Hormonal Regulation of Vaginal Mucosa

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Abstract

Vaginal atrophy symptoms such as dryness, irritation, and itching, are common after menopause. Vaginal estrogen therapy is the most effective treatment but not appropriate for all women. Women with estrogen-responsive breast cancer treated with aromatase inhibitor (AI) treatment, suppressing estrogen levels, often suffer from more pronounced vaginal atrophy symptoms. However, vaginal estrogen treatment is not recommended, leaving them without effective treatment options. The aim of this thesis was to study the effect of long-term anti-estrogen therapy on circulating estrogen levels and biochemical factors in vaginal mucosa in relation to morphological changes and clinical signs of vaginal atrophy.

Circulating estrogen levels were analyzed by use of mass spectrometry and radioimmunoassay. Immunohistochemistry was used to study vaginal proliferation and steroid hormone receptors in vaginal mucosa. Vaginal gene expression was studied by use of microarray technology and bioinformatic tools, and validated by use of quantitative real-time PCR and immunohistochemistry. An estrogenic regulation of aquaporins and a possible role in vaginal dryness was investigated in vaginal mucosa and in Vk2E6E7 cells.

Aromatase inhibitor-treated women had higher than expected estradiol and estrone levels but still significantly lower than other postmenopausal women. Aromatase was detected in vaginal tissue, the slightly stronger staining in vaginal mucosa from AI-treated women, suggest a local inhibition of vaginal aromatase in addition to the systemic suppression. Vaginal mucosa from AI-treated women had weak progesterone receptor, and strong androgen receptor staining intensity. Low estrogen levels lead to low expression of genes involved in cell adhesion, proliferation, and differentiation as well as weak aquaporin 3 protein immunostaining.

The higher than expected estrogen levels in AI-treated women suggest that estrogen levels might previously have been underestimated. Systemic estrogen suppression by treatment with AIs, and possibly also by local inhibition of vaginal aromatase, results in reduced cell adhesion, proliferation, differentiation, and weak aquaporin 3 protein staining. Low proliferation and poor differentiation leads to fewer and less differentiated superficial cells affecting epithelial function and possibly also causing vaginal symptoms. Aquaporin 3 with a possible role in vaginal dryness, cell proliferation, and differentiation should be further explored for the development of non-hormonal treatment options for vaginal symptoms.

Keywords: aromatase inhibitors, vaginal atrophy, estrogen, proliferation, steroid hormone receptors, cell differentiation, cell adhesion, aquaporin 3

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To my loved ones
List of Papers

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


*Authors contributed equally to the work*

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Abbreviations

AI Aromatase inhibitor
ALOX12 Arachidonate 12-Lipoxygenase
AQP Aquaporin
AR Androgen receptor
BC Breast cancer
BMI Body mass index
cDNA Complementary deoxyribonucleic acid
CHST9 Carbohydrate sulfotransferase 9
DAB 3,3-diaminobenzidine
DAVID Database for Annotation, Visualization, and Integrated Discovery
DHEA Dehydroepiandrosterone
DSC2 Desmocollin 2
DSG1 Desmoglein 1
ER α Estrogen receptor alpha
ER β Estrogen receptor beta
ERR Estrogen related receptor
ET Estrogen therapy
FLG Filaggrin
FSH Follicle stimulating hormone
GC-MS/MS Gas chromatography tandem mass spectrometry
GSTM1 Glutathione S-transferase mu 1
IPA Ingenuity Pathway Analysis
IVL Involucrin
JUP Junction plakoglobin
KRT8 Keratin 8
LC-MS/MS Liquid chromatography tandem mass spectrometry
LH Luteinizing hormone
mRNA Messenger Ribonucleic acid
PBS Phosphate-buffered saline
PCDH8 Protocadherin 8
PR A Progesterone receptor A
PR B Progesterone receptor B
qRT-PCR Quantitative real-time polymerase chain reaction
RIA Radioimmunoassay
SERM Selective estrogen receptor modulator
<table>
<thead>
<tr>
<th>Acronym</th>
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<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
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<tr>
<td>VMI</td>
<td>Vaginal maturation index</td>
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Introduction

About 40% of postmenopausal women experience vaginal atrophy symptoms caused by low estrogen levels. Common symptoms are vaginal dryness, itching, irritation, and pain during intercourse, all affecting the quality of life [1]. Estrogens are important for maintaining a healthy vaginal mucosa. Low estrogen levels caused either by the menopausal transition or by anti-estrogen therapy have negative effects on the vaginal mucosa [2]. Treatment by way of vaginal estrogen therapy (ET) offers healthy postmenopausal women great relief from vaginal atrophy symptoms. Not all women, however, are eligible for vaginal ET [2]. Postmenopausal women with estrogen-responsive breast cancer (BC) treated with aromatase inhibitor (AI) treatment are a particularly vulnerable group of women. Such treatment suppresses estrogen production and results in extremely low estrogen levels [3], with subsequent vaginal atrophy symptoms and sexual dysfunction [4-5]. Their treatment options are limited because vaginal ET is considered contra-indicated [2] and other options are not sufficient. Therefore, new non-estrogen treatments for vaginal atrophy symptoms are urgently needed. This requires more knowledge about underlying mechanisms of vaginal atrophy symptoms caused by estrogen deprivation. Hence, the overall aims of the current work were to study the effects of long-term anti-estrogen therapy on circulating estrogen levels and biochemical factors in the vaginal mucosa in relation to morphological changes and clinical signs of vaginal atrophy.

Estrogens and other sex steroid hormones

Sex steroid hormones includes three different classes; estrogens, progestins, and androgens. All of these hormones are synthesized from cholesterol by reactions controlled by enzymes. Cholesterol can be synthesized in situ from acetate or obtained from the blood system [6].

Estrogens are important for the development and function of many different tissues, for instance the reproductive system, breast tissue, the nervous system, and the cardiovascular system. There are three major estrogens; estrone, estradiol, and estriol, which are all synthesized from androgens via enzymatic aromatization [7]. Estrone and estradiol are synthesized from androstenedione and testosterone, respectively (Figure 1). Estriol is a less biologically active peripheral metabolite of estrone and estradiol. The pro-
duction of estrogens occurs in the ovaries until menopause, when depletion of ovarian follicles leads to a steady decline of estrogen production. Ovarian estrogen production is controlled by feedback systems involving follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which in turn are regulated by pulsatile release of gonadotropin-releasing hormone [6].

Figure 1. Steroidogenesis with special focus on the role of aromatase in estrogen production.

The decrease in estrogen levels after menopause and the structural similarities between estrogens and other sex steroid hormones have led to the development of highly sensitive and specific immunoassays and mass spectrometric methods for determination of hormone levels [8]. Immunoassays depend on an antibody-antigen interaction for measurement of hormone levels. In mass spectrometry the hormones are ionized, separated, and analyzed according to their mass-to-charge ratio [9].
Sex steroid hormone receptors

The effects of estrogens and other sex steroid hormones are mediated by steroid hormone receptors. The majority of them belong to the nuclear receptor super-family, which includes 18 receptors divided into class I and class II. Estrogen receptor alpha (ER α), estrogen receptor beta (ER β), progesterone receptor A (PR A), progesterone receptor B (PR B), and the androgen receptor (AR), all belong to the nuclear receptor class I family [10-11]. These receptors are inducible transcription factors. Sex steroid hormones diffuse through the cellular membrane and interact with the receptor proteins. This interaction induces conformational change and release of heat-shock proteins, which in turn reveals the nucleus translocation signal and the steroid hormone-receptor complex is translocated to the nucleus. The steroid hormone-receptor complex acts on DNA together with co-activators and/or co-repressors, influencing messenger RNA (mRNA) transcription and thereby affecting subsequent gene expression and cellular response [6, 10]. Steroid hormone receptors have high sequence homology and they all have a transactivational domain, a DNA-binding domain, and a ligand-binding domain [10-11].

The two ER subtypes, ER α and ER β are encoded by two different genes on different chromosomes but share DNA-binding domains interacting with estrogen-responsive elements. The transactivational and ligand-binding domains on the other hand, differ between the subtypes, resulting in different effects on gene expression [10] and binding affinities to different ligands [7]. Estrogen receptor β has several isoforms, e.g. ER β1, ER β2, and ER β5 resulting from alternative splicing and leading to truncated forms of the receptor [12]. In addition to the ER α and ER β subtypes, there are also orphan estrogen related-receptors (ERRs) named ERR α, ERR β, and ERR γ. The ERRs share some sequence homology with the ERs but they differ significantly in the ligand binding-domain and cannot interact with estradiol [13].

The two PR subtypes, in contrast to ER subtypes, are transcribed from the same gene. They are the result of transcription from different initiation sites in the promoter region and also from independent translational start sites. As a result, PR A differs from PR B only by being 156 amino acid residues shorter, this making it difficult to distinguish PR A from PR B by use of immunohistochemistry. Progesterone receptor A can act as an inhibitor of PR B and other steroid hormone receptors. The expression of PR A often leads to gene repression while PR B expression often leads to gene activation [10]. Both receptor subtypes are regulated by estrogens in humans [14] and the presence of PR is a marker of estrogen action [11].

Androgen receptor is activated by testosterone and by the more active form 5α-dihydrotestosterone, a reduced form of testosterone. The expression of AR is stabilized by binding to androgens [11]. There are two isoforms of AR, A and B, and also several splice variants. Androgen receptor B is the
full-lengths isoform and also the most intensely studied, whilst the AR A isoform and other splice variants are less well studied [15].

Steroid hormones can also act on membrane-bound receptors such as G-protein-coupled receptor 30, also called G-protein-coupled estrogen receptor (GPER), and progesterone receptor membrane component 1. The actions and roles of these receptors are less well studied, but they do contribute to the complex effect of steroid hormones on target tissues [16].

Menopause

Menopause is defined as the time period of 12 months after the last menstruation [17] and it usually occurs around 51 years of age [18]. It is associated with a drop in circulating estrogen levels [7]. Circulating FSH and LH levels, on the other hand, increase [6]. Circulating estradiol levels drop more than tenfold during the menopausal transition from approximately 800 pmol/L in premenopausal women to below 74 pmol/L in postmenopausal women [7]. A number of studies reviewed by Santen in 2014 revealed median circulating postmenopausal estradiol levels to be between 11 to above 110 pmol/L. This discrepancy is most likely to be a result of differences in sensitivity and specificity between analysis methods [19]. Postmenopausal estradiol and estrone levels vary between immunoassays and mass spectrometry methods [19-21]. Measurement of low estrogen levels is still a challenge and more methodological studies are needed [22].

Estrogen production in postmenopausal women occurs in tissues expressing aromatase, e.g. breast, mesenchymal cells in adipose tissue, and in osteoblasts and chondrocytes in bone. This production is dependent on the presence of circulating androgens as precursors [7, 23]. Local production of steroid hormones in peripheral tissues (intracrinology) is of great importance for local effects, with limited effects on systemic hormone levels [24]. Circulating estrogen levels are often low in postmenopausal women but local levels in tissues such as the breast are often higher and more important as regards biological effects [23]. Estrone is the major estrogen after menopause [7].

Low estrogen levels are associated with several menopausal symptoms, such as hot flushes, cold sweats, and vaginal atrophy symptoms [17]. Vasomotor symptoms are most likely to subside over time but vaginal symptoms usually progress over time and seldom resolve without treatment [2].

The vagina

The vagina extends from the vulva to the uterine cervix in the female genital tract. The vaginal wall is composed of vaginal mucosa, lamina propria, also
called stroma, a smooth muscle layer, and an adventitia layer, all controlled by estrogens [25]. Steroid hormone receptors are expressed in the vaginal wall and the highest staining intensity is found in the vaginal mucosa [26]. Estrogen levels influence steroid hormone receptor expression in vaginal tissue [27-30]. Vaginal ER α and ER β mRNA expression is low in post-menopausal women, when circulating estrogen levels are low [27-28]. No difference in ER protein staining intensity has been observed, however, suggesting that vaginal mucosa is receptive to estrogen irrespective of estrogen status [29]. Progesterone receptor A+B protein staining is strong in vaginal mucosa when estrogen levels are high [29], while AR protein staining is weak [30].

Aromatase mRNA has been observed in vaginal tissue [30-31] and the presence of aromatase protein has been investigated by way of immunohistochemistry in vaginal tissue from cynomolgus monkeys [31]. The local production of steroid hormones in peripheral tissues [24] and the expression of vaginal aromatase might suggest that there is aromatase activity in vaginal tissue which may affect the expression of steroid hormone receptors.

The vaginal mucosa, a non-keratinized stratified squamous epithelium, can be subdivided into three layers; the basal, intermediate, and superficial epithelial layer (Figure 2) [32-33]. It undergoes continuous renewal through proliferation, differentiation, and maturation [25, 32]. The specific mechanism behind vaginal epithelial cell differentiation and maturation is still unclear [32], but it has been shown to be regulated by estrogens [34]. Stromal ER α expression is important for vaginal cell proliferation, whilst both stromal and epithelial ER α expression are imperative for proper cell differentiation and maturation [34]. The intermediate and superficial layers vary in thickness [32-33] and this is much dependent on proliferation [35-36]. High-level proliferation is reflected in high presence of the proliferation marker Ki67 coinciding with high serum estrogen levels [36].

The vaginal mucosa is moist and important for barrier function, secretion, and prevention of water loss. Glycogen is produced in the vaginal mucosa, under the influence of estrogens, and is metabolized by vaginal microflora generating a low vaginal pH. Maintenance of a low vaginal pH is important for protection against pathogens [25]. Intercellular adhesion is also important for barrier function [37] as well as for a functioning epithelium [25]. Components of intercellular adhesion such as desmosomes, adherence junctions, and tight junctions are most common in the basal and intermediate epithelial layers and diminishes in the superficial epithelial layer [37].

With sexual arousal, the central nervous system, and genital and extra-genital mechanisms come into play with vaginal lubrication as the end point [33]. Vaginal lubricant is a mixture of fluid resulting from transudation from blood vessels in vaginal tissue, influenced by cell permeability, and cervical mucus. Estrogen affects the maintenance of vaginal smooth muscle bundle density, blood vessels, and nerve ending morphology and density [33].
Estrogens also affect genes involved in the immune response and many other factors involved in maintaining a healthy condition and moisture in the vaginal mucosa [38].

![Figure 2. Vaginal tissue section stained with Mayer’s hematoxylin. The vaginal mucosa lies on the stroma and consist of a basal, intermediate and superficial epithelial layer.](image)

**Vaginal atrophy**

Atrophy occurs in vaginal mucosa during the menopause. Vaginal symptoms associated with estrogen deprivation during the menopause include vaginal dryness, irritation, itching, and dyspareunia (pain during intercourse). They all may have a negative impact on quality of life [2] through emotional and physical effects on postmenopausal women and their partners [39].

The clinical appearance of an atrophic vaginal wall is thin, pale, dry and occasionally inflamed. The occurrence of vaginal symptoms after menopause varies between postmenopausal women [2]. Low estrogen levels also lead to low glycogen content [32] and changes in microflora, ultimately increasing the vaginal pH [33]. The decrease of estrogen associated with menopause is followed by morphological and cytological changes. The mucosa becomes thinner, with fewer intermediate and superficial cells. The ratio between basal, intermediate, and superficial cells in the vaginal mucosa changes, fewer superficial cells and more basal and intermediate cells are...
observed [35, 40]. Cytology and calculation of the vaginal maturation index (VMI), i.e. the percentage of basal/intermediate/superficial cells, in the vaginal mucosa or determination of vaginal pH are the most appropriate assessments of vaginal atrophy [35].

Treatment of vaginal symptoms

The main purpose of vaginal atrophy treatment is to relieve symptoms and to reverse atrophic changes. Clinical challenges include the limited knowledge of vaginal symptoms, hesitancy to discuss symptoms with health practitioners, safety concerns, and insufficient relief of symptoms by way of available treatment options [1].

Systemic estrogen replacement therapy can be used for healthy postmenopausal women who, in addition to vaginal symptoms, also experience vasomotor symptoms [2, 41]. However, when vaginal symptoms are the main complaint, locally administered vaginal ET is the recommended treatment option [2]. Vaginal ET (estriol or estradiol) administered via cream, a vaginal ring, or vaginal tablets has proven sufficient in alleviating vaginal symptoms, with limited effects associated with systemic ET [2, 42].

The effect on circulating estrogen levels of vaginally administered estrogens is dependent on several factors (reviewed by Santen (2014) [19]). The analysis method, time point of analysis, delivery system, dose, and the condition of the vaginal mucosa all affect the systemic estradiol levels observed. Low-dose vaginal estradiol (a vaginal ring releasing 7.5 μg per day or a 10 μg vaginal tablet), does increase estradiol levels, but not above the normal menopausal range. Treatment with an intermediate dose (a 25 μg vaginal estradiol tablet) however, often results in estradiol levels closer to or above 74 pmol/L. High-dose treatment (50 μg or more) leads to estradiol concentrations close to premenopausal levels [19].

In a systematic review of clinical trials on vaginal ET it was concluded that the treatment results in symptom relief and positive effects on vaginal cytology and pH, with no statistical difference between administration routes [42]. However, notably, the low dose 10 μg vaginal estradiol tablet was not included in this review. Positive effects on vaginal symptoms and atrophy parameters with limited systemic effects have been observed with the 10 μg vaginal estradiol tablet, and it is currently the only dose available on the market [2, 19]. Safety concerns concerning vaginal ET include endometrial hyperplasia, breast cancer, and venous thromboembolism [2]. Endometrial hyperplasia was rare with the previously used intermediate dose of vaginal ET and current studies on low-dose treatments show no effect on endometrial hyperplasia, but long-term studies are needed. The effects on breast cancer and venous thromboembolism also need further investigation [2, 19]. Vaginal ET creams and tablets are initially administered daily for one to two weeks and then once weekly in order to maintain the effect. Vaginal rings
are inserted and then have continuous release of estrogen. Positive effects should be observed within a few weeks; otherwise, conditions such as dermatitis, vulvodynia, and vaginismus should be investigated [2].

Non-hormonal therapies with vaginal moisturizers, lubricants, and oils together with regular sexual activity, are often recommended for vaginal symptoms [2]. However, treatment with vaginal moisturizers neither improves VMI [43] nor reduces vaginal pH [44], but can show some relief of mild symptoms without reversing atrophic changes [2].

Biochemical factors in the vagina

The biochemical milieu in the estrogen deficient vagina as a result of menopause or anti-estrogen treatment is not well studied and specific biochemical factors involved in the vaginal symptoms are not known.

The effect of systemic ET on vaginal gene expression in postmenopausal women has been studied by use of a microarray technique. The mRNA expression patterns of genes involved in cell proliferation, differentiation, apoptosis, and the pathogenic response were affected by systemic ET and associated with improvement of vaginal pH and VMI. The investigators, however, did not report any vaginal atrophy assessment or symptoms before or after treatment [38]. Vaginal symptoms do not always concur with measurement of vaginal pH and VMI [45] and systemic ET is not always adequate for vaginal symptom relief [2]. A study including women suffering from vaginal dryness has shown down-regulation of genes involved in epithelial structure and barrier function, whilst genes involved in inflammation were up-regulated [46]. The differential gene expression concurs with the clinical symptoms associated with vaginal atrophy caused by estrogen deprivation [2]. Estrogen levels, however, were not reported in this study [46] and it is therefore difficult to assess how these differences relate to them. In addition, neither of the above-mentioned studies [38, 46] were further validated by way of immunohistochemistry or western blots. Protein and mRNA expression does not always correspond [47]. Hence, the effect of estrogen deprivation on biochemical factors and the association with vaginal symptoms need further investigations.

Aquaporins

Members of the aquaporin (AQP) family are membrane-bound proteins involved in the transport of water and small solutes across plasma membranes. They facilitate the transport of water and sometimes glycerol in response to osmotic gradients created by transport of ions and solutes. This AQP-facilitated transport is important for epithelial fluid secretion [48]. Aquaporins have been suggested to be involved in vaginal lubrication and dryness [49-50].
So far, the AQP family has 13 members named AQP0 to AQP12, organized as tetramers in cell membranes. The monomeric units of AQPs are ~30 kDa and many of them have glycosylation sites in the extracellular entrance. The pore is amphipathic, containing both hydrophilic and hydrophobic properties, important for facilitating transport of water and glycerol and also preventing transport of larger molecules and protons [48].

Aquaporin 1 is expressed in blood vessels, and AQP2, AQP3, AQP5, and AQP6 are expressed in epithelial cells in vaginal tissue from premenopausal women [51]. The localization and expression of AQPs in vaginal tissue from postmenopausal women remains to be investigated. Studies performed on rodents have shown translocation of AQPs to the plasma membrane after pelvic nerve stimulation [49]. The expression of AQPs was also decreased in ovariectomized rodents and restored by estradiol treatment, suggesting possible estrogenic regulation of AQP expression and a role in vaginal lubrication [49-50]. The involvement of AQPs in the vaginal symptoms associated with estrogen deprivation remains to be elucidated.

A subset of AQPs called aquaglyceroporins, transport glycerol as well as water [48]. Glycerol is a water-retaining humectant important for skin hydration. One member of the aquaglyceroporins is AQP3. Low expression of AQP3 results in low glycerol content and dry skin, which can be reversed by glycerol via systemic or topical application [52]. Glycerol and water transport via AQP3 in addition to skin hydration, is also important for cell proliferation and wound healing [53]. Aquaporin 3 has also been suggested to be involved in epithelial cell differentiation in the epidermis via a negative feedback loop with the transmembrane protein Notch 1. The expression of AQP3 has been shown to decrease when Notch 1 increases in differentiated cells in the superficial layer in epidermis. The decrease of AQP3 coincided with an increase in differentiation markers [54]. Epithelial cell differentiation in the epidermis is affected by estrogens [55]. Vaginal epithelial cell differentiation is also strongly regulated by estrogens [32, 34]. The resemblance between skin epidermis and vaginal mucosa might imply a role for AQPs in vaginal lubrication and possibly also in cell differentiation.

Breast cancer

There are more than one million women diagnosed with BC yearly worldwide [56] and 7000 women are diagnosed each year in Sweden alone [57]. It is the most common female cancer [58], mainly affecting women after menopause. There is firm evidence that long-term estrogen exposure increases the risk of developing BC [58]. Roughly 80% of BC tumors diagnosed in postmenopausal women are ER-positive [56]. These tumors are estrogen-dependent which is the reason why these women benefit from adjuvant endocrine treatments; tamoxifen inhibiting ERs or AI treatment inhib-
iting estrogen synthesis. Both treatments are aimed at impeding the effect of estrogens, improving survival, and reducing BC recurrence [59].

Tamoxifen
Tamoxifen is a selective estrogen receptor modulator (SERM). It inhibits the ligand-binding domain in the ER and has antagonistic or agonistic effects depending on the tissue [6]. It has an antagonistic effect in breast tissue which is beneficial as regards reduction of BC recurrence and mortality in both pre- and postmenopausal women with ER-positive tumors [60]. Tamoxifen has been shown to have an agonistic effect in the vagina [61-62] and uterus [58], leading to increased risk of endometrial cancer as a result of an undesirable increase in endometrial proliferation [10].

Circulating estrogen levels are unchanged or elevated during treatment with tamoxifen [63-64]. Women treated with tamoxifen do, however, often experience vaginal discharge and dyspareunia [4-5, 65].

Aromatase inhibitors
The AIs currently available are either reversible non-steroid inhibitors such as anastrozole and letrozole or non-reversible steroid inhibitors such as exemestane [66]. All three are effective inhibitors of estrogen synthesis and improve disease-free survival of women with BC [59]. However, any possible increase in overall survival rate compared with tamoxifen-treated women has to our knowledge not yet been ascertained [56, 59]. Aromatase inhibitors are not recommended for premenopausal women, as the resulting estrogen deprivation may stimulate endogenous estrogen synthesis via feedback systems if the ovaries are left intact [67].

Effective inhibition of estrogen synthesis by AIs has been shown in several longitudinal studies, with undetectable estradiol levels or concentrations between 0.6–3.7 pmol/L after three months of treatment [3, 68]. However, there are to the best of my knowledge no studies in which estrogen levels have been investigated during long-term AI-treatment, or comparative studies involving healthy postmenopausal women. There are inter-individual differences in estrogen levels during anastrozole treatment, suggested to be caused by differences in BMI [69]. The effect of BMI on the outcome of AI treatment, however, is still inconclusive [70-71].

The depletion of estrogens during AI treatment is associated with increased risks of bone fractures, cardiovascular events, [72], and musculoskeletal and joint pain [73]. Women treated with AIs also have an increased prevalence of menopausal symptoms [4, 74-76]. According to previous results from our group, more than two-thirds of women treated with AIs experience moderate or severe vaginal dryness and dyspareunia [4]. Clinical evaluations of vaginal atrophy, vaginal pH, and VMI indicate more profound
vaginal atrophy in AI-treated women compared with tamoxifen-treated women and postmenopausal controls with or without vaginal ET [4]. Vaginal symptoms have negative effects on sexual function [5, 76] and often affect the quality of life [1-2].

The vaginal atrophy symptoms that are so common in AI-treated women would of course be alleviated by vaginal ET [2]. However, this is considered to be contraindicated for AI-treated women [77-79]. This precautionary attitude was at first based on the results of a small study in which serum estradiol levels were examined at 0, 2, 4, 7 and 12 weeks after initiation of vaginal estradiol tablet treatment in AI-treated women, where an initial increase in estradiol levels (to approximately 200 pmol/L) was noted [77]. Serum estradiol levels subsequently decreased over time to pre-treatment levels, presumably as a result of vaginal maturation [40, 77]. However, long-term vaginal ET treatment with a vaginal ring in AI-treated women also showed increasing estradiol levels [78]. The results of these studies strongly suggest that vaginal ET, with estradiol in particular, is not an option for AI-treated women [77-79]. Hence, most women who are on treatment with AIs and suffer from vaginal atrophy symptoms are prescribed various non-hormonal alternatives. While these preparations may be associated with some symptom relief, especially if symptoms are mild [2], they are, however, not adequate for improving VMI [43] or reducing vaginal pH in healthy postmenopausal women or women with BC not treated with AIs [44].

Given the increasing number of women living with BC, there is an urgent need for development of effective non-hormonal treatment options for vaginal atrophy. The effect of estrogen deprivation on biochemical factors and the association with vaginal symptoms needs further investigation.
Aims

The overall objective of the present work was to study the effect of long-term anti-estrogen therapy on circulating estrogen levels and biochemical factors in the vaginal mucosa in relation to morphological changes and clinical signs of vaginal atrophy.

Specific aims

I To measure circulating estrogens and to calculate the aromatase index in postmenopausal women with breast cancer on long-term aromatase inhibitor or tamoxifen treatment and to compare the results with those among postmenopausal women with and without vaginal estrogen therapy.

II To study cell proliferation and the presence and distribution of steroid hormone receptors in vaginal mucosa in relation to vaginal atrophy and estrogen levels in postmenopausal women with breast cancer treated with aromatase inhibitors or tamoxifen and in postmenopausal controls.

III To investigate gene expression in vaginal tissue from postmenopausal women treated with aromatase inhibitors and to compare with vaginal tissue from postmenopausal women treated with vaginal estrogen therapy.

IV To examine the effects of aromatase inhibitor treatment on the presence, expression, and distribution of aquaporins in the vagina in vivo and in vitro.
Materials and methods

Study population

This was a cross-sectional population-based study. The Swedish Cancer Registry, with a population-based register that covers more than 96% of all BC cases in Sweden, was used to identify eligible postmenopausal women with BC in the Uppsala/Örebro Region. Inclusion criteria were: postmenopausal women with BC in the Uppsala or Örebro Region, being between 55–70 years of age, and having ER-positive BC diagnosed between 2003–2006. Age-matched healthy postmenopausal controls were selected from the Population Registry and invited by letter to participate. Postmenopausal status was defined as 12 months since last menstruation. Women with BC with ongoing primary therapy, such as radiation therapy or chemotherapy, patients with recurrent cancer, as well as women with other cancers were excluded. In addition, all women with severe diseases, such as neurological or rheumatoid disorders, and women hospitalized with depression or psychosis that could interfere with the study outcomes were excluded. In addition, postmenopausal control women took no part if they had any prior breast cancer diagnosis. All participants gave written informed consent and the study was approved by the Regional Ethical Review Board, Uppsala, Sweden.

In all, 97 women with BC and 105 postmenopausal control women fulfilled the inclusion/exclusion criteria and accepted the invitation to participate in the study. All women attended a gynecologic and somatic health examination at the Department of Women’s and Children’s Health, Uppsala University Hospital, or the Department of Obstetrics and Gynecology, Örebro University Hospital, between November 2008 and March 2009. Breast cancer history, including previous and current adjuvant endocrine therapy, was obtained from the medical records and directly from the women by means of a questionnaire. Compliance with adjuvant endocrine therapy was requested (never missed/missed once a week/missed once a month). Data was collected on medical history, drug therapy, and previous and current hormonal treatment. Weight and height were measured in a standardized manner, and body mass index (BMI) was calculated. Blood samples were gathered for hormone analyses in serum and plasma and stored at -20°C.

Of the 97 women with BC included in the study, 19 with no endocrine adjuvant treatment and 11 using vaginal estradiol or estriol treatment were
excluded from the final analysis. Thirteen of the postmenopausal control women were on systemic hormone replacement therapy and were also excluded from the final analysis (Figure 3). The populations in each study are shown below. Paper I included all women with BC and controls fulfilling all inclusion and exclusion criteria described above and in more detail in Paper I. Papers II-IV were smaller studies including a limited number of women with BC and controls.

**Paper I**

The study population in Paper I included AI-treated women with BC (n=33), with the subgroups anastrozole/letrozole-treated women (n = 24) and exemestane-treated women (n = 9), postmenopausal tamoxifen-treated women with BC (n = 34), postmenopausal controls without treatment (n = 56), postmenopausal controls with vaginal estradiol (n = 25), and postmenopausal controls with vaginal estriol (n = 11).

**Figure 3.** Population investigated in Paper I. Out of the 97 postmenopausal women with breast cancer, 19 had no adjuvant endocrine therapy and 11 were using vaginal estrogen therapy and these 30 were therefore excluded. Thirteen of the postmenopausal controls were using systemic hormone replacement therapy and therefore excluded.

**Paper II**

The study population in Paper II consisted of four groups. Primary inclusion criteria were that women should be sexually active and have a vaginal biopsy sample available for immunohistochemical analysis. Among eligible women, a random selection was made. All women with BC had high-level
self-reported adjuvant endocrine compliance (never missed). The four study groups consisted of postmenopausal women with BC treated with AIs (n = 15) or tamoxifen (n = 16) and postmenopausal controls without treatment (n = 19) or with vaginal ET (estradiol or estriol) (n = 16).

**Papers III and IV**
In Papers III and IV, there were two groups in the study population. Aromatase inhibitor-treated women with BC, with high vaginal atrophy scores, were compared with postmenopausal women treated with vaginal ET, with low vaginal atrophy scores. Therefore, women with BC without adjuvant therapy, tamoxifen-treated women, AI-treated women on vaginal/systemic ET, and controls without vaginal ET or with systemic ET were excluded.

In Paper III, AI-treated women with BC (n = 28) and postmenopausal controls treated with vaginal ET (estradiol or estriol) (n = 35) were available for immunohistochemical analysis and included in the study. Out of these, RNA quality was sufficient for mRNA gene expression analysis for 15 AI-treated women and 22 controls, where four and five respectively, were selected for microarray analysis.

In Paper IV, AI-treated women with BC (n = 30) and postmenopausal controls treated with vaginal ET (estradiol or estriol) (n = 35) were available for immunohistochemical analysis and included. Out of these, RNA quality was sufficient for mRNA gene expression analysis for 13 AI-treated women and 18 controls.

**Vk2E6E7 vaginal epithelial cells**
Vk2E6E7 cells (ATCC CRL-2616) were used in Paper IV. They are a non-tumor epithelial cell line established from normal vaginal tissue [80]. The cells were provided as a kind gift from Helena Aro, Department of Genetics, Microbiology and Toxicology, Stockholm University. They were cultured in cell-culture vessels in keratinocyte serum free medium supplemented with recombinant epidermal growth factor, bovine pituitary extract, and calcium chloride. The cells were incubated with estradiol, anastrozole, or vehicle for 48h. The effects on cell viability and proliferation were studied by use of WST-1 Reagent and Cell Proliferation ELISA BrdU colorimetric, respectively.
Methods

Vaginal assessment and biopsy samples

The degree of vaginal atrophy was noted by assessing the vaginal epithelium according to thickness, color, and presence of petechial bleeding. A 4-grad scale was used for classification of atrophy (none, mild, moderate, and severe). Vaginal pH was measured with a paper indicator on the lateral vaginal wall. Vaginal punch biopsy samples were taken from the lateral upper part of the vagina and divided into three parts longitudinally. The three parts were either fixed in formalin for immunohistochemistry or placed in RNA-later reagent at -70 °C for use in quantitative real-time polymerase chain reaction (qRT-PCR) or snap frozen in liquid nitrogen.

Steroid hormone analysis

Extraction radioimmunoassay (RIA) performed at the Pediatric Growth Research Center, Sahlgrenska Academy, University of Gothenburg, was used for measuring serum estradiol concentrations. The limit of quantification (LOQ) was 4 pmol/L [81]. Liquid chromatography tandem mass spectrometry (LC-MS/MS), performed at the ARUP Institute for Clinical and Experimental Pathology, was used for determination of estradiol, estrone, estriol, androstenedione, 5α-dihydrotestosterone and testosterone. Limits of quantification were 3.7 pmol/L, 3.7 pmol/L, and 3.5 pmol/L for estradiol, estrone and estriol respectively, while the LOQs for androstenedione, 5α-dihydrotestosterone, and testosterone were 0.035 nmol/L, 0.017 nmol/L, and 0.035 nmol/L respectively [21, 82-84] when measured by LC-MS/MS.

RNA extraction and complementary DNA synthesis

Total RNA was isolated from vaginal biopsy samples by use of miRNeasy mini kits or the TRIzol method. RNeasy mini kits were used for RNA isolation from Vk2E6E7 cells. The RNA was stored at -70°C until required. RNA quality and integrity was determined using 2100 Agilent Bioanalyzer. All samples included in the study had a RNA integrity number (RIN) > 5. RNA concentrations were measured by use of Nanodrop equipment. Complementary DNA (cDNA) was synthesized by use of SuperScript III Reverse Transcriptase or SuperScript VILO Master Mix for RNA isolated from biopsy samples and cells, respectively.

Microarrays

Gene expression was studied by use of an Affymetrix Gene Chip Gene 1.0 ST Array system. The protocol is described in Paper III. Genes with an ad-
justed p-value of < 0.05 and an average log₂-fold change of at least 1 were considered differently expressed. Cluster analysis was performed by use of Genesis [85] to visualize differentially expressed genes. Functional analysis and gene ontology were studied by use of the Database for Annotation, Visualization, and Integrated Discovery (DAVID), with the whole genome as background [86]. Networks of differentially expressed genes were further analyzed by way of Ingenuity Pathway Analysis (IPA) and visualized with dendrograms.

Quantitative real time polymerase chain reaction

If not stated otherwise, all qRT-PCRs were performed according to manufacturer’s instructions. TaqMan Gene Expression assays (Table 1) were carried out with TaqMan Fast Universal PCR Master Mix using the following cycling conditions: an initial holding stage at 95 °C for 20 s followed by 55 amplification cycles of 95 °C for 1 s and 60 °C for 20 s using a StepOnePlus Real-time PCR system. Mean primer efficiency was calculated for each plate by use of LinRegPCR [87-88] and used for calculating the efficiency-adjusted cycle threshold (Ct) [89]. Fold change between groups was calculated by the comparative Ct method and normalized gene expression ($2^{-\Delta\Delta\text{Ct}}$) was used for statistical comparisons [90].

Table 1. TaqMan Gene Expression assays in quantitative real-time PCRs.

<table>
<thead>
<tr>
<th>Gene of interest</th>
<th>TaqMan Gene Expression Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arachidonate 12-Lipoxygenase</td>
<td>Hs00167524_m1</td>
</tr>
<tr>
<td>Desmoglein 1</td>
<td>Hs00355084_m1</td>
</tr>
<tr>
<td>Carbohydrate sulfotransferase 9</td>
<td>Hs01089074_m1</td>
</tr>
<tr>
<td>Glutathione S-transferase mu 1</td>
<td>Hs01683722_gh</td>
</tr>
<tr>
<td>Junction plakoglobin</td>
<td>Hs00158404_m1</td>
</tr>
<tr>
<td>Desmocollin 2</td>
<td>Hs00951428_m1</td>
</tr>
<tr>
<td>Keratin 8</td>
<td>Hs01595539_g1</td>
</tr>
<tr>
<td>Protocadherin 8</td>
<td>Hs04187285_g1</td>
</tr>
<tr>
<td>Aquaporin 3</td>
<td>Hs01105469_g1</td>
</tr>
<tr>
<td>Involucrin</td>
<td>Hs00846307_s1</td>
</tr>
<tr>
<td>18S</td>
<td>1319413E</td>
</tr>
</tbody>
</table>

Immunohistochemistry

Antibodies used for studying the presence, localization, distribution, and staining intensity of proteins in vaginal tissue are listed in Table 2.

Briefly, slides were de-paraffinized in xylene and rehydrated in decreasing concentrations of ethanol and in de-ionized water. The slides were finally washed in 1×Tris-buffered saline (TBS) or phosphate-buffered saline (PBS) pH 7.4, depending on the antibody used. Antigen retrieval was per-
formed by heating the tissue sections in citrate buffer. Tissue sections were blocked and then incubated with a primary antibody. Thereafter, the sections were incubated with the secondary biotinylated antibody and subsequent incubation with horseradish peroxidase avidin D was carried out, with repeated washings with 0.1% Tween 20 in TBS or 0.1% Tween 20 in PBS between incubations. Immunoreactivity was detected by use of a chromogen solution containing 3,3-diaminobenzidine (DAB). The sections were then counterstained by use of Mayer’s hematoxylin before being mounted with pertex.

Omission of the primary antibody was used as negative control. Staining intensities were evaluated without knowing the identities of the slides. The slides were graded on a scale of 0 = no staining, 1 = faint staining, 2 = moderate staining, and 3 = strong staining. The Ki67 index was calculated as the number of stained cells divided by the total number of cells in one vaginal tissue layer, using Axio Observer. Z1 Microscope.

Table 2. Primary antibodies used in immunohistochemistry and western blot.

<table>
<thead>
<tr>
<th>Protein of interest</th>
<th>Supplier</th>
<th>Method and dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrogen receptor α</td>
<td>M7047 Dako</td>
<td>IHC 1:300</td>
</tr>
<tr>
<td>Estrogen receptor β₁</td>
<td>MCA1974S AbD Serotec</td>
<td>IHC 1:100</td>
</tr>
<tr>
<td>Estrogen receptor β₂</td>
<td>MCA2279 AbD Serotec</td>
<td>IHC 1:200</td>
</tr>
<tr>
<td>Estrogen receptor β₅</td>
<td>MCA4676GA AbD Serotec</td>
<td>IHC 1:250</td>
</tr>
<tr>
<td>Progesterone receptor A+B</td>
<td>M3569 Dako</td>
<td>IHC 1:400</td>
</tr>
<tr>
<td>Progesterone receptor B</td>
<td>MA1-411 Thermo Scientific</td>
<td>IHC 1:12.5</td>
</tr>
<tr>
<td>Androgen receptor</td>
<td>M3562 Dako</td>
<td>IHC 1:50</td>
</tr>
<tr>
<td>Ki67</td>
<td>NA59 Oncogene Research Products</td>
<td>IHC 1:500</td>
</tr>
<tr>
<td>Arachidonate 12-Lipoxygenase</td>
<td>HPA010691 Sigma Aldrich</td>
<td>IHC 1:50</td>
</tr>
<tr>
<td>Junction plakoglobin</td>
<td>NCL-G-CAT Leica Biosystems</td>
<td>IHC 1:40</td>
</tr>
<tr>
<td>Aquaporin 1</td>
<td>ab9566 Abcam</td>
<td>IHC 1:100</td>
</tr>
<tr>
<td>Aquaporin 3</td>
<td>ab125045 Abcam</td>
<td>IHC 1:1000</td>
</tr>
<tr>
<td>Aquaporin 3</td>
<td>HPA014924 Atlas antibodies</td>
<td>WB 1:250</td>
</tr>
<tr>
<td>Involucin</td>
<td>HPA055211 Atlas antibodies</td>
<td>IHC 1:200</td>
</tr>
<tr>
<td>Involucin</td>
<td>ab53112</td>
<td>WB 1:300</td>
</tr>
<tr>
<td>Filaggrin</td>
<td>HPA030188 Atlas antibodies</td>
<td>IHC 1:2500</td>
</tr>
<tr>
<td>Beta actin</td>
<td>Sc-47778 Santa Cruz Biotechnology</td>
<td>WB 1:1000</td>
</tr>
</tbody>
</table>

Primary antibodies used in immunohistochemistry (IHC) and western blot (WB) in Papers II–IV.

**Western blot**

Proteins were isolated from Vk2E6E7 cells by use of RIPA buffer and used for western blots. The samples were separated and transferred to PVDF membrane by use of a Novex NuPAGE gel system. The membranes were blocked in Licor blocking buffer and then incubated with the primary antibodies (Table 2) at 4 °C over night. The membranes were thereafter incu-
bated with fluorescently labeled secondary antibodies and visualized by use of an Odyssey Infrared imaging device.

Statistics
Statistical analysis was performed by use of IBM SPSS 20 Statistics unless stated otherwise. Power calculation for Paper I was based on a publication by Geisler and colleagues [91]. Our study had 95% power to detect a difference in estradiol levels of 15 pmol/L between AI-treated women with BC and postmenopausal controls without ET treatment, if 10 subjects were included in each group [91]. In Paper II, power calculation was based on the difference in proliferation between postmenopausal women with or without vaginal ET [35]. Since there were no preliminary studies on vaginal proliferation in AI-treated women, we assumed that the AI-treated women would resemble postmenopausal women without vaginal ET. The study had 85% power to detect a difference in proliferation. Normally distributed data were analyzed by ANOVA with post hoc testing using Tukey’s honest significant difference (HSD) test, and skewed data by means of the Kruskal-Wallis test followed by the Mann-Whitney U-test if significant. Frequencies were compared by use of Chi square tests or Fisher’s exact test. Parametric correlations were tested according to Pearson’s correlation method and non-parametric correlations were tested according to Spearman’s rank correlation. Values of p < 0.05 were considered significant. Estradiol levels measured by extraction RIA and LC-MS/MS were compared by use of Deming regression [92]. Vaginal atrophy scores and vaginal dryness are presented as frequencies within each study group, and statistical analysis was performed by using both frequencies (atrophy/dryness: yes or no) and ordinal values (clinical atrophy scores or vaginal dryness scores).
Summary of results

Paper I

Estrogens were measured in women with BC treated with AIs or tamoxifen, and in postmenopausal controls without treatment or treated with vaginal estradiol or estriol. There were no significant differences in age, BMI, or time since menopause among the groups. Both adjuvant endocrine therapy in women with BC, and vaginal ET in postmenopausal control women had lasted for longer than 6 months. Self-reported compliance with AIs and tamoxifen treatment, with 96.7% women reporting never missing and 3.3% reporting missing no more than once a month was considered to be high.

Women treated with AIs had lower circulating estradiol and estrone levels compared with those in all other groups (p < 0.001) (Table 3). The differences remained when sub-grouping to anastrozole/letrozole-treated and exemestane-treated women when using LC-MS/MS, but not as regards estradiol levels measured by extraction RIA. In addition, there was a wider than expected range of estradiol and estrone levels in the AI-treated group of women. Estradiol levels were measured by use of both extraction RIA and LC-MS/MS and the two methods correlated well with each other (Figure 4).

Estrone levels were significantly higher in the tamoxifen-treated women with BC compared with those in all other groups (p < 0.05). Estriol levels were below LOQ (3.5 pmol/L) in all study groups.

The aromatase index, based on the estradiol/testosterone or the estrone/androstenedione ratio, was also lower in AI-treated women compared with those in all other groups. Tamoxifen-treated women had higher estrone/androstenedione ratios compared with control women without treatment (p < 0.05), as a result of higher estrone levels.
Table 3. Circulating estradiol and estrone levels and calculated aromatase index

<table>
<thead>
<tr>
<th></th>
<th>Postmenopausal women with breast cancer</th>
<th>Postmenopausal control women</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All AI (n=33)</td>
<td>Anastrozole /Letrozole (n=24)</td>
</tr>
<tr>
<td>Estradiol Extraction RIA</td>
<td>10.6a (2.3-182.0)</td>
<td>9.4a (2.3-182.0)</td>
</tr>
<tr>
<td>Estradiol LC-MS/MS</td>
<td>16.7a (2.4-162.6)</td>
<td>16.7a (2.4-162.6)</td>
</tr>
<tr>
<td>Estrone LC-MS/MS</td>
<td>17.0a (5.7-153.9)</td>
<td>17.3a (7.8-153.9)</td>
</tr>
<tr>
<td>Estradiol/Testosterone ratio</td>
<td>0.025c (0.007-0.232)</td>
<td>0.022a (0.007-0.232)</td>
</tr>
<tr>
<td>Estrone/Androstenedione ratio</td>
<td>0.010a (0.002-0.037)</td>
<td>0.010a (0.002-0.037)</td>
</tr>
</tbody>
</table>

Estradiol and estrone levels in pmol/L are displayed as median (range) measured by extraction radioimmunoassay (RIA) and liquid chromatography tandem mass spectrometry (LC-MS/MS). The calculated aromatase index is displayed as the median (range) of estradiol/testosterone or estrone/androstenedione ratios. Significant differences (p < 0.05) are indicated as follows: a) significantly lower than in all other groups, b) significantly higher than in postmenopausal controls without treatment, c) significantly higher than in postmenopausal women without treatment and estriol-treated women, d) significantly higher than in all other groups, e) significantly lower than in all control groups, f) significantly higher than in tamoxifen-treated women with breast cancer and postmenopausal controls without treatment.
Figure 4. Method comparison between extraction RIA and LC-MS/MS. Estradiol levels measured with extraction RIA and LC-MS/MS were compared and evaluated by using Deming regression. Resulting in a regression line corresponding to Extraction RIA = 1.022 × LC-MS/MS - 8.9, a correlation coefficient of $r = 0.879$, and a standard error of $S_{y/x} = 11.74$, showing significant correlation between the two methods ($p < 0.001$). The data is displayed using a Bland-Altman plot. The solid line shows the mean of the two methods and the dotted lines show the upper and lower limits of agreement.

Paper II

Vaginal cell proliferation and steroid hormone receptors were studied in vaginal tissue from women with BC treated with AIs or tamoxifen, and postmenopausal controls without or with vaginal ET.

A representative histological illustration of vaginal tissue from AI-treated women and control woman treated with vaginal ET is displayed in Figure 5. AI-treated women often had a thinner vaginal mucosa dominated by basal and intermediate cells. Control women generally had a thicker vaginal mucosa dominated by superficial cells.

Evaluation of clinical vaginal atrophy scores showed that AI-treated women more often showed signs of moderate and severe atrophy than both postmenopausal control groups ($p < 0.05$). In addition, AI-treated women had a higher vaginal pH compared with tamoxifen-treated women ($p = 0.001$) and postmenopausal controls treated with vaginal ET ($p = 0.001$). Tamoxifen-treated women displayed more moderate atrophy scores and a lower pH than postmenopausal controls without treatment ($p < 0.05$). Vagi-
nal atrophy scores and vaginal pH correlated negatively with circulating estrogen levels.

Figure 5. Representative immunohistochemical staining of the proliferation marker Ki67 and a demonstration of morphology in vaginal tissue from A) aromatase inhibitor-treated women with breast cancer and B) postmenopausal control women treated with vaginal estrogen therapy.

Proliferation in vaginal epithelium

Cell proliferation, i.e. the Ki67 index, was determined by immunohistochemistry and was seen as intense nuclear staining in the intermediate epithelial layer (Figure 5). Aromatase inhibitor-treated women showed low cell proliferation compared with tamoxifen-treated women (p = 0.002) and controls with vaginal ET (p = 0.037). Tamoxifen-treated women, on the other hand, showed greater cell proliferation compared with controls without treatment (p = 0.018).

Proliferation in the intermediate epithelial layer was negatively correlated with clinical vaginal atrophy evaluation scores and vaginal pH, while positively correlating with estrone levels.

Steroid hormone receptors

Immunohistochemical staining of steroid hormone receptors was nuclear and localized in vaginal epithelial and stromal cells. The most intense staining of ER α was found in the basal epithelial layer, whilst the ER β isoforms stained more intense in the stromal cells. The protein staining intensity of ERs did not, however, differ between study groups.

Progesterone receptor A+B staining was most intense in the basal and intermediate epithelial layers. Staining intensity of PR A+B was weaker in vaginal epithelium from AI-treated women compared with tamoxifen-treated women (p = 0.001) and control women with vaginal ET (p = 0.023), as shown in Figure 6. Tamoxifen-treated women showed stronger staining intensity in the vaginal epithelium than those in both control groups (p < 0.05) and also stronger staining intensity of PR B compared with women in all other groups (p < 0.05).
The strongest AR staining was found in the basal epithelial layer. Aromatase inhibitor-treated women showed stronger epithelial staining intensity compared with control women treated with vaginal ET (p < 0.05) shown in Figure 6.

Staining intensity of PR A+B correlated negatively with vaginal atrophy scores and positively with circulating estrone levels. The staining intensity of AR correlated positively with vaginal atrophy scores and vaginal pH but not with circulating hormone levels.

![Box plots](image)

**Figure 6.** Box plots, displaying the group median, minimum, and maximum of immunohistochemical staining intensity of A) progesterone receptor A+B and B) androgen receptor in vaginal epithelium from aromatase inhibitor-treated women with breast cancer, tamoxifen-treated women with breast cancer, and postmenopausal control women without or with vaginal estrogen therapy. Significant differences are indicated *p < 0.05, **p < 0.005, and ***p < 0.001.

**Paper III**

Vaginal gene expression was compared in AI-treated women and control women treated with vaginal ET by use of microarray technology. The results were validated in larger study groups by use of qRT-PCR and immunohistochemistry. The presence, localization and staining intensity of aromatase in vaginal tissue from postmenopausal women was also studied.

In total, 279 genes differed significantly between the two groups; 196 genes showed lower expression and 83 genes showed higher expression in vaginal tissue from AI-treated women compared with controls treated with vaginal ET. Genes with low expression in AI-treated women were overrepresented in connection with biological processes such as epidermis develop-
ment, keratinization, epithelial cell differentiation, and epidermal cell differentiation. They were also involved in cellular components such as cornified envelopes, cell-cell junctions, and desmosomes.

Network analysis by use of IPA, allowed us to identify differentially expressed genes in indirect interaction with the ER (Figure 7) that were chosen for array validation. Well annotated genes with the largest log2-fold change were identified by use of IPA and were also selected for array validation by use of qRT-PCR. Genes chosen for array validation by qRT-PCR are shown in Figure 8.

Figure 7. The highest scoring ingenuity pathway analysis network. Differentially expressed genes were connected with dermatological diseases and conditions, embryonic development, and hair and skin development and function. Junction plakoglobin (JUP), desmocollin 2 (DSC2), keratin 8 (KRT8), and protocadherin 8 (PCDH8) were found to have indirect interactions with estrogen receptor alpha. Down-regulated genes are green and up-regulated genes are red.
Figure 8. Microarray validation performed with real-time quantitative PCR (qRT-PCR). Log₂-fold change results from the microarray (black) and qRT-PCR (white) in aromatase inhibitor-treated women with breast cancer compared with postmenopausal control women treated with vaginal estrogen therapy. Shown are the expressions of arachidonate 12-lipoxygenase (ALOX12), desmoglein 1 (DSG1), carbohydrate sulfotransferase 9 (CHST9), glutathione S-transferase mu 1 (GSTM1), junction plakoglobin (JUP), desmocollin 2 (DSC2), keratin 8 (KRT8), and protocadherin 8 (PCDH8). All of the genes differed significantly in the microarray (log₂-fold change > 1 and p-value < 0.05) and showed similar expression in qPCR. The expression of ALOX12, DSG1, GSTM1, JUP and DSC2 differed significantly between groups (*p < 0.05) when studied in larger study groups by means of qRT-PCR.

The expression patterns of arachidonate 12-lipoxygenase (ALOX12), desmoglein (DSG1), junction plakoglobin (JUP), and desmocollin 2 (DSC2) were significantly lower in AI-treated women when studied by means of both microarray and qRT-PCR analysis. Expression was correlated with circulating estrogen levels, vaginal atrophy scores, and vaginal pH.

The localization and staining intensity of ALOX12 and JUP were further investigated by use of immunohistochemistry. Protein staining of ALOX12 was localized in the vaginal epithelium, blood vessels, and occasionally also in stromal cells. The strongest staining intensity of ALOX12 was in the basal epithelial layer. On the other hand, JUP protein staining was exclusively localized to the vaginal epithelium. It was the strongest in the plasma membrane and less intense in the cytoplasm. Women treated with AIs tended to have less intense staining of ALOX12 (p = 0.08) and more intense staining of JUP (p = 0.053) in the basal epithelial layer compared with controls treated with vaginal ET.
Vaginal aromatase

The presence, localization, and staining intensity of aromatase in vaginal tissue were studied to add to the understanding of a possible local effect of AIs. Aromatase staining was observed in the basal and intermediate epithelial layers, and also in stromal cells. Slightly stronger staining intensity in the intermediate epithelial layer was evident in vaginal tissue from AI-treated women compared with controls (p < 0.05) (Figure 9).

![Figure 9. Representative immunohistochemical staining of aromatase in vaginal tissue from A) aromatase inhibitor-treated women with breast cancer and from B) controls treated with vaginal estrogen therapy.](image)

Paper IV

The effect of AIs on the presence, expression and distribution of AQPs in the vagina in vivo and in vitro was examined. Associations between AQPs and vaginal lubrication and cell differentiation were also studied. Low estrogen levels, higher vaginal atrophy scores, high vaginal pH, and insufficient vaginal lubrication were observed in AI-treated women compared with controls treated with vaginal ET.

Aquaporins

The mRNA expression of AQP 1–3 was detected by use of qRT-PCR in vaginal tissue. The presence, localization, and staining intensity of AQP1 and AQP3 were further investigated by use of immunohistochemistry.

Aquaporin 1 protein staining was primarily observed in blood vessels, with more intense staining in the nuclei of endothelial cells in AI-treated women than in controls (p < 0.001). Occasionally, weak staining of AQP1 was observed in the cytoplasm of vaginal epithelial cells. This occurred more often in AI-treated women than in controls (p < 0.05).

Aquaporin 3 protein staining was evident in the vaginal epithelium, and strongest in the plasma membranes. Women treated with AIs had less intense staining intensity in the intermediate epithelial layer (p < 0.01), but more intense staining in the superficial epithelial layer compared with con-
controls (p < 0.05, Figure 10). Aquaporin 3 staining intensity in the intermediate epithelial layer was positively correlated with circulating estrone levels, and negatively correlated with vaginal pH and insufficient vaginal lubrication.

![Image](image1.png)

Figure 10. Representative immunohistochemical staining in vaginal tissue of aquaporin 3 in A) aromatase inhibitor-treated women and B) control women treated with vaginal estrogen.

Vk2E6E7 cells (a vaginal epithelial cell line) treated with estradiol showed increased AQP3 mRNA (p < 0.05) and also slightly higher protein expression compared with vehicle treated-cells. Anastrozole treatment did not affect AQP3 mRNA expression but increased protein expression compared with vehicle-treated cells.

Differentiation markers

Immunohistochemical staining of the differentiation markers involucrin (IVL) and filaggrin (FLG) in vaginal tissue was performed to study any association with AQPs and cell differentiation. Women treated with AIs had less intense IVL membrane staining in the intermediate epithelial layer (p < 0.05) and more intense staining in the cytoplasm of the superficial layer (p < 0.01) compared with controls. This IVL staining pattern resembles the pattern observed for AQP3. Filaggrin staining was observed as small spots in the superficial epithelial layer. Vaginal epithelium from AI-treated women had FLG spots less often than controls. The presence of FLG spots was associated with strong staining of both AQP3 and IVL in the intermediate epithelial layer.
Discussion

Methodological considerations

The populations in Papers I and II included women with BC treated with AIs or tamoxifen and postmenopausal women without treatment or treated with vaginal ET. All study groups were included to study the effects of anti-estrogen therapy on circulating estrogen levels, vaginal proliferation, and steroid hormone receptors in vaginal tissue, in relation to morphological changes and clinical signs of vaginal atrophy. Papers I and II showed that AI-treated women have suppressed estrogen levels and also low vaginal cell proliferation, weak PR A+B, and strong AR staining intensities in the vaginal mucosa. These observations correlated with vaginal atrophy scores and vaginal pH and demonstrate the strong estrogen deprivation occurring in vaginal tissue from AI-treated women. The effects on biochemical factors in vaginal mucosa brought about by AI treatment were further investigated in Papers III and IV. Postmenopausal women with vaginal ET, a homogeneous and hormonally controlled group, were chosen as the control group. They have more absent/light vaginal atrophy scores, lower vaginal pH, and more superficial vaginal epithelial cells compared with postmenopausal women without treatment. Women with vaginal ET have more self-reported vaginal symptoms, which is counterintuitive, but most likely the result of a cross-sectional design [4]. The results of a clinical examination with atrophy scoring, calculation of VMI, and measurement of vaginal pH do not always agree with self-reported symptoms [2]. Women experiencing vaginal symptoms most likely seek treatment, whilst women not affected by their atrophic vaginal mucosa do not seek treatment [4]. In Papers II and III only clinical evaluation of vaginal atrophy and measurements of vaginal pH were used in correlation analysis, whereas in Paper IV, self-reported insufficient lubrication was used in addition.

The vaginal epithelial cell line Vk2E6E7 was used in addition to vaginal biopsy samples in Paper IV, to evaluate the possible estrogenic regulation of AQP5s. Vk2E6E7 cells originate from normal vaginal tissue and resemble primary vaginal epithelial cells [80]. They express steroid hormone receptors [93] and epithelial markers [80] and have previously been used to study the effects of different hormones [93-94]. Vk2E6E7 cells offer a good in vitro model of vaginal epithelial cells. It is important, however, never to extrapolate the results obtained in vitro. The use of both Vk2E6E7 cells and vaginal
biopsy samples offers the possibility to study estrogenic regulation in vitro which could explain the difference in gene expression observed between AI-treated women and controls.

Measurement of ultra-low postmenopausal circulating estrogen levels is difficult, especially in AI-treated women. Immunoassays, LC-MS/MS and gas chromatography tandem mass spectrometry (GC-MS/MS) are the three main assays currently used for measurement of ultra-low estradiol concentrations [8]. Immunoassays used in the past have been associated with problems with detection limits and the distinction of estradiol from its metabolites and conjugated estrogens which may interfere with the antigen-antibody interaction [8]. There can also be cross-reactivity between estradiol and exemestane [95] in exemestane-treated women. An initial extraction step increases assay sensitivity [3, 81] but the recovery rate of the extraction method may also affect the results [8]. Extraction RIA, however, has good correlation to GC-MS/MS when analyzing low estradiol levels in postmenopausal women [20]. Mass spectrometry methods are considered to be the reference methods [8]. The results obtained by use of mass spectrometric methods are much dependent on standardization of reagents, ionization techniques, hardware, etc [96]. Liquid chromatography tandem mass spectrometry with dansyl derivatization has good sensitivity and specificity for low concentrations of estradiol and estrone with established reference values for postmenopausal women [82].

The difference in vaginal epithelial thickness between AI-treated women and control women in vaginal biopsy samples has been an issue in gene expression studies performed with microarrays and qRT-PCRs. The difference in epithelial thickness makes it difficult to distinguish between a difference in gene expression and a difference in the number of cells expressing the genes. However, the differentially expressed genes found in Paper III are consistent with results found when investigating gene expression in vaginal epithelial cell smears from women suffering from vaginal dryness [46]. Estrogen receptors in stromal cells are imperative for estrogenic regulation of epithelial cell differentiation and stratification [34]. In addition, stromal cells also stain positive for aromatase [31], and therefore it can be suggested that stromal cells are also important when studying hormonal regulation of the vaginal mucosa. Furthermore, the most superficial cells most likely do not express any mRNA as a result of cornification [32].

The microarray technology used in Paper III is hypothesis-generating. Together with bioinformatic analysis performed by using DAVID and IPA, it can provide information on how a disease or hormonal condition affects genes that are involved in biological processes, cellular components, and molecular functions, and how they interact with each other [86, 97]. Results obtained by use of microarrays should be validated in larger study populations by use of qRT-PCRs and differential protein expression can also be studied by use of western blots and immunohistochemistry. Protein and
mRNA expression does not always concur and many events occur before the protein is expressed and a cellular response occurs [6, 47].

Immunohistochemistry was used to study the presence, distribution and staining intensity of various proteins in vaginal tissue. It is a semi-quantitative technique which relies on specific binding of an antibody to an epitope on a target protein. This interaction is complex and can be affected by alternative splicing and posttranslational modifications of the target protein [98]. The antibodies used in Paper II have previously been used for studies on steroid hormone receptor expression in vagina, vulva, and endometrium [99-104]. Steroid hormone receptors show the strongest staining in the cell nucleus but staining in the cytoplasm has also been observed [10]. Use of the Ki67 antibody to study proliferation has been validated in other tissues [105-106]. The antibodies used for microarray validation in Paper III and the antibodies used for the differentiation markers described in Paper IV, were identified in the Human Protein Atlas and they have been validated in vaginal tissue [107]. The aquaporin antibodies used in Paper IV had not at the time been evaluated in the Human Protein Atlas, and were therefore identified in the literature [108] and by reviewing available antibodies.

General discussion

In Paper I we showed that circulating estradiol and estrone levels in AI-treated women can be higher in clinical practice than previously observed when measured by use of RIA in clinical studies [3, 68, 91]. Somewhat elevated estrogen levels have also been observed in other studies [69, 109-110] and most likely reflect clinical practice. However, AI-treated women did have lower levels compared with all other groups, and high self-reported compliance, reflecting good treatment efficacy with AIs. We also observed a wide range of estradiol and estrone levels, which has been suggested to be due to differences in BMI [69], but this effect varies between studies [70-71]. Body mass index appears not to be a contributing factor in our population sample, as there was no correlation between estrogen levels and BMI, or any difference in BMI between groups. The wide estrogen concentration range could also not be explained by lack of self-reported compliance or time since menopause. This range may also be due to variability in AI metabolism, affecting the suppression of estrogen production [109]. However, AI metabolism was not assessed.

The effect of AIs on circulating estrogens is often assessed in order to evaluate BC treatment efficacy, even though this has been questioned [111]. As previously stated, low estrogen levels are difficult to measure and gold-standard methods have not been implemented in clinical practice [8]. In addition, plasma estrogen levels provide good estimates of estrogens produced in different body compartments, but estradiol levels are higher in breast tis-
sue compared with plasma [111-113] and there is no correlation between the two [111]. Breast tissue estrogen levels are not correlated with aromatase protein staining, suggesting a circulatory uptake [114]. The overall effect on the breast depends on numerous factors; ERs, growth factors, and other receptors in addition to local and circulating estrogens [111]. Hence the relationship between circulating estrogen levels during treatment with AIs and breast cancer recurrence needs further investigation.

In the light of our findings, i.e. the unexpectedly high levels of circulating estrogen and the wide range of estradiol concentrations in AI-treated women, we revived the question of whether or not it would be possible to treat vaginal atrophy symptoms with low-dose vaginal estradiol or estriol. However, we did not pursue this route of investigation, for several reasons. First, it is well-known that vaginal ET results in estradiol concentrations above normal postmenopausal ranges at initiation of treatment, but as the vaginal mucosa gradually matures, the systemic absorption decreases [40]. Thus, resulting levels of circulating estradiol are in the postmenopausal range, which has been considered to be acceptable in healthy women. However, with more accurate estradiol assays, this supposition has somewhat changed. According to a review by Santen, published in 2014, low-dose vaginal ET, (i.e. a vaginal ring releasing 7.5 micrograms per day or a 10-microgram tablet) increased plasma estradiol levels during chronic administration, but levels were still within the normal range of below 74 pmol/L. Intermediate vaginal estrogen doses, i.e. 25 micrograms of estradiol, on the other hand, resulted in plasma estradiol levels approaching or exceeding 74 pmol/L [19]. Secondly, there was some suggestion in the literature that a proportion of women who used both an AI and vaginal ET would end up having long-term estradiol levels that exceed 100 pmol/L, i.e. unpredictable serum concentrations clearly outside the postmenopausal range [78]. Furthermore, our own finding of somewhat elevated estradiol levels in users of estriol precluded use of the latter treatment option. Finally, it was also evident from the women with BC who we met in the study that they were not eager to try any treatment that contained the slightest amount of estrogen.

All in all, the findings of Paper I made it more evident that the actions of estrogens in connection with vaginal atrophy need further investigation in order to improve treatment options.

The main finding in Paper II is that low estrogen levels, most importantly low estrone levels, leads to a low rate of proliferation, weak PR staining intensity, and stronger AR staining in vaginal mucosa. The effect on vaginal tissue may be caused by the particularly low estrogen levels during AI treatment, but the presence of vaginal aromatase [30-31] suggests that it might also be its inhibition that gives rise to the more pronounced symptoms.

Estrogenic regulation, shown as low-level vaginal proliferation in AI-treated women and higher proliferation in estrogen-treated controls is in agreement with the results of previous studies [36]. The correlations between
estrone, vaginal atrophy, and vaginal pH support the involvement of proliferation in vaginal atrophy symptoms and the thickness of vaginal mucosa, as previously shown [33, 35].

The strong ER staining in the basal and intermediate epithelial layers and the lack of difference between study groups concur with previous results [29] and show estrogenic responsiveness irrespective of estrogen status. It is a limitation that we did not investigate ERRs or (membrane-bound) GPER, as it would have given a better understanding of the complex effect of estrogens in vaginal tissue.

Progesterone receptors are influenced by estrogens [11] and are also regulated by estrogen status in the vagina [29]. This was also seen in Paper II as weak PR A+B staining in samples of vaginal mucosa from AI-treated women whilst stronger staining was observed in vaginal tissue from tamoxifen-treated women and controls treated with vaginal ET. The low PR A+B protein staining intensity seen in AI-treated women and the correlations with estrone, vaginal atrophy evaluation scores, and the proliferation marker Ki67, suggest that low levels of PRs might serve as a marker of vaginal atrophy symptoms regulated by estrogens.

The strong AR staining in AI-treated women and weak staining in controls with vaginal ET reflects negative regulation of ARs by high estrogen levels. This has previously been seen in vaginal tissue from postmenopausal women [30], but is in contrast to a suggested decrease of ARs with increasing age [115]. Testosterone treatment has been shown to increased vaginal AR expression [115] and improve vaginal atrophy symptoms [116]. In our study however, AR staining did not correlate with circulating estrogen or androgens levels, but only with atrophy evaluation scores and vaginal pH. This suggests that it is not circulating hormones per se, but instead local inhibition of aromatase in the vagina, causing an increase in ARs, which in turn might have a negative impact on vaginal atrophy. Only a small percentage of circulating androgens undergoes aromatization and local inhibition of aromatase will probably only lead to an insubstantial increase in androgens [114] affecting AR levels, but not enough to improve vaginal atrophy.

In Paper III, we investigated the expression of aromatase in postmenopausal vaginal tissue. Aromatase staining intensity was more intense in vaginal tissue from AI-treated women compared with controls treated with vaginal ET, which is in agreement with findings reported in Paper II. Aromatase inhibitors have been shown to stabilize and thereby reduce protein turnover of aromatase, leading to increased protein expression but with inhibition of estrogen synthesis [117-118]. Aromatase has tissue-specific promoters that might enable production of selective AIs [23], but the equilibrium between plasma and breast tissue estrogen levels makes this difficult to achieve [114]. Currently it is difficult to make a distinction between active and inhibited aromatase by use of immunohistochemistry [119], leaving the question of whether or not vaginal aromatase is inhibited in AI-treated women unsolved.
However, the more prominent vaginal atrophy symptoms in AI-treated women [4], seen even though estrogen levels were higher than expected, strengthens the theory of a local effect of AIs in the vagina, resulting in lower local estrogen levels and more detrimental effects.

However, regardless of whether or not AIs act only via systemic or possibly also via local inhibition of vaginal aromatase, the low estrogen levels affect the vaginal expression of genes involved in cell proliferation, cell adhesion, and cell differentiation. Similar effects have been observed in women treated with systemic ET, where the expression of genes involved in these processes increased [38]. The low expression of genes involved in cell proliferation agrees with the low proliferation observed in Paper II. Some of the differentially expressed genes in our study were found by use of IPA to be in in-direct interaction with the unchanged ER. Estrogenic regulation of genes important for cell adhesion has been observed in mammary cells [120] and the importance of estrogen for cell permeability through regulation of cell adhesion has been shown in vaginal cell cultures [121]. The low expression of genes involved in cell adhesion might therefore affect cell transudation and vaginal dryness. Cell adhesion is also of great importance for barrier function and epithelial function [25, 37]. Estrogens acting via ERs are of great importance for vaginal cell differentiation [34] but the exact mechanism is not known.

Poor differentiation in vaginal mucosa from AI-treated women was also observed in Paper IV. Previous studies of AQP3 in skin epidermis and vaginal tissue have shown strong AQP3 expression in the basal and intermediate epithelial layers and diminished staining in the more differentiated superficial cells [51, 54]. This was observed in vaginal mucosa from postmenopausal women treated with vaginal ET. Vaginal mucosa from AI-treated women, on the other hand, showed weak AQP3 protein staining in the intermediate epithelial layer but stronger staining in the superficial epithelial layer. A similar staining pattern was also observed for the differentiation marker IVL in vaginal mucosa from AI-treated women. They also less frequently had FLG spots in the superficial epithelial layer. Taken together, these findings suggest poor differentiation of mature superficial cells in estrogen-deprived vaginal mucosa. Aquaporin 3 has been suggested to be involved in the control of cell differentiation in the skin. The expression of AQP3 in skin has been reported to be low in differentiated cells whilst the induction of higher AQP3 expression coincided with decreased levels of differentiation markers and impaired barrier function [54]. The role of AQP3 in cell differentiation, however, is under discussion [54, 122]. It is difficult to elucidate if AQP3 is involved in the control of vaginal epithelial differentiation or if proper differentiation is needed for AQP3 expression. In one study it was shown that epithelial differentiation was independent of AQP3 in skin [122]. However, cell differentiation is most likely to be dependent on
numerous biochemical factors and this does not exclude a possible role of AQP3 in cell differentiation.

The transport of water and glycerol by AQP3 in the epidermis is of great importance for cell proliferation, skin hydration [52-53], and possibly cell differentiation. The weak staining intensity of AQP3 in the intermediate epithelial layer is in line with the low level of cell proliferation observed in samples of vaginal mucosa from AI-treated women. This might suggest a role for AQP3 in vaginal cell proliferation, where adequate hydration via glycerol and water is needed for proliferation.

Vaginal dryness is one of the most bothersome vaginal atrophy symptoms in postmenopausal women [2] and in women undergoing treatment with AIs [4]. The less intense AQP3 staining intensity in the intermediate epithelial layer in AI-treated women was associated with self-reported insufficient vaginal lubrication. This weak AQP3 staining intensity was also correlated with low circulating estrone levels and high vaginal pH. Our in vitro studies showed that estradiol increased the expression of AQP3, whilst anastrozole decreased it in Vk2E6E7 cells. This, together with previous findings in rodents and vaginal tissue from premenopausal women [49, 51], suggests estrogenic regulation of AQP3 in vaginal epithelium. Low AQP3 expression in the skin is associated with low glycerol permeability and reduced skin hydration [52]. Insufficient transport of water and glycerol, caused by a low AQP3 content, may also have a negative impact on vaginal lubrication. Possibly, epithelial hydration via AQP3 and glycerol is needed for proper cell proliferation and differentiation in vaginal epithelial cells.

**Clinical implications**

Vaginal health is receiving more attention and recent studies show that vaginal atrophy symptoms have a great effect on the quality of life of women and their partners [1, 39]. Vaginal symptoms caused by estrogen deprivation are a clinical problem, not only for women in their menopausal transition and anti-estrogen-treated women, but also for young women breast feeding or taking oral contraceptives, where vaginal dryness affects compliance [2, 5, 123-124].

The need for effective treatment options and hesitancy towards vaginal ET, not only in women with BC but also in healthy postmenopausal women, have led to the development of a range of new treatment alternatives [2]. Suggested therapies include the use of ospemifene [125-126], dehydroepiandrosterone (DHEA) [127], testosterone [116, 128], oxytocin [129], microablative CO₂ treatment [130], high-molecular-weight hyaluronic acid [131], and also a combination of estriol with *Lactobacillus acidophilus* to restore the microflora [132].
Ospemifene is a SERM, administered orally, with agonistic effects on ERs in vaginal tissue. It has been shown to improve dyspareunia, vaginal dryness symptoms, increase VMI, and decrease vaginal pH [125-126]. Treatment with ospemifene is currently not recommended for women with estrogen-responsive BC [2], but ospemifene does have an anti-estrogenic effect in the breast and further studies are warranted [133]. Treatment with intravaginal DHEA, through local androgen and estrogen formation, has positive effects on the most bothersome vaginal symptoms, and it increases VMI and decreases vaginal pH [127]. Daily dosing with DHEA, however, has been questioned because of a compliance concern [134]. Likewise, treatment with vaginal testosterone has shown positive effects on vaginal symptoms, VMI, and vaginal pH. However, further investigations are needed with regard to efficacy and safety issues [116, 128]. Circulating estrogen and androgen levels show a slight increase during vaginal DHEA or testosterone treatment but remain within normal postmenopausal ranges [116, 135]. While treatment with androgens might be of interest for AI-treated women, given the strong staining of AR in the vaginal mucosa in these cases, it is also possible that vaginal inhibition of aromatase might decrease treatment efficacy. In addition, the slight increase in estrogen and androgen levels might have negative effects in AI-treated women and must be taken in consideration [111, 136]. In a pilot study, treatment with vaginal oxytocin was associated with improvements in vaginal atrophy evaluations and some aspects of vaginal histology. However, the study included only a limited number of patients, which is why it failed to demonstrate any significant effect on symptom relief [129]. Oxytocin is important for labor and lactation in that it brings about muscle contractions [6], and it has also been shown to aid wound healing [137]. Further investigations of oxytocin are needed to establish if it is a suitable treatment option for AI-treated women. Microablative CO₂ laser treatment, by inducing new collagen and elastic fibers, has positive effects on vaginal symptoms, sexual satisfaction, and quality of life. However, further studies with control subjects to rule out any placebo effects are needed [130].

Other researchers have investigated the use of vaginal estriol, in combination with *Lactobacillus acidophilus*, as a treatment option for AI-treated women [132]. The treatment results are promising, with improved vaginal symptoms, VMI, and vaginal pH [138]. However, again, the slight increase in estriol levels observed [132] might be a concern in AI-treated women. Estriol has been shown to have similar results as estradiol in BC cell lines [139]. The effect of slightly higher estrogen levels on BC is still unclear, but higher circulating levels will lead to higher levels in breast tissue [111, 113].
Future research

The prospect of influencing cell proliferation, epithelial hydration, and possibly also cell differentiation by use of AQP3 as a future treatment target is interesting. Increasing the expression of AQP3 in vaginal epithelium might be of benefit for treating vaginal dryness and other symptoms of vaginal atrophy caused by estrogen deprivation. However, high AQP3 expression has been observed in skin cancer and absence of AQP3 seems to be protective against this disease [48], but how these findings relate to risk of vaginal cancer remains to be investigated. Glycerol application has been successful in improving skin hydration [52] and cell proliferation [53] when AQP3 expression is low. Vaginal moisturizers containing glycerol do offer some symptom relief [44] but do not improve VMI or vaginal pH [43-44]. However, the vaginal moisturizers available today have only a small glycerol content and increasing the amount in these vaginal moisturizers might be beneficial for cell proliferation, epithelial hydration and possibly also cell differentiation. This might serve as a possible treatment option for all those women who are not eligible for vaginal ET.

In addition to evaluation of self-reported vaginal symptoms, VMI, and vaginal pH, it also may be of interest to evaluate future treatment options by use of vaginal biopsy samples. The benefits of taking biopsy samples, i.e. histological examination and investigation of markers of cell proliferation and cell differentiation, might possibly outweigh the pain the procedure might cause. Biopsy sampling may enable better understanding of treatment mechanisms which would be of great benefit.

Concluding remarks

The higher than expected circulating levels of estradiol and estrone in AI-treated women observed in Paper I show that circulating estrogen levels might have been underestimated in previous studies. Systemic estrogen suppression by treatment with AIs, and possibly also by local inhibition of vaginal aromatase, results in less vaginal cell proliferation, low expression of genes involved in cell adhesion and differentiation, and also decreased amount of AQP3 in vaginal epithelial cells. Vaginal dryness seems to be associated with a low AQP3 content in the intermediate epithelial cell layers, and possibly also low-level expression of genes important for cell adhesion, possibly affecting vaginal transudation. Epithelial hydration via AQP3 and glycerol together with adequate cell adhesion might be needed for proper cell proliferation and differentiation in vaginal epithelial cells. Atrophy is defined as the wasting away of normally developed tissue as a result of degeneration of cells [140]. The poor differentiation and low-level cell proliferation observed in estrogen-deprived vaginal mucosa suggest that the ob-
served changes depend on lack of proper regeneration of vaginal epithelial cells rather than degeneration of cells. This results in a vaginal mucosa consisting of fewer cells that matures but does not differentiate properly, leading to defective epithelial function and ultimately causing vaginal symptoms. The estrogenic regulation of AQP3 and its possible role in vaginal dryness, cell differentiation, and cell proliferation, is of great interest and should be further explored. Deeper knowledge of the complexity of vaginal symptoms and the importance of vaginal hydration could possibly lead to the discovery of new non-hormonal therapies to alleviate vaginal symptoms caused by low estrogen levels.

för att utveckla nya hormonfria behandlingsalternativ. Syftet med denna avhandling var att studera effekten av antiöstrogenbehandling på östrogennivåer i blodet samt att se hur antiöstrogenbehandling påverkar biokemiska faktorer i vaginalslemhinnan i relation till vaginalslemhinnans uppbyggnad, vaginal atrofi och underlivsbesvär.

I den första delstudien analyserades östrogennivåer hos kvinnor som genomgått klimakteriet med bröstcancer behandlade med aromatashämmare eller tamoxifen, samt hos kvinnor som genomgått klimakteriet utan behandling eller behandlade med vaginalt östrogen. Resultaten visade att kvinnor behandlade med aromatashämmare hade lägre östrogennivåer än andra kvinnor efter klimakteriet. Trots det så hade de oförväntat höga östrogennivåer och stora individuella skillnader inom gruppen konstaterades.


I delstudie IV studerades aquaporiner, som är involverade i fuktighetsbalansen, i vaginalslemhinnan. Aquaporiner transporterar vatten och andra små molekyler så som glycerol genom cellens membran. De är viktiga för epitellellers vätskesekretion samt celldelning, nybildning och utmognad [48, 54]. Vaginalslemhinnan från kvinnor behandlade med aromatashämmare hade en mindre mängd aquaporin 3, vilket var associerat med låga östrogennivåer, mer vaginal torrhet och även sämre utmognad av celler i vaginalslemhinnan. Sammanfattningsvis kan denna avhandling påvisa att kvinnor behandlade med aromatashämmare har låga östrogennivåer, dock högre än förväntat. Dessutom kan vi påvisa en potentiell hämning av aromatas i vaginalslemhinnan hos kvinnor behandlade med aromatashämmare. Låga östrogennivåer leder till lågt uttryck av gener viktiga för vaginalslemhinnans nybildning och utmognadprocess. Aquaporin 3 verkar vara en viktig faktor för vaginal torrhet och kan möjligtvis även ha en viktig roll för cellernas nybildning och utmognad. Avsaknad av nybildning och sämre utmognad av celler, snarare
än en tillbakabildning, leder till sämre funktion hos vaginalslemhinnan vilket troligtvis ger upphov till de symptom som uppstår i underlivet vid låga östrogennivåer. En östrogenreglering av aquaporin 3, som är viktig för vaginalslemhinnans fuktighetsbalans, cellernas nybildning och utmognad bör studeras vidare för att utveckla hormonfria behandlingsalternativ mot underlivsbesvär.
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