopausal women. Data on testosterone effects on the breast is scarce and interpretation is complicated by incoherent testosterone measurements. Addition of testosterone may counteract breast cell proliferation as induced by continuous combined estrogen/progestogen therapy. The apparent inhibition occurs in both epithelial and stromal cells. The effects of testosterone alone on breast cell proliferation and apoptosis require further study.

- The effects of testosterone on mammographic breast density will need further clarification. The present results suggest however that the addition of testosterone to a common estrogen/progestogen regimen will not further increase mammographic breast density. Whether testosterone alone or given in higher doses could even reduce this well known risk factor for breast cancer is still unclear.

- According to clinical experience a significant proportion of women may experience breast symptoms during hormonal treatment. Breast symptoms of soreness and pain were found to be most intense after two months of hormonal treatment and thereafter to decline. These common symptoms are associated with an increase in mammographic density.

- In a well established experimental monkey model, alternative regimens for hormonal therapy displayed apparent differences in their influence on two markers of possible importance for the development of breast cancer. AR expression was strongly increased by tibolone and suppressed by CEE/MPA. Syndecan-1 is a proteoglycan and believed to act as a co-receptor for growth factors and extra cellular matrix interactions. Both treatments increased Syndecan-1 in breast stromal tissue. In this model CEE alone had a neutral effect which did not differ from placebo. These experimental findings may be relevant for the ongoing clinical discussion on the long-term safety of different hormonal treatments.
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Det som var stort

Det som var stort visade sig litet.
Kungadömen bleknade som översnöad brons.
Det som bländade bländar inte mer.
Himlakroppar rullar och lyser.
Utsträckt i gräset vid stranden av en flod,
Som då, som då, släpper jag iväg barkbåtar.

Czeslaw Milosz
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