Modulation of Intracrine Estrogen in Menopausal Women:
Implications for Women’s Reproductive Health and Breast Cancer Risk

By

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Institute of Medical Science
Faculty of Medicine
University of Toronto

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Doctor of Philosophy, 2010

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Abstract

Extensive research and clinical observations in the past 20 years confirmed that the cessation of ovarian function at menopause does not stop the process of sex steroid hormone synthesis in females. Indeed, multiple extra-ovarian tissues contain the same enzymatic machinery the ovary uses which can maintain a significant rate of local hormonal synthesis sufficient to cause pathological outcomes. This is commonly termed “intracrine”. However, two obstacles face intracrinology. Firstly, wide clinical appreciation of this mechanism in causing disease and in targeting it with therapy does not currently exist. Secondly, blood hormonal assays are used in the clinic to diagnose and manage intracrine based disorders. This could be entirely misleading, since hormonal synthesis, action and metabolism occur within the tissue and, therefore, measuring blood levels is not reflective of the actual disease environment.

This thesis presents evidence of significant intracrine based disorders in menopausal women that could be effectively managed by tackling the core intracrine mechanism. Three protocols are investigated emphasizing the usefulness of menopausal intracrine estrogen inhibition. The first presents a joint objective of treating menopausal symptoms using estrogenic replacement therapy while reducing breast cancer risk using long-term aromatase
inhibitors. Aromatase inhibition is used to suppress the local estrogen synthesis in the breast. The second protocol is a new method of acute inhibition of breast estrogens to improve the accuracy of breast imaging techniques. This method showed a benefit in reducing the benign parenchymal enhancement during breast MRI indicating a potential improvement in specificity. The third protocol involves using aromatase inhibitors in the treatment of severe endometriosis that did not respond to oophorectomy. The pathogenesis of breast cancer, endometriosis and fibroids are believed to involve intracrine estrogen activity.

Another significant contribution presented in this thesis is the development of a new technique that enables minimally invasive tissue assays of hormones in their genuine site of synthesis rather than indirectly in the blood. The new assay requires only microliter volumes of sample and employs a novel digital microfluidics technology. Estrogen and other sex steroids were extracted from droplet-scale breast tissue and blood samples.
To My Husband and My Parents
Acknowledgement and Overview of Co-Authors

The work presented in this thesis could never be accomplished without the help of many people. The following is a list of colleagues who helped me carry out the research projects of my thesis:

First and foremost, I am indebted to my supervisor and mentor, Dr. Robert Casper. Dr Casper has guided me throughout the years of my PhD research in the smoothest and finest way of supervision possible. He gave me total independence in steering my research which increased my self confidence and motivated me to think, plan and work freely. I was always treated as his colleague rather than his student. His endless guidance, support and encouragement were invaluable. I am impressed by his innovative ideas and entrepreneurial mentality. Working with him and his patients in several clinical studies, I could see also how an exemplary doctor-patient relationship could exist and last. I believe I was really fortunate to know and work closely with Dr Casper, an experience that will definitely have a long-lasting effect in my memory, future career and my professional development.

I would like to thank Dr Aaron Wheeler for his exceptional contribution toward the estrogen digital microfluidic assay study (Chapter 5). Dr Wheeler has encouraged me to convert an imaginary idea into a real achievement. He has hosted me in his lab as one of his own team members over the past 2 years. He provided me with continuous guidance in the field of microfluidics and analytical chemistry. Dr Wheeler supplied unlimited resources from the expertise of his group and the facilities and equipment of his lab. His special style of group leadership was also very inspiring for me.

I would like to express my gratitude to Mais Jebrail who worked with me to develop the estrogen microfluidic assay (Chapter 5). I truly appreciate his patience and hard work on
this project. His friendly personality and team-work manners made this part of my research work a real enjoyable experience. I am also grateful to Hao Yang and Mohamed Abdelgawad for their help in the microfluidic assay study (Chapter 5). Hao Yang helped me in the Mass Spectrometry evaluation of estrogens. Working with him, I gained important experience in using nanoLiquid Chromatography and Mass Spectrometry for quantitative and qualitative hormonal assays. Through Mohamed, I learned about the principle of the digital microfluidics and he designed and fabricated the original devices we used in this work.

I like to thank Dr Pavel Crystal for his assistance in the breast imaging analysis in two studies (Chapter 3 and Chapter 4). He was an extremely accessible collaborator and provided valuable advice in study design, critical evaluation of the results and help in understanding technical details. I also thank Dr Riham Eiada who was very helpful in the analysis of the breast MRI (Chapter 4). She spent full days of her holiday time and weekends patiently working with me on image analysis of hundred of MRI images. I have learned a lot about breast MRI from her.

I also thank Professors Theodore Brown, Eleftherios Diamandis and Steven Narod for their valuable advice, time, guidance and helpful discussions throughout the years of my PhD program as members of my supervising committee at the Institute of Medical Science.

I am grateful to Dr Mohamed Bedaiwy for guiding me at the beginning of my PhD especially in developing my scientific writing skills and I thank Dr Wendy Wolfman for her help in the study presented in Chapter 3. She provided access to cases that formed the control group of the study. She has been also my supervisor in a clinical fellowship at the Department of Obstetrics and Gynecology. Working with her has significantly enhanced my
appreciation of the hormonal and gynecologic disorders in the perimenopausal stage of women’s life.

I would like to thank Frances Kelly, Dr Casper’s assistant for her help in the clinical studies. She has been kindly providing immediate help with details related to patients’ records and recruitment.

At last, I would like to acknowledge the funding sources that supported my research throughout the PhD program including: The University of Toronto Open Fellowship, the Institute of Medical Science entry award, the Ontario Graduate Scholarship (OGS) and the Canadian Institute of Health Research (CIHR) through the Health Professional Fellowship and the Bisby Award.
Overview of Chapters and Related Publications

Chapter 1 is a general overview of estrogen and its role in health and disease. It introduces the intracrine mechanism of estrogen biosynthesis and its contribution to the pathogenesis of various hormone-sensitive disorders in postmenopausal women. It also presents evidence regarding the value of inhibition of the aromatase enzyme in treating these disorders and the rationale for extending its use for prophylactic purposes.

This chapter has been published in part as sections of a review article in the *Journal of Minimally Invasive Gynecology*.


Chapter 2 presents a hypothesis about the role of multiple intracrine factors in the hormonal carcinogenesis of breast cancer after menopause, particularly in women exposed to high estrogenic stimuli during their reproductive life. The hypothesis is supported by clinical, epidemiological, and fundamental research evidence. In the chapters that follow, I discuss my research that supports aspects of this hypothesis.

This chapter was published in *Bioscience Hypotheses*.


Chapter 3 presents a retrospective controlled study that investigated a new protocol using a combination of aromatase inhibitors and hormone replacement therapy in the long-term management of menopause.

This chapter was published in *Menopause*.

Chapter 4 presents a prospective pilot clinical trial that investigated a new protocol for using aromatase inhibitors in modulating the breast MRI parenchymal enhancement in postmenopausal women.

This chapter was submitted for publication in *Radiology*.


Chapter 5 presents a new technique for estrogen assay requiring micro-droplets of clinical samples and utilizing a “lab on chip” technology called digital microfluidics. Additionally, a preliminary study showing the utility of the assay in extracting other sex steroid hormones is discussed.

The estrogen assay study was published in *Science Translational Medicine*.


Chapter 6 presents the protocol and design of a double-blinded randomized clinical trial recently initiated to investigate the effect of aromatase inhibitors and hormone therapy in reducing breast cancer risk in menopausal women. This clinical trial encompassed the knowledge and methodology developed through the studies presented in this thesis.
Chapter 7 is summary of the thesis and presents potential future directions.

In the appendix, I present a case report study on the use of aromatase inhibitors in the treatment of a resistant case of endometriosis in a postmenopausal patient who had severe pelvic pain.

This was published in *Obstetrics and Gynecology*.

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<td>MBD</td>
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Appendix I: Aromatase Inhibitors in the Treatment of Severe Postmenopausal Endometriosis
Chapter 1 Introduction

1.1 Estrogen

Estrogens are the primary female sex hormones. They are responsible for the characteristic secondary sexual changes in females at puberty. Normal levels of estrogens are requisite for normal growth [1], menstrual cycle [2, 3], proper ovulation [4, 5], conception [6, 7] and normal pregnancy [8, 9]. Synthetic estrogens alone or with other sex steroid hormones such as progesterone or androgens, are used widely for contraception and hormone replacement for different therapeutic purposes [10-12]. A role for estrogens in the physiology of non-reproductive systems has been increasingly highlighted in the past 50 years. For example, estrogens reduce the risk of osteopenia, osteoporosis and fractures [13-16]. Their role in the cardiovascular system has been also emphasized [17, 18], although recent controversy occurred about its use as a long-term cardioprotective agent [19]. The effect on improving memory and cognition [20-24] as well as reducing colon cancer [25, 26] have been also controversial.

1.2 Estrogen Biochemistry

The Estrogens encompass a large number of naturally occurring and synthetic steroid compounds and their metabolites. All of these compounds have a characteristic phenolic ring which makes the compound more polar as compared to other steroids. Estradiol (E2) refers to the 17-beta-isomer of estradiol, an aromatized C18 steroid with hydroxyl group at the 3-beta- and 17-beta-positions. It is the most potent form of mammalian estrogens. The 17-alpha isomer of estradiol binds weakly to estrogen receptors and has weak estrogenic activity.
Estrone (E1) is another common form of naturally occurring estrogens. It is an aromatized C18 with a 3-hydroxyl group and a 17-ketone. Estriol (E3) has a hydroxyl group at the C3-beta, 16-alpha, and 17-beta positions. Estriol is produced via hydroxylation of estrone or estradiol. It is mainly found in high concentrations during pregnancy (Figure 1-1)[27].

![Estradiol (E2) Estrone (E1) Estriol (E3)](image)

MW = 272.38  MW = 270.36  MW = 288.38

Figure 1-1: Three common naturally occurring estrogens, estradiol (most potent), estrone and estriol. Adapted with modifications from PubChem Compounds [27]

Estradiol and estrone are synthesized from their androgenic precursors (testosterone and androstenedione respectively), which in turn are synthesized through a long series of biochemical reactions starting with the parent molecule cholesterol. This process is commonly referred to as “steroidogenesis” and results in the formation of five major classes of steroid hormones including glucocorticoids (such as cortisol), mineralocorticoids (such as aldosterone), androgens, estrogens and progesterone (Figure 1-2).
Figure 1-2: Diagram illustrating a simplified process of steroidogenesis. Chemical structure of the individual compounds obtained from PubChem database [27].

Estrogens act through binding to mainly nuclear receptors which are primarily ligand-activated transcription factors. There has been consistent evidence supporting a role for membrane activated estrogen receptors, however significantly more nuclear receptors are available than cell membrane receptors [28]. Two types of estrogen receptors (beta and alpha
or ER-β and ER-α) are expressed variably in various tissues. Distinct genetic regulation, functions and ligand preference have been attributed to each one of these receptors, with a general impression of the beneficial and protective role of ER-β especially in cancer development [29-31]. Activation of estrogen receptors leads to increased expression of large number of proteins such as vascular endothelial growth factor (VEGF), progesterone receptor (PR) and insulin like growth factor -1 (IGF-1) [32].

Active estrogens are also metabolized by multiple enzymes into a large group of metabolites with variable activities and biological effects (Figure 1-3). Most common metabolites include 16-alpha-hydroxy estrone (16α-OH-E1), which was shown to have a genotoxic effect and to be related to increased risk of breast cancer [33], and catechol-estrogens such as 4-hydroxyestradiol (4-OHE2) and 2-hydroxyestradiol (2-OHE2), which were also shown to form reactive oxygen species and induce endometrial cancer [34].
Several enzymes of clinical significance are involved in the estrogenic biosynthesis process including aromatase, which is the final rate-limiting enzyme that converts testosterone and androstenedione – through methyl oxidation and hydroxylation reactions- into the C-18 estradiol and estrone respectively. Other important enzymes include 17-β hydroxysteroid dehydrogenase (17β-HSD) which converts estradiol to estrone and vice versa by its two isomers (17β-HSD 1 and 2) and steroid sulphatase (STS) that hydrolyzes estrone sulphate to estrone and dehydroepiandrosterone sulphate (DHEA-sulphate) to DHEA which in turn is converted to testosterone catalyzed by 3-βHSD isomerase [36].

Circulatory levels of estrogens in females change in a cyclic monthly pattern during their reproductive years with a pre-ovulatory rise and higher levels in the luteal phase than the follicular phase. A significant elevation of blood estrogen levels is maintained throughout pregnancy (Figure 1-4). During menopause, with the decrease in the number of functioning ovarian follicles, estrogen concentration in the blood declines markedly, leading to a feedback rise of the pituitary follicle stimulating hormone (FSH) (Figure 1-5).
1.3 Pathologic Roles of Estrogen: Overview

Abnormal levels of estrogens have been associated with a broad range of hormonal disorders. In females, endometriosis (10% incidence rate in women of reproductive age) and fibroids (incidence of 70-80%) have been long known for their estrogenic dependency [39-41]. Polycystic ovary syndrome has several hormonal imbalances in the ovary including a disordered follicular production of androgens and estrogens [42]. Endometrial cancer and breast cancer are both coined as estrogen sensitive cancers. The role of estrogen in ovarian cancer pathogenesis and progression has been investigated in several research studies, though not yet utilized in routine clinical management [43-46]. Furthermore, a link between estrogen and non gynecologic cancers, such as colon cancer, has been demonstrated in a number of studies with some studies showing estrogen as a protective factor while others indicating an
increased colon cancer risk by estrogen deprivation [47, 48]. The role of estrogen in the pathogenesis of gastric and lung cancers has been also suggested [49-51].

On the other hand, estrogen deficiency is a hallmark in several female congenital amenorrhea syndromes, premature ovarian failure and menopausal symptoms and it increases the risk for osteoporosis as discussed above. Estrogen deficiency contribution to impaired cognition and dementia has been long debated [52].

In males, estrogen seems to be an important hormone for proper spermatogenesis. Abnormal estrogen levels or imbalanced estrogen to androgen ratio could result in several male reproductive disorders such as gynecomastia, hypogonadism (both congenital and late-onset) and infertility [53-55]. The role of estrogen in causing and treating prostate cancer has been also widely investigated [56].

1.4 The Intracrine Concept of Estrogen Synthesis and Its Implications for Breast Cancer Risk

Estrogens and their metabolites have multiple crucial roles in breast cancer pathophysiology acting as genotoxic agents [57-59], epigenetic pro-carcinogens and occasional inducers of genetic mutations [59-61]. Although, the majority of breast cancers (~ 75%) are estrogen receptor positive [62], there is no decrease in breast cancer risk at menopause when circulating levels of ovarian estrogens dramatically fall. On the contrary, breast cancer rates continue to increase with age with 80% of breast cancer cases in women over 50 years of age and around one third in women over 70 years old [63]. It is now accepted that estrogen plays its role as a significant intracrine and paracrine factor (rather than a circulatory hormone) in menopausal women in many tissues including the breast [64-66]. This non-ovarian in situ
biosynthesis of estrogen could contribute to around 75% of the total estrogen produced in premenopausal women and almost 100% in menopausal women [64].

1.5 Aromatase

Aromatase is the rate-limiting enzyme catalyzing the final step of estrogen synthesis from androgens. It is the protein product of the CYP 19 gene and a cytochrome P450 superfamily member. The gene is located at chromosome 15q21.1 in humans (Figure 1-6), and contains 9 coding exons. Aromatase gene expression is regulated by different tissue specific promoters, these represent regions of the DNA that facilitate the transcription of the gene [67]. The enzyme is identified in multiple tissues and organs including ovaries, placenta, adipose tissue, skin, bone, vascular endothelium, muscles and others. In adipose tissue, promoters 1.4, 1.3 and II account for all of the aromatase transcripts. Promoter 1.4, which down regulates aromatase expression is the predominant promoter in the normal breast and in abdominal fat tissue, while there is a reduced activity of promoter 1.4 and increased activity of promoters II and 1.3 in the malignant breast tissue [68, 69].
The increased aromatase activity leads to a significant build-up of estrogens within the breast and subsequent development of pre-neoplastic disorders [72-75]. Expression of the aromatase gene is significantly elevated in the adipose tissue of healthy menopausal women compared to cycling women [76]. In postmenopausal breast cancer patients, intratumoral estrogen levels are significantly higher than the blood levels, consistent with aromatase activity and aromatase gene expression being considerably increased in this group [72, 74, 75]. Furthermore, evidence exists for the relation between polymorphisms in the aromatase CYP19 gene and the risk of breast cancer in females. Some of these genetic variations could include a switch from activation of the adipose tissue promoter to the ovary promoter [77, 78]. Based on these findings and extensive other research, the disruption of in-situ estrogen synthesis has been a cornerstone in the management of breast cancer.
1.6 Aromatase Inhibitors (AIs)

Aromatase is a good target for selective inhibition because estrogen production is a terminal step in the biosynthetic sequence. Three generations of the aromatase inhibitors have been developed. Currently, third generation aromatase inhibitors including anastrozole, letrozole and exemestane have replaced the first (aminogluthethimide) and second generation (formestane and fadrozole) aromatase inhibitors in the clinical application due to their greater selectivity in blocking the aromatase enzyme [79]. Anastrozole (Arimidex®), letrozole (Femara®) and exemestane (Aromasin®) are available for clinical use in North America, Europe and other parts of the world for treatment of postmenopausal breast cancer. Letrozole and anastrozole are triazole (antifungal) derivatives that are reversible, competitive aromatase inhibitors that bind to the heme unit of the aromatase enzyme and, at doses of 1-5 mg/day, inhibit total body aromatization and blood estrogen levels by 96% to > 99%. They could inhibit various local tissue estrogens levels by 73 to 89.0% [80]. Exemestane is a steroidal, irreversible (suicidal) inhibitor of aromatase that binds to the substrate site of the aromatase enzyme and inactivates it with similar potency for estrogen inhibition at a dose of 25 mg/day [81]. Adverse effects of these drugs are gastrointestinal upset, hot flashes, headache, and back pain based on studies in postmenopausal breast cancer patients [82].

1.7 Aromatase Inhibitors in Breast Cancer Therapy

During the past decade, AIs have been competing with the selective estrogen receptor modulators (SERMs) - especially tamoxifen - as endocrine therapy for breast cancer. They gained ground successively as first-line treatment for postmenopausal patients with hormone receptor-positive metastatic breast cancer [83] and as an FDA approved primary adjuvant treatment of early breast cancer in postmenopausal women [84-86]. They are now considered
the gold standard for the endocrine treatment of hormone receptor positive menopausal breast cancer [87]. Another emerging indication (previously a contraindication) is the use of AIs in pre-menopausal breast cancer patients with hormone sensitive tumors [88, 89]. This could revolutionize current concepts of breast cancer management in young women although years of research are needed prior to wide clinical application.

1.8 Breast Cancer Prevention: The Need

There is a current intense interest in the notion of breast cancer prevention, mainly due to the growing public awareness of the high incidence of breast cancer. One in nine women in North America is diagnosed with breast cancer [90]. Additionally, the spread of familial breast cancer screening programs, the successive discoveries in genetic diagnosis of deleterious mutations in breast cancer susceptibility genes and the development of epidemiological tools that can calculate breast cancer risk of an individual have together created a large population of healthy women with a strong perception of their high breast cancer risk. Nonetheless, no effective and tolerable preventive measures are presently available. For instance, BRCA1/2 mutation carriers who have a drastically elevated risk of breast cancer (up to 87% by the age of 70) [91, 92] are left with very invasive procedures such as bilateral prophylactic mastectomy or bilateral salpingo-oophorectomy or both [93, 94].
1.9 AIs Reduce the Risk of Breast Cancer in Menopausal Women, What is the Rationale?

- The established superiority of AIs over selective estrogen receptor modulators (SERMs) in the therapeutic setting made them candidates for primary prevention of breast cancer [95-99].

- The potential of AIs in breast cancer prevention has been substantiated by the large breast cancer adjuvant trials in which AIs significantly reduced the incidence of cancer in the contralateral breast compared to tamoxifen (43%, 46% and 56% greater reduction than tamoxifen in the ATAC, the MA-17, and the exemestane trials respectively). It is therefore, thought that AIs could prevent breast cancer incidence by more than 70% [100, 101]. This is higher than the 48% reduction of breast cancer risk caused by tamoxifen, which led to the FDA approval of tamoxifen for primary prevention of breast cancer in high-risk women [102]. However, the significant increase in venous thromboembolic events (RR = 1.9) and of endometrial cancer (RR = 2.4) have limited the use of tamoxifen for prevention. Currently, many women refuse to take tamoxifen because of its toxicity [103-105].

- Unlike SERMs, AIs have not been associated with thromboembolism or endometrial cancer and have no known adverse effects other than those expected from estrogen deprivation. [95, 106]. Tamoxifen was associated with a 4-fold increase in hysterectomies compared to anastrozole in the ATAC study, while anastrozole showed significantly less gynecologic side effects [107].

- Unlike SERMs which block estrogen receptors in the breast, AIs directly prevent estrogen synthesis. Evidence that the carcinogenic effect of estrogen is not solely
receptor mediated but may be related to genotoxic metabolites of estrogen suggests a preventive advantage of AIs over tamoxifen [33].

- Moreover, AIs increase the breast levels of androgens which may play an anti-proliferative role through activation of androgen receptors [108] resulting in increased ER beta and reduced ER alpha, leading to suppression of cellular proliferation [109, 110]

1.10 Why Aromatase Inhibitors Alone Are Not Used for Breast Cancer Prevention?

- AIs are known to create a global hypoestrogenic state that typically produces symptoms of menopause including hot flashes, skeletal and joint pains, mood swings and vaginal dryness. In postmenopausal women who commonly have such symptoms, giving AIs could be an aggravating factor. A drop-out rate of 20% was shown in women treated for early breast cancer due to these symptoms [111].

- The aromatase inhibitor-associated arthralgia syndrome is an increasingly recognized estrogen deprivation phenomenon that might occur in 30-40% of women and be disabling in 5-10% [112-114].

- Chronic AI therapy increases the risk of osteoporosis by depriving the bone of the protective effect of estrogen [115]. For example, exemestane given for 2 years significantly reduced bone mineral density in the femoral neck [116] while letrozole significantly reduced the hip and lumbar bone mineral density after 24 months with significant increase bone turn-over markers at 3-6 months of initiating the treatment.[117, 118]. In fact, bone fracture risk with all third generation AIs is believed to be up to 60% [119]. Multiple therapies were investigated to reduce the
AI-induced bone loss including tibolone, oral (risedronate and clodronate) and intravenous (zoledronic acid) bisphosphonates [120]. However, tibolone was shown to significantly increase the recurrence of breast cancer [121]. Such data are likely to discourage the use of tibolone in women with elevated risk of breast cancer. Adding bisphosphonates to aromatase inhibitors was shown to reduce bone loss in breast cancer patients. For example, six months of either risedronate or zoledronic acid led to significant increase in lumbar bone mineral density in breast cancer patients receiving AIs [122, 123]. As well, new therapies that showed a bone enhancing effect such as AMG-162 (Denosumab) acting as a receptor activator of nuclear factor-kB ligand inhibitor might be used in combination with AIs to counteract their negative effects on bones [124]. However, bisphosphonates are costly, have several side effects, especially on the gastrointestinal tract [125]. Both of the bisphosphonates and denosumab could only alleviate the bone associated adverse effects of AIs but are not designed to help the other aromatase inhibitors-induced hypoestrogenic symptoms including hot flashes.

- Furthermore, there is the evidence that the near total estrogen deprivation caused by AI could be a leading factor in the acquired resistance of breast cancer cells to AIs [126]. This resistance is believed to be due to activation of estrogen dependant gene transcription [127]. Concomitant estrogen therapy was able to restore the sensitivity of the cells to AIs and enhanced apoptosis [128, 129]. Similarly, this resistance mechanism might develop in normal breast cells with long-term preventive AI protocol in healthy women. The exhaustive deprivation of the natural ligand (estrogen) could lead to ER-alpha dependent activation of downstream cross talk
pathways such as mitogen activator protein kinase (MAPK), ErbB-2, and insulin like growth factors, which all lead to enhanced cellular proliferation [130]. This means the total absence of estrogen may lead to paradoxical consequences.

- Therefore, keeping a background of low estrogen exposure in the cells could actually prolong and enhance the effectiveness of the AI as a long-term preventive treatment. Unfortunately, ongoing phase III clinical trials investigating AIs in primary breast cancer chemoprevention, including the IBIS-II and the NCIC CTG MAP.3 trial [105, 131], have excluded women who are using hormone replacement therapy.

1.11 Aromatase Inhibitors in the Treatment of Endometriosis

Endometriosis has been treated by different modalities with the primary aim of minimizing ovarian estrogen production or antagonizing estrogen action that should ultimately deprive the endometriotic implants of the estrogen that enhances its growth and maintenance. However, it has been recently established that the endometriotic implants can produce significant amounts of estrogen locally through an independent intracrine process [39, 132, 133]. Prostaglandin E2, several cytokines and peritoneal hormones could stimulate both aromatase overexpression and activity in the endometriotic tissue, which results in the local production of estrogens from androgens [134-136]. Consequently, aromatase inhibitors have been used successfully as an alternative management for the treatment of endometriosis. Aromatase inhibitors could target both local estrogen formation within the endometriotic implants themselves, as well as systemic estrogen production in the ovary and adipose tissue [137]. Aromatase inhibitors have been presented as the next generation of drugs for the management of endometriosis [138, 139].
Several reports showed that aromatase inhibitors have been successful in the treatment of severe endometriosis in postmenopausal women [140, 141]. Aromatase inhibitor co-treatment significantly reduced pain compared with GnRH agonist alone [142]. The largest study so far is a prospective randomized trial that included 97 women with severe endometriosis. Patients were randomized to receive either a GnRH agonist (goserelin acetate implant) plus anastrozole versus the GnRH agonist alone. Fewer patients had recurrent pain with combination therapy during the study duration of 24 months [143]. The use of aromatase inhibitors as a single agent is recommended for women who are menopausal naturally or who had their ovaries removed (i.e. surgical menopause). Their use in premenopausal women is associated with stimulation of FSH release which might cause multi-follicular cyst formation. However, AIs can be used in this population if combined with a GnRH agonist, an oral contraceptive or less commonly a high dose of progesterone [144, 145].

1.12 Aromatase Inhibitors and Uterine Fibroids

Uterine fibroids are the most common benign tumor in women, with an approximate prevalence of 70% in Caucasians and more than 80% in African-American women by age 50 years [146]. Symptomatic uterine fibroids are often associated with significant uterine bleeding and pelvic pain and pressure symptoms. Surgical treatment is still the mainstay for symptomatic uterine fibroids. This in part is due to the absence of a simple, inexpensive, and safe long-term medical treatment.

Similar to endometriotic tissue, fibroids have been demonstrated to have aromatase and active local estrogen production [147, 148]. Aromatase was highly expressed in the fibroids of African-American women who have the highest incidence of this pathology [71].
Accordingly, AIs might be more effective than Gonadotrophin Releasing Hormone Agonists (GnRH-a) for the short-term treatment of uterine fibroids due to their profound and effective inhibition of local and peripheral estrogen synthesis (in contrast with GnRH-a which act mainly through the pituitary-ovarian axis). In premenopausal women, they have been used for short-term management [149-152]. Several case series have shown improved symptoms and reductions in the size of fibroids with the use of aromatase inhibitors [153, 154]. Reduced vascularity of the myoma and the uterus and a reduction in size of both are expected to occur in approximately 8-12 weeks of GnRH-a treatment [155, 156]. In a recent randomized clinical trial, letrozole was more effective than GnRH-a in reducing the volume of the fibroid without producing hypoestrogenic symptoms or changes in the blood hormonal profile [157].

However, the value of the in-situ estrogen inhibition by AIs becomes more obvious in postmenopausal women, since the persistence of the significant fibroid size or symptoms after the cessation of ovarian function indicates a local mechanism and apparently GnRH agonists will be ineffective in this age group. Further research is necessary to confirm the clinical effectiveness of AIs in long-term treatment of fibroids.

1.13 Other Gynecologic Applications for Aromatase Inhibitors

Recently, AIs have been investigated in other common gynecologic disorders of possible hormonal dependency with variable outcomes. Examples include their role in the treatment of ovarian cancer (with moderate therapeutic outcome and particular benefit in endometrioid ovarian cancer) [158], endometrial cancer [159] and endometrial hyperplasia [160, 161]. AIs have been also used in the treatment of advanced uterine leiomyosarcomas with no significant effect [162].
Figure 1-7: Current Clinical Applications of Aromatase Inhibitors: A schematic of the indications of aromatase inhibitors in premenopausal and postmenopausal women.
Chapter 2  A Hypothetical Model of Increased Local Estrogen Biosynthesis after Menopause and its Relation to Breast Cancer Development

2.1 Introduction

In the menopausal breast, estrogen acts as a key intracrine-paracrine molecule rather than a circulatory endocrine hormone [64-66]. The breast and the adipose tissue are capable of maintaining a significant level of local estrogens that could have a role in inducing or maintaining the development of cancer cells. Meanwhile, growing data supports the presence of normal breast stem cells with potential for malignant transformation into cancer stem cells that are capable of further driving the tumor growth and metastasis; generally referred to as the “cancer stem cell theory” [163].

In this chapter I present a hypothetical model for menopausal breast cancer development based on these two significant etiological pathways and the research referring to the link between them.

2.2 Model

Steroid sensitive stem cells (3SCells) exist in the normal breast comprising a side population of cells rich in stem cell properties and steroid receptors (including estrogen receptors). The estrogen receptors on these stem cells are upregulated by a positive feedback mechanism especially in women who have been exposed to high cumulative estrogen throughout their reproductive life (from both endogenous and exogenous sources). Around menopause, those women become hypersensitive to the considerable circulatory estrogen deprivation. The
sensitized stem cells and their abundant estrogen receptors signal feedback stimulation for local estrogen synthesis, in an effort to restore the high premenopausal estrogenic milieu they used to. This is mainly achieved by the activation of breast aromatase and other relevant enzymes. The subsequent local build-up of estrogen, a potent mitogenic factor, induces the multiplication of the 3SCells. Simultaneously, the hydroxy metabolites of estrogen induce DNA damage, by adduct formation and other epigenetic and mutational effects, to help establish a cancer stem cell (CSC) that is capable of tumor initiation and progression (Figure 2-1). Interruption of this cascade by either preventing the acute estrogenic withdrawal effect on already sensitized receptors (using estrogen therapy) or by blocking the synthesis of estrogen (using aromatase inhibitors therapy) could be a successful strategy for breast cancer prevention in high risk women.

2.3 Rationale

2.3.1 Evidence from In-Vitro and In-Vivo Studies

- **Characterization of Normal and Malignant Breast Stem Cells:** There is substantial evidence that cancer is generated by a tumor-initiating cell that has properties similar to those of other stem cells [164-166]. Breast cancer–initiating cells with several stem cell properties have also been identified [167, 168].

- **Evidence of Normal Breast Stem Cells Positive for Steroid Receptors:** Most breast cancers express steroidal hormone receptors [62, 101]. A series of recent studies by Clarke et al demonstrated a correlation between stem cell properties and the expression of steroid receptors (ER-alpha and PR) amongst normal human breast
cells. They proposed a model of a side population of stem cells that act as “steroid sensors” and could be the cancer initiating cells [165, 169, 170].

- **Estrogen as a Negative Feedback Regulator of Its Own Biosynthesis**: Estradiol was shown, in several studies, to significantly inhibit the activity of both aromatase and sulfatase (the enzyme catalyzing the production of active estrogens from estrogen sulphates) in breast cancer cell lines. A negative feed back of estrogen on its own production, therefore, has been suggested [171-173]. Likewise, breast cancer cells that were deprived of estrogen long-term have significantly higher aromatase expression compared to cells that were not estrogen deprived. The enzyme was inhibited upon re-exposure of the cells to estrogen [174]. In baboons, bilateral ovariectomy increased the breast aromatase activity while treatment with exogenous estrogens resulted in significant reduction of the aromatase mRNA and the activity of the enzyme. Based on these results, increased aromatase activity in menopausal women was suggested to maintain high local estrogen concentrations inside the breast [175].

### 2.3.2 Evidence from Clinical Studies

- Aromatase activity was slightly elevated in breast cancer patients who were not using estrogenic hormone replacement therapy (HRT) compared to HRT users [174]. Also, when estradiol was added to cancerous and normal tissues obtained from breast cancer patients, it expressed strong anti-sulfatase activity [173].

- According to the initial report of the Women’s Health Initiative study (WHI), women who received estrogen alone replacement therapy (CEE arm) for an average follow up period of 7.1 years had an over all non-significant decrease of breast cancer incidence
compared to women who were given placebo (P <0.06)[176]. In a sub-analysis of these results, it was found that estrogen therapy caused a significant reduction of breast cancer incidence in sub-groups of women including women who were first time users during the trial, women who were adherent to the treatment and those who had no family history of breast cancer and no past history of benign breast disease [177].

Figure 2-1: Steroid Sensitive Stem Cell Hypothesis: A normal breast stem cell functions as a steroid sensor (3SC). Estrogen receptors are up-regulated over the span of the premenopausal period when exposed to prolonged unopposed estrogen. At menopause this sensitized stem cell responds to the systemic estrogen decline by activation of aromatase which induces estrogen synthesis inside the breast. Locally accumulated estrogen increases the proliferation of the stem cells and the estrogen hydroxy metabolites are injurious to their DNA. A steroid sensitive cancer stem cell (CSC) eventually arises.

E = Estrogen

3SC= Steroid sensitive stem cell

CSC= Cancer stem cell
2.4 Discussion and Implications

Based on this model, a low dose estrogen therapy could be of potential benefit for breast cancer prevention, particularly when given to menopausal women (early around menopause) who have considerably higher cumulative exposure to estrogen during their reproductive life. The exogenous estrogen could act through a negative feedback mechanism to sustain the pre-menopausal suppression of local breast aromatase. Alternatively, AIs can lead to the reduction of breast cancer risk in such women. A combination of AIs and estrogen might work synergistically to increase the protective value of both agents compared to using either one alone. Earlier lifestyle strategies during the reproductive years could also help by reducing chances of sensitization of the 3SCells and the upregulation of estrogen receptors.

This theory integrates two independently well-supported hypotheses for breast cancer development; the cancer stem cell theory and the increase in local breast tissue estrogens. Both of these concepts are sustained by strong scientific evidence over recent decades. Furthermore, it puts common clinical breast cancer risk factors into reasonable perspective. For instance, intracrine-based estrogen synthesis in the breast explains why breast cancer risk does not decrease at menopause when the circulating levels of ovarian estrogens drop. On the contrary, the majority of breast cancers occur after the discontinuation of ovarian estrogen production [63]. As well, there is consistent epidemiological evidence of elevated breast cancer risk with prolonged and uninterrupted exposure to ovarian estrogens during the reproductive period. For example, early menarche, late menopause, nulliparity and low parity, late first term pregnancy; all result in prolonged or unopposed estrogen exposure and are known risk factors for breast cancer. Also, obesity after menopause, by providing expanded adipose sites for estrogen synthesis is also associated with higher risk of breast cancer [178].
The WHI study outcome described above agrees with this model. There was a statistically significant reduction of breast cancer incidence in the sub-groups of women who received estrogen-alone therapy. In women who have a low inherited risk for breast cancer, estrogen therapy might be able to provide significant protection since the carcinogenic mechanism in these women is likely due to the increase in local breast estrogen synthesis. However, women at high inherited risk might also benefit from an estrogen-AI combination therapy by reducing the additional impact of the rise in breast estrogens at menopause on top of existing genetic defects. This is further supported by data referring to an association between BRCA1 gene mutation and the upregulation of the aromatase gene [179, 180] and the role of BRCA1 gene in the regulation of estrogen and progesterone receptors [181-183].

This model also agrees with significant outcomes in the field of breast cancer prevention research. For example, tamoxifen significantly reduced breast cancer risk in postmenopausal women leading to approval of tamoxifen as a primary chemo-preventative drug [102] and to investigation of AIs for the same purpose [105]. Tamoxifen may have blocked the sensitized 3SC estrogen receptors so they are not activated by estrogen decline at menopause, whereas AIs directly block estrogen synthesis. However, AIs additionally prevent the formation of the genotoxic hydroxy metabolites of estrogen. Moreover, AIs increase breast levels of androgens which may have an anti-proliferative role through activation of androgen receptors [108] resulting in increased ER beta and reduced ER alpha leading to suppression of cellular proliferation [109].

Additionally, this hypothesis might also explain the role of progesterone – at least partially- in the puzzle of breast cancer pathogenesis. The results of the WHI suggested an elevated breast cancer risk with a combination of estrogen and progestin therapy for 5 years
after menopause. This may be attributed to an interfering effect from the continuous progesterone receptor stimulation on the functionality of estrogen receptors in a way that mimics or enhances the effect of menopausal estrogen deprivation, leading to further activation of local estrogen synthesis pathways. For instance, progesterone receptor transfection inhibited the effect of estradiol on estrogen receptors and the binding of estrogen receptor to the estrogen response element [184]. However, on the other hand, some studies showed a negative feedback effect of progesterone metabolites on aromatase [185]. Others have suggested that a combination of estrogen and progesterone early in reproductive life could mimic the protective effect of early pregnancy against breast cancer [186]. It seems that the critical balance between progesterone and estrogen concentrations and the timing of administering these hormones are together the deciding factor in modulating breast cancer risk. However, even if it is possible that estrogen-alone therapy could be protective against breast cancer in postmenopausal women, estrogen therapy alone increases the risk of endometrial cancer. An estrogen only protocol, therefore, would be ideal for women who have had hysterectomy. Women whose risk of breast cancer is considerably elevated due to familial and genetic factors could be considered for estrogen therapy plus a hysterectomy or a progesterone-releasing IUD to avoid the unfavorable systemic and breast effects of progesterone. In women with an intact uterus, adding aromatase inhibitors to estrogen ± progestin therapy could be effective in reducing the breast cancer risk associated with the prolonged use of this combined therapy.

2.5 Preliminary Data in Support of the Hypothesis

In the following 0, I present a retrospective cohort study in which a significant reduction in mammographic breast density occurred in healthy menopausal women who were given AIs
plus HRT (mostly combined estrogen-progestin therapy) for a median duration of 24 months compared to a similar group of women who used HRT alone. These data support the hypothesis and its implications for prevention.

2.6 Further Hypothesis Validation

Several investigations could be pursued to test this model. Examples include the characterization of breast cancer stem cells with elevated expression of the aromatase gene. Measuring elevated local breast estrogens or isolation of stem cells rich in estrogen receptors among menopausal women with significant past estrogen exposure (compared to women with low exposure) will also support this hypothesis. The identification of normal breast stem cells with reduced expression of the aromatase gene or aromatase activity in women who started estrogen therapy (early in their menopause) compared to women who have not used estrogen would be an additional support for the hypothesis.
Hypotheses

The hypotheses tested in the following chapters include:

- Aromatase inhibition in postmenopausal women could reduce mammographic breast density, a marker of breast cancer risk. Using aromatase inhibitors together with estrogen replacement therapy can minimize their hypoestrogenic side effects and allow for long-term use in preventative protocols.

- Aromatase inhibitors could reduce the breast background enhancement in breast MRI in postmenopausal women. The reduction in the breast parenchymal enhancement can be of value for diagnostic purposes.

- Developing minimially invasive assays for measuring local estrogens and other steroid hormones in the breast and other tissues is essential for evaluating the actual exposure of the breast tissue to locally produced hormones. Microfluidics is a new technology that can allow quantifying hormones extracted from minute volumes of biological samples.
Chapter 3  Aromatase Inhibitors and Mammographic Breast Density in Postmenopausal Women Receiving Hormone Replacement Therapy

One of the main concerns regarding long-term use of hormone replacement therapy (HRT) in symptomatic menopausal women is the perceived increased risk of breast cancer. A new protocol to reduce breast cancer risk in this population of women is investigated in this study. The hypothesis tested is that adding AIs to HRT would reduce local breast estrogen exposure and breast cancer risk without altering the beneficial systemic effects of HRT on menopausal symptoms or bone density. A retrospective cohort study is presented. The study group included postmenopausal women who received a low dose HRT daily plus letrozole 2.5 mg three times weekly. Postmenopausal women on HRT alone served controls. Mammographic breast density (MBD), the primary outcome, was measured using quantitative image analysis software and by visual analysis by a radiologist. Hypoestrogenic effects, adverse reactions, and bone mineral densities were secondary outcome measures.

3.1 Introduction

Breast cancer is the most common cancer in women comprising 10% of all cancers diagnosed and 7% of all cancer-related deaths around the world. In North America, it has been estimated that one in nine women will be diagnosed with breast cancer [90, 187]. Epidemiological and clinical studies demonstrated an increased risk of breast cancer with prolonged exposure to estrogen. Early menarche, delayed menopause, nulliparity, late age at first term pregnancy and obesity after menopause are known risk factors for breast cancer
In fact, the contribution of estrogen to breast cancer is well established and was recognized more than a century ago, when bilateral oophorectomy was first suggested as a method of treatment of advanced breast cancer [189]. Blockade of estrogenic signaling has been the core of the treatment of ER-positive and PR-positive breast cancer with the use of selective estrogen receptor modulators (SERMs) [101].

Estrogen plays multiple roles in breast cancer pathophysiology [60]. It is a mitogenic hormone that increases the rate of cell division, and thus reduces the probability for DNA repair [190]. The hydroxy metabolites of estrogens, which have been found in breast cancer in amounts significantly greater than those in the normal breast, are thought to exert direct damage to DNA. The free radicals produced from the catecholestrogens can cause single strand breaks, hydroxylation of the guanine bases and DNA adducts [33, 191].

Although breast cancer is estrogen dependent, there is no decrease in breast cancer risk at the time of menopause when circulating levels of estrogen fall [63]. Estrogen, therefore, in both postmenopausal women and in men plays a role as an intracrine and paracrine factor (rather than a circulatory hormone) [66]. Some authors, therefore, regard the serum levels of estrogens in postmenopausal women as a reflection of the non-metabolized excess of estrogen which has escaped from the intracellular compartment [192].

Aromatase is the key enzyme that catalyzes the final step of estrogen synthesis from androgens. Overexpression of aromatase in the mammary gland of transgenic mice leads to a significant increase of pre-neoplastic events such as hyperplasia, dysplasia, fibroadenomas and nuclear abnormalities [73]. Additionally, the expression of the aromatase gene is significantly higher in the adipose tissue of postmenopausal women compared to premenopausal women [76]. In breast cancer, approximately half of the intratumoral
estrogens are believed to be produced within the tumor itself or from the surrounding tissues. In postmenopausal breast cancer patients, the intratumoral estrogens reach up to 20 times the corresponding serum levels, consistent with local aromatase activity. The transcripts of the aromatase producing gene (CYP19) are markedly increased in postmenopausal breast cancer patients compared to premenopausal patients [72, 74, 75]. Aromatase activity was also significantly higher in breast tumor tissues and tissues adjacent to the tumors than in healthy tissues [193]. Despite the fact that the total amount of locally produced estrogen is low, the presence of aromatase in a large mass of breast tissues helps provide a concentration that can cause biological effects [192].

Aromatase inhibitors (AIs) are effective in reducing circulating estrogen concentrations in menopausal women [194] with a very good safety profile [95]. AIs are now the gold standard for the adjuvant endocrine therapy of ER positive breast cancer patients [84]. They have been tested in several phase III trials and impart a significant clinical advantage over previous drugs such as tamoxifen and megestrol acetate. The AI anastrozole was shown to be superior to megestrol acetate in the treatment of advanced breast carcinoma resistant to tamoxifen [195, 196]. In a consensus, the Central European Cooperative Oncology Group (CECOG) recommended the use of AIs as first-line treatment for postmenopausal patients with hormone receptor-positive metastatic breast cancer [83]. Moreover, letrozole was more effective than tamoxifen for ErbB-1– and/or ErbB-2–positive, estrogen receptor–positive primary breast cancer [84]. AIs have also resulted in significant improvement of overall survival and disease free survival in patients with ER/PR positive tumors [197].
Nonetheless, AIs are known to create a systemic hypoestrogenic state that typically produces or aggravates the symptoms of menopause such as hot flashes, skeletal and joint pains, mood swings and vaginal dryness. In the long term, osteoporosis and an increased risk of fractures are also expected.

The objective of this study was to investigate the effect of AIs on mammographic breast density in postmenopausal women receiving hormone replacement therapy.

3.2 Patients and Methods

3.2.1 Study Design

This was a preliminary retrospective cohort study that was conducted from December 2006 until August 2007. Ethics approval for this study was obtained from the Committee for Research on Human Subjects at Mount Sinai Hospital (06-0264-C). Women provided written consent after the study was explained to them.

3.2.2 Selection of Study Subjects

Study subjects were chosen from healthy postmenopausal women being treated with HRT in the Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, University of Toronto, Canada. The study group comprised 28 postmenopausal women who were prescribed the aromatase inhibitor, letrozole, (2.5 mg daily on Monday, Wednesday and Friday) in addition to their current HRT. These women had at least 2 annual mammograms; one within a year prior to the administration of the aromatase inhibitor and the most recent mammogram during the study. The women's initial use of AIs was not related to participation in the study, although all women were counseled regarding the theoretical and off-label nature of administering AIs to reduce their risk for breast cancer.
prior to receiving letrozole. Patients who had a present or past history of breast cancer were excluded.

The mammograms of the study group were compared with 2 control groups for mammographic breast density. One control group included a comparison between two annual mammograms of the study patients while on HRT alone prior to starting the AI. This control was to determine the trend of change in MBD with advancing of age in the same study population while taking hormone therapy only. The second control group included two annual mammograms of postmenopausal women who used HRT alone for one or more years and who had at least 2 regular mammograms during this therapy. This second control group was to verify the effects of age on MBD in a separate but similar group of women.

3.2.3 Assessment and Outcome Measures

3.2.3.1 Chart review

Clinical characteristics of the study and control subjects were recorded. Additionally, risk factors for breast cancer in both groups were analyzed. A validated Breast Cancer Risk Assessment Tool available online from the National Cancer Institute (NCI) was used to calculate the 5 years breast cancer risk based on the Gail model [198]. However, the Gail model does not take into account risk factors such as second degree relatives with breast cancer, family history of ovarian cancer, and breast cancer susceptibility genetic mutations which are considered in other risk models and considerably elevate the individual absolute risk for breast cancer [199]. Therefore, these additional risk factors were included in the evaluation of breast cancer risk in the subjects. Menopausal symptoms before and after the treatment with AIs were included as well as side effects and causes of treatment discontinuation. Bone mineral density records were also evaluated when available.
3.2.3.2 Breast density

Two mammogram formats were used in this study:

**Digital mammograms**: Obtained by Direct Digital Acquisition (DDA) and archived through the Picture Archiving and Communication Systems (PACS). The digital mammograms were burned on CDs with a DICOM viewer (eFilm (TM) Lite (TM) software Copyright ©1998-2005, Merge eMed, Milwaukee, WI USA) that allowed manipulation of the images and exporting them to other computer software.

**Analog mammograms**: Obtained by a conventional film-screen X-ray system using an analog acquisition technique. Digitization was done to convert the analog mammograms into digital formats suitable for further computer analysis. Kodak LS85 film digitizer (Eastman Kodak Co., Rochester, NY), a high-resolution film digitizer that produces digital signals as accurate as 0.001 optical densities, was used. For some patients, flatbed scanners; Agfa DuoScan (Agfa-Gevaert Group- Mortsel, Belgium) and ScanMaker i900 (Microtek International, Inc., Carson, CA) were used. To have identical scanning settings, each subject’s mammograms were compared using the same scanner and the same scanning parameters set in the corresponding scanning software. Only mammograms produced using the same format were able to be usefully compared. That is, digital vs. digital or analogue vs. analogue formats were compared pre and post treatment.

3.2.4 Quantitative Image Analysis of Breast Density

Available projections of both breasts were obtained. For the mammographic breast density (MBD) analysis by computer software program, the right craniocaudal (RCC) projections, or the left craniocaudal (LCC) projections were used - when the former were not available - based on our own data (unpublished) and previous data of the high symmetry existing
between different projections [200-202]. One film for each woman on HRT before the start of the AI treatment and the most recent mammogram during the combination of HRT and AI were compared. In this study, two image analysis software programs were used to assess MBD:

3.2.4.1 ImageQuant

ImageQuant (IQ) Version 5 (Copyright ©1998 Molecular Dynamics Sunnyvale, CA). This is a multi-purpose program which offers different modules for various types of samples. Each set of mammograms assigned for comparison was viewed simultaneously and the gray scale was adjusted to clearly visualize the breast edge. The same outline of the total area of the breast was used in both images. Chest wall structures were excluded from the outline. IQ calculates the area using the number of pixels for the outlined region. Then it assigns each pixel a numerical value called pixel intensity according to the absorbance of the image at that point, which in turn is an indication of breast tissue density. Finally, pixel intensity is integrated over all the pixels in the outlined area and presented as the integrated pixel intensity (IPI), (Figure 3-1).

**Background and breast compression correction**: IQ includes various types of background correction methods that subtract the background noise from the IPI to result in the approximate volume estimate of the MBD. The “Local Average” option in all measurements was selected so that the program calculated a background value equal to the average of all the points beneath the outline.
3.2.4.2 **ImageJ**

This is a free public domain Java image processing program offered by the National Institutes of Health (NIH). It can be used, either as an online applet or can be downloaded from (http://rsb.info.nih.gov/ij/). Using Image J, the integrated intensities of the pixels inside an outlined area was measured similar to IQ. In addition, Image J provided a visual representation of the changes in breast density by its “Surface Plot” function. Interactive 3-D surface plotting is also available as a plug-in. MBD in grayscale images could be additionally represented in a broad selection of colors that can be assigned for each of 256 possible displayed pixel values (Figure 3-2).
3.2.5 Visual Analysis of Breast Density

An experienced breast radiologist (CP) was responsible for the analysis of the MBD by visual inspection. The radiologist was blinded to time points, treatment status and any clinical data related to the mammograms. To assess the intra-observer reliability, all projections (RCC, LCC), and right and left mediolateral oblique (RMLO, LMLO) of the same patient were evaluated by the radiologist. In addition, random samples (around 20%) of the films were re-blinded and shown again to the same radiologist. The correlation coefficient for the intra-observer reliability was 0.92 for the visual scores of breast density.

Measurements of breast density were scored based on the Breast Imaging Reporting and Data System (BI-RADS) which is a qualitative method developed by the American College of
Radiology[203]. In addition, the two projections compared by the software analysis were also compared blindly by the radiologist by assigning (increase, decrease or unable to detect significant changes) of MBD for each pair of mammograms.

3.2.6 Data Recording and Statistical Analysis

IQ IPI data output was numerical and exported to a spreadsheet (Microsoft Office Excel 2003). The statistical tests were performed using SPSS 14.00 for Windows (Release 14.01, SPSS Inc., Chicago, IL, USA). The non-parametric Wilcoxon’s signed-rank test was used to analyze related continuous variables and the non-parametric Mann–Whitney U-test to analyze independent continuous variables. The chi-square ($\chi^2$) test was used to analyze categorical variables, and Spearman’s test was used in measuring bivariate correlations. $P < 0.05$ was considered statistically significant.

3.3 Results

3.3.1 Clinical Characteristics and Menopausal Symptoms

The characteristics of 28 postmenopausal women in the study group and 28 postmenopausal women in the control group are shown in Table 3-1. Study subjects all received the AI letrozole (Femara™, Novartis Pharmaceuticals, East Hanover, NJ) 2.5 mg orally three times weekly (Monday, Wednesday and Friday) with the exception of 2 women who were given anastrozole (Arimidex® Astrazenca Pharmaceuticals, LP) 1 mg daily because of headaches with letrozole. The women received HRT together with AI for a median duration of 24 months (range 2-63) months. Women received either estrogen and progestin if they had an intact uterus (about 75%) or estrogen alone if they had had a previous hysterectomy (about 25%). The estrogen was predominantly micronized estradiol (Estrace 1 mg), in the majority
of patients. The others received transdermal 17β estradiol. The progestin used was norethindrone 0.35 mg daily or micronized progesterone 100 mg daily by the majority of women. The remaining patients received medroxyprogesterone acetate 2.5 mg daily. In all cases, the HRT was not changed during the course of the study. Twenty-five of the 28 study subjects (89%) were at increased risk of breast cancer. During the study, six women had experienced one or more symptoms indicative of hypoestrogenism. Only one woman had a mild increase of hot flashes, 2 described an increase in joint and muscle aches, 2 complained of insomnia without night sweats or hot flashes and one complained of decreased libido. Three patients discontinued AIs after 2 months of use because of adverse effects including (headaches, nausea, vomiting, and diarrhea). We could retrieve bone mineral density measurements done before and after addition of an AI in 7 patients shown Table 3-2. In these seven women there was no decrease in BMD in the lumbar spine, the femoral neck or the total hip measurement.
Table 3-1: Clinical data of women in the treatment and the control groups: 28 women were included in each group with no significant differences in these characteristics.

<table>
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<th>AI+HRT (n= 28)</th>
<th>HRT controls (n=28)</th>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HRT total duration (Years)</td>
<td>14.48±6.69</td>
<td>1.31</td>
<td>13.91±7.35</td>
</tr>
<tr>
<td>Menopause (Natural/Surgical) :</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(E+P/E)</td>
<td>21 (75%)/7(25%)</td>
<td>HRT Indications</td>
<td></td>
</tr>
<tr>
<td>Vasomotor symptoms (Hot flashes, night sweats)</td>
<td>6 (21.43%)</td>
<td>4 (14.3%)</td>
<td></td>
</tr>
<tr>
<td>Sexual symptoms (Vaginal dryness, dyspareunia, decrease libido)</td>
<td>2(7.14%)</td>
<td>0(0%)</td>
<td></td>
</tr>
<tr>
<td>Psychological symptoms (Depression, mood swings)</td>
<td>2(7.14%)</td>
<td>0(0%)</td>
<td></td>
</tr>
<tr>
<td>Mixed menopausal symptoms</td>
<td>10 (35.71%)</td>
<td>18 (64.3%)</td>
<td></td>
</tr>
<tr>
<td>Osteoporosis</td>
<td>3 (10.71%)</td>
<td>3 (10.7%)</td>
<td></td>
</tr>
<tr>
<td>Premature menopause</td>
<td>5 (17.86%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### AIs indications

<table>
<thead>
<tr>
<th>Indications</th>
<th>Count (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>To reduce breast cancer risk</td>
<td>25(89.29%)</td>
</tr>
<tr>
<td>Severe endometriosis</td>
<td>2(7.14%)</td>
</tr>
<tr>
<td>Breast tenderness</td>
<td>1(3.57%)</td>
</tr>
</tbody>
</table>

### Breast Cancer Risk

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gail score of 1.67% or higher</td>
<td>10</td>
</tr>
<tr>
<td>Second degree relatives with breast cancer</td>
<td>6</td>
</tr>
<tr>
<td>Family history of ovarian cancer</td>
<td>4</td>
</tr>
<tr>
<td>BRCA mutation carriers</td>
<td>2</td>
</tr>
<tr>
<td>Others (High breast density, Combined HRT, Age)</td>
<td>3</td>
</tr>
</tbody>
</table>

### Menopausal symptoms after using AIs

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Count (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vasomotor symptoms</td>
<td>1 (3.57%)</td>
</tr>
<tr>
<td>Joint and skeletal pains</td>
<td>2 (7.14%)</td>
</tr>
<tr>
<td>Sexual symptoms</td>
<td>1 (3.57%)</td>
</tr>
<tr>
<td>Psychological symptoms</td>
<td>2 (7.14%)</td>
</tr>
<tr>
<td>Occasional night sweats</td>
<td></td>
</tr>
<tr>
<td>Joint and muscle aches</td>
<td></td>
</tr>
<tr>
<td>Decrease libido</td>
<td></td>
</tr>
<tr>
<td>Insomnia</td>
<td></td>
</tr>
</tbody>
</table>

### Bone mineral density (N=7)

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Mean± SD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMD-Lumbar before AI</td>
<td>0.97±0.12</td>
<td>0.735</td>
</tr>
<tr>
<td>BMD-Lumbar after AI</td>
<td>0.98±0.14</td>
<td></td>
</tr>
<tr>
<td>BMD-Femoral before AI</td>
<td>0.73±0.07</td>
<td>0.866</td>
</tr>
<tr>
<td>BMD-Femoral after AI</td>
<td>0.73±0.09</td>
<td></td>
</tr>
<tr>
<td>BMD-Hip before AI</td>
<td>0.86±0.07</td>
<td>0.028</td>
</tr>
<tr>
<td>BMD-Hip after AI</td>
<td>0.89±0.09</td>
<td></td>
</tr>
</tbody>
</table>

Table 3-2: AIs in the study group: The majority of AIs inhibitors were originally given to the patients to reduce breast cancer risk that was elevated due to factors mentioned in this table. Menopausal symptoms after starting AI and throughout the treatment period are also shown. Bone mineral density measurements in 7 patients show no statistically significant difference between the AI+ HRT and the HRT groups in the density of the lumbar and femoral neck. There was a significant increase in the total hip bone density.

### 3.3.2 Mammographic Breast Density

A total of 203 films were digitized and another 235 mammograms were obtained as digital images. The mammograms of eighteen (18 out of 28) postmenopausal women on AI and
HRT were eventually entered into the statistical analysis (Figure 3-3). The remaining 10 women were excluded from the MBD assessment for the following reasons. There were six women in whom one of the pre-treatment or post-treatment mammograms was not available or one mammogram was analog and the other was digital, precluding comparison since previous data showed that MBD is significantly lower in the case of digital compared to analog acquisition [204]. One high risk patient had a prophylactic bilateral mastectomy. Three other women used AIs for two months only and were excluded from the mammogram analysis. The minimal duration of AI use was 5 months in the study group.

Figure 3-3: The mammograms of sixteen of the 18 patients in the (AI+ HRT) group are compared before and after the treatment: Picture (a) in each pair is the mammogram before the treatment whereas picture (b) is after the treatment.
Table 3-3 shows the characteristics of women entered in the mammogram analysis comparing the study with the control group.

There were no statistically significant differences between the study and the control women regarding their age, body mass index (BMI), risk of breast cancer, duration of HRT and the interval between the compared mammograms.

<table>
<thead>
<tr>
<th></th>
<th>AI+HRT (N= 18)</th>
<th>HRT controls (N=22)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean± SD</td>
<td>SEM</td>
<td>Mean± SD/N</td>
</tr>
<tr>
<td>Age (Years old)</td>
<td>63.06±6.99</td>
<td>1.75</td>
<td>65.45±8.28</td>
</tr>
<tr>
<td>Age at the 1st mammogram (Years old)</td>
<td>59.72±7.45</td>
<td>1.86</td>
<td>60.86±8.75</td>
</tr>
<tr>
<td>BMI (Kg/m²)</td>
<td>25.57±5.44</td>
<td>1.57</td>
<td>24.40±4.07</td>
</tr>
<tr>
<td>Duration between the 1st and the 2nd mammograms (Years)</td>
<td>2.44±1.24</td>
<td>0.29</td>
<td>3.00±1.41</td>
</tr>
<tr>
<td>HRT total duration (Years)</td>
<td>14.13±7.97</td>
<td>1.99</td>
<td>15.4±7.4</td>
</tr>
<tr>
<td>Type of HRT product (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estradiol 1mg</td>
<td>88.9%</td>
<td></td>
<td>68.2 %</td>
</tr>
<tr>
<td>Norethindrone 0.35 mg or Progesterone 100 mg</td>
<td>100.0%</td>
<td></td>
<td>81.3%</td>
</tr>
<tr>
<td>Breast cancer risk factors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gail Risk Score (Mean± SD)</td>
<td>1.82±0.96</td>
<td>0.22</td>
<td>1.7± 0.66</td>
</tr>
<tr>
<td>Gail score higher than the estimated risk for the same age/race (N/%)</td>
<td>6 (33.3%)</td>
<td></td>
<td>6 (27.3%)</td>
</tr>
<tr>
<td>Second degree relatives with breast cancer</td>
<td>5 (27.8%)</td>
<td></td>
<td>3 (13.6%)</td>
</tr>
<tr>
<td>Family history of ovarian cancer</td>
<td>3 (16.7%)</td>
<td></td>
<td>1(4.5%)</td>
</tr>
<tr>
<td>BRCA mutations</td>
<td>1(5.6%)</td>
<td></td>
<td>0(0%)</td>
</tr>
</tbody>
</table>

Table 3-3: Characteristics of women in the treatment and the control groups in the mammographic breast density analysis: 18 women and 22 women in the treatment and the control groups were compared. No statistically significant difference was found in regard to these characteristics.
3.3.3 Image Analysis of MBD

The software image analysis of MBD showed a statistically significant reduction of the total integrated pixel intensity (IPI) as well as the percentage of dense IPI in the women who used an AI plus HRT, whereas there were no significant changes of the IPI observed between either the two films in the control group or between the two control films in the study group prior to starting on an AI (Table 3-4 and Figure 3-4).

<table>
<thead>
<tr>
<th></th>
<th>AI+HRT (n= 18)</th>
<th>HRT alone (n= 22)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean + SD</td>
<td>SEM</td>
</tr>
<tr>
<td>Total IPI 1st Mammogram</td>
<td>$2.1 \times 10^{10} \pm 2.6 \times 10^{10}$</td>
<td>0.62 x$10^{10}$</td>
</tr>
<tr>
<td>Total IPI 2nd Mammogram</td>
<td>$1.2 \times 10^{10} \pm 1.2 \times 10^{10}$</td>
<td>0.27 x$10^{10}$</td>
</tr>
<tr>
<td>Δ Total IPI</td>
<td>$-0.85 \times 10^{10} \pm 1.5 \times 10^{10}$</td>
<td>0.36 x$10^{10}$</td>
</tr>
<tr>
<td>Percent of total IPI change from the baseline IPI</td>
<td>$-17.48 \pm 24.32$</td>
<td>5.73</td>
</tr>
<tr>
<td>Percentage Dense IPI 1st Mammogram</td>
<td>$50.44 \pm 23.33$</td>
<td>5.49</td>
</tr>
<tr>
<td>Δ Percentage Dense IPI 2nd Mammogram</td>
<td>$43.60 \pm 22.65$</td>
<td>5.34</td>
</tr>
<tr>
<td>Δ Percentage Dense IPI</td>
<td>$-6.84 \pm 9.00$</td>
<td>2.12</td>
</tr>
</tbody>
</table>

Table 3-4: Integrated pixel intensity (IPI) of mammographic breast density in the treatment and the control groups. Statistically significant difference is shown in the total IPI, delta IPI as well as in the percentage of IPI in the dense areas of the breast to the whole breast and the delta of the dense IPI between the 1st and the 2nd mammograms of the comparison.
Figure 3-4: Mammographic breast density in the AIs plus HRT group represented by the ImageJ software-Surface Plotting: Interactive 3-D surface plotting (second column) shows MBD in gray scale. Broad selection of colors that can be assigned for each of 256 possible displayed pixel values (third column) can be obtained by the look up table tool.

3.3.4 Radiologist Assessment of MBD

The radiologist detected a significant change in 21 of the 56 pairs of mammograms evaluated (37.5%). In these cases, the radiologist was able to confirm significant changes in the MBD between the two films in each subject when the mean change from the baseline IPI on image analysis was 35.6% with a median value of 27.9%. When the mean change of the total IPI on image analysis was less than 24.2% and the median was 11.3%, the radiologist was unable to visually confirm a significant change in breast density. Whenever the radiologist confirmed a
significant change in MBD, there was a significant correlation (P= 0.027, R = 0.482) between the radiologist’s trend of change and the trend shown by the image analysis software (i.e. increase or decrease in MBD). The BI-RADS scores of dense areas to the total area of the breast seemed to be less sensitive than the image analysis assessment and there was a non-significant change of BI-RAD scores in both the study and the control groups.

3.4 Discussion

It is well established that breast density is one of the strongest risk factors for breast cancer, only surpassed by age and breast cancer susceptibility genetic mutations [205, 206]. Women with the highest mammographic breast density (MBD) have a 4 to 6 fold increase in breast cancer risk compared to women who have the lowest MBD [207]. Breast cancer risk also increases in BRCA mutation carriers with an increase in MBD [208]. Mammographically dense breasts have been associated with an increase in proliferative lesions such as hyperplasia, atypia and carcinoma in situ [209]. Reduction of MBD was associated with a reduction of breast cancer risk [210, 211]. Additionally, high breast density tends to decrease the sensitivity of mammograms for cancer detection [212] and a reduction of breast density should make mammograms more sensitive for earlier detection [213]. Therefore, recognizing or developing factors that can modify breast density is an important approach in breast cancer prevention [214].

The present study findings agree with other studies that examined various strategies to reduce MBD. Tamoxifen caused significant reduction of MBD in women with high breast cancer risk [215, 216]. Gonadotrophin releasing hormone agonists (GnRH-a) reduced MBD in six BRCA1 mutation carriers. Patients were put on a low dose of estrogen, intermittent progesterone and testosterone to alleviate the marked hypoestrogenic effects of the GnRH-a
Likewise, tibolone, given for one year, demonstrated a significant decrease in MBD in postmenopausal women with dense breasts [218]. Another study demonstrated a non-significant reduction in the MBD of 30 premenopausal women who used isoflavones for one year [219]. Apart from pharmacological interventions, there has been one study that demonstrated that dietary changes such as adopting a low-fat high-carbohydrate diet could significantly reduce breast density [220].

On the other hand, a large number of studies investigated the effect of HRT in different regimens, doses and durations on MBD in menopausal women. Combined HRT, especially with continuous progestin regimens, significantly increased MBD [221-224] and the MBD changes decreased upon HRT discontinuation [225]. HRT-induced increase in MBD is known to reduce the sensitivity and the specificity of mammograms [212, 226].

In one study, MBD measured by ImageQuant software and a previously validated computer-assisted thresholding technique (Cumulus 108 software, University of Toronto) and by subjective visual analysis all had a positive correlation with Gail risk for breast cancer [227]. However, the planimetry and thresholding methods commonly used to measure changes in MBD calculate the percentage of the dense to the total area of the breast, considering the breast a two dimensional surface. This methodology applies an all or none rule (dense vs. non dense) to the calculation so that changes in density above or below the reader-defined threshold, a subjective decision, are missed. Additionally, these methods do not account for the total size of the breast nor the total amount of fibroglandular tissue. Therefore, a small breast could be judged as having a larger percentage of dense areas although it does not have much fibroglandular tissue in absolute terms [228].
In this regard, the advantage of the approach used in this study is the calculation of the total densities of all the breast tissue in the mammogram. IQ and ImageJ software both take into consideration the breast as a three dimensional structure so that each pixel in the image represents a volume rather than an area unit of the breast. In other words, the same region of the breast could be recognized as being slightly more or less dense in a serial section of films of the same patient (Figure 3-4). As well, having several background correction and subtraction tools that can adjust for dissimilarities in the dose of radiation and the processing of the films enabled more objective and consistent analysis of the MBD. Differences in positioning and compression of the breast can be also managed by adjusting the outline tools in the software.
Figure 3-5: Mammographic breast density in the AIs plus HRT group represented by the ImageJ software - Image Subtraction: Image subtraction shows the sites of increased or decreased breast densities between the 2 images before and after the AI.

**Hormone Replacement Therapy: Current Application:**

The concerns raised by the earlier reports of WHI of the increased risk of breast cancer in women who were using combined HRT and the increased risk of cardio-vascular accidents[229] led to current clinical guidelines recommending the use of the lowest HRT dose for the shortest duration. However, these guidelines only reduced the mass use of HRT
as a life-long cardio-protective treatment but have not significantly changed the prevalence or
the clinical indications for using HRT to treat menopausal symptoms. A recent report
showed that 97% of 600 physicians in Europe and US believe that HRT was beneficial for
their patients and 92% would prescribe it for their family while 90% believe the benefits of
HRT outweigh the risks in indicated patients. In fact 78% thought the WHI media
interpretation was not justified [177].

According to a recent analysis of the estrogen alone (CEE) arm of the Women’s
Health Initiative (WHI) study, the effect of estrogen therapy for 7.1 years included a
significant reduction of the total fracture risk as well as of fractures of the hip, vertebra and
wrist bones. There was a modest albeit consistent increase in bone mineral density as well
[230, 231]. A positive correlation between serum estradiol and the bone density as well as its
ability to increase collagen deposition and bone mass in postmenopausal women with
osteoporosis, led to the recommended use of estrogen in the treatment of osteoporosis [232,
233]. Further, progesterone may augment the osteoblastic activity to synergize with estrogen
[234].

Although there have been various alternative treatments for the relief of menopausal
symptoms, no treatment so far has been shown to provide the same level of symptom relief
as estrogen [235]. Also, recent analyses of WHI subgroup data have demonstrated
cardioprotection of HRT in women between 50 and 59 years of age [236].

From the evidence above, it seems that the estrogen therapy in postmenopausal
women could play a protective role in many tissues. Suppression of local estrogen in the
breast by using a potent aromatase inhibitor and adding back systemic estrogen to allow a
physiologic circulatory level has been suggested – in theory – as a novel approach that could
enhance the acceptability of aromatase inhibitors in a long term prevention protocol [97, 102, 237]. This strategy could offset the increased breast cancer risk reported in the WHI study in similar women using HRT regimens that contain progesterone.

Although there was a decrease in breast cancer risk in the estrogen alone arm of the WHI study [176], patients on estrogen alone therapy were also included in the present study since many of these women had an elevated breast cancer risk due to other factors such as family history of breast cancer. The number of women who used the combined or the estrogen alone HRT was comparable in both the study and control groups.

One recent pilot study of letrozole given for 6 months to women on HRT has shown a reduction in the breast proliferation marker Ki67, although there was not a significant reduction in MBD [238], perhaps because of the shorter duration of AI use compared to the present study.

The present study was the first investigation to report the use of an aromatase inhibitor plus HRT for extended durations of up to 5 years, and to show a significant reduction in mammographic breast density with AI plus HRT in healthy women with no previous history of breast cancer. This protocol was well tolerated with few hypoestrogenic side effects. Therefore, the use of AIs in women who receive HRT could reduce their breast density without causing hypoestrogenic symptoms. Similarly, a low dose add-back HT could be added to aromatase inhibitors in a chemopreventative protocol to reduce any negative effect on bone mineral density in women at high risk for breast cancer.

Nevertheless, this study is limited by being an observational study in a small number of patients. Prospective randomized studies are needed to ensure that all the technical factors
that might interfere with accurate measurements of MBD are controlled for and to replicate our findings before clinical recommendations can be made.

In Chapter 6, I outline the design and protocol of a current double-blinded randomized placebo controlled trial that was developed based on the findings of this preliminary study to explore the effects of combined AI and HT on breast density and on other markers of breast cancer risk as well as markers of safety of this protocol.

3.5 Conclusion

Using aromatase inhibitors could reduce mammographic breast density in postmenopausal women using hormone replacement therapy without producing significant hypoestrogenic symptoms.
Chapter 4 The Effect of Acute Aromatase Inhibition on Breast Parenchymal Enhancement in Magnetic Resonance Imaging in Postmenopausal Women

In this chapter, I present results of a prospective pilot clinical trial investigating the effect of a new protocol of an acute inhibition of aromatase in postmenopausal women. The tested hypothesis is that estrogen deprivation therapy could reduce breast gadolinium enhancement in breast MRI and thereby improve the performance of this technique. The implications for patient care includes: 1) by reducing the non-specific breast background enhancement, patients would have less call backs and unnecessary invasive procedures for false positive results of breast MRI. 2) Theoretically (not assessed in this study), lowering the background parenchymal enhancement might enable the detection of small malignant areas of enhancement that would be otherwise masked.

4.1 Introduction

Breast magnetic resonance imaging (MRI) is increasingly utilized in diagnosing and staging breast cancer [239]. It has been shown to be more sensitive than mammogram in detecting multiple cancerous foci especially in patients with dense breasts [240]. It is also more accurate in detecting high grade tumors [241] and is more sensitive than mammograms and breast ultrasound in diagnosing breast cancer in BRCA mutation carriers [242, 243]. Mammograms appear to be inadequate as a screening method for the BRCA mutation carrier high risk group [244].
The main difference between MRI and other routinely used breast imaging techniques is that the magnetic field produced by the MRI results in detailed cross-sectional images of the breast which have very good soft tissue contrast. This contrast between various types of breast tissues such as between fat and glandular tissue is determined by the mobility and magnetic environment of the hydrogen atoms in water or fat which is reflected in the form of brightness of tissues that can be quantified as a signal intensity [245]. Therefore, a peculiar MRI feature is its ability to demonstrate physiological changes in addition to detection of the anatomic abnormalities. The fundamental diagnostic ability of breast MRI is based on the difference in angiogenesis and patterns of vascularization in breast carcinoma and benign breast tissue. Specifically, this information is obtained after intravenous injection of a contrast dye, commonly gadolinium, which is a non radioactive material typically taken up by highly vascular and permeable tissues leading to image enhancement of this tissue that distinguishes it from the surrounding less vascular breast areas. Malignant breast tissue is known to enhance faster and to a greater degree than normal breast tissue or benign lesions, possibly due to its increased vascularity. Also, the malignant tissue is less able to retain the dye due to increased vessel permeability and abnormal structure of blood vessels causing a rapid wash out of the enhancement. On the other hand, the normal breast parenchyma typically should show no, or minimal, enhancement that is maintained longer.

Despite the high sensitivity of MRI (80-95%), its specificity is relatively low (65%-72%) [240, 246-249]. This means that normal breast tissue could also significantly enhance leading to false positive callbacks of patients and a low positive predictive value of the technique. Understanding that breast vascularity [250] and composition [251] could be significantly affected by hormonal variations; therefore, the objective of the present study
was to determine the effect of estrogen deprivation therapy using a high dose of an aromatase inhibitor on the normal breast tissue enhancement in postmenopausal women undergoing breast MRI.

**4.2 Patients and Methods**

**4.2.1 Study Design**

This was a prospective pilot interventional clinical trial. Sixteen women were recruited for the study between October 2008 and September, 2009. Research ethics approvals were obtained from Health Canada and Mount Sinai Hospital Ethics Board (REB # 08-0116-A).

Volunteers were eligible to participate if they were 35 years or older and had induced or natural menopause (had no menstrual bleeding during the past 12 months). Women who had a history of bilateral mastectomy, osteoporosis, and renal impairment were excluded. The aromatase inhibitor letrozole (Femara TM Novartis Pharmaceuticals Canada Inc., Dorval, QC) was given to each patient in a dose of 12.5 mg per day (five 2.5 mg tablets) for 3 successive days immediately prior to breast MRI. Patients were counseled thoroughly about the investigational nature of using the medication for this new indication and the possible side effects of the letrozole in this relatively high dose.

Each patient had two standard bilateral breast MRI studies. The duration between the 2 MRI studies ranged between 3-5 weeks. The patient was excluded from continuing in the study if incidental breast cancer was found on the pre-treatment scan. Women were asked to take the medication 3 full days prior to the 2nd MRI study. They were asked to record any adverse effect they noticed during these days or in the following week.
All MRIs were performed at a tertiary academic hospital with an established high-volume clinical breast MRI service. A routine clinical breast MRI protocol was used as described below. Women were scanned in the prone position with a dedicated breast coil. The MRI protocol was standardized in all cases. Diagnostic enhanced breast MRI was performed using a 1.5T magnet (SIGNA LX Echo speed, GE Healthcare) with a phased-array coil. The protocol included an axial localizer sequence followed by sagittal and axial fast spin-echo T2-weighted sequences. Pre and post gadolinium-enhanced sagittal 3D fast spoiled gradient echo (FSPGR) sequences were done. Gadopentetate dimeglumine was injected IV at a dose of 0.16 mmol/kg body weight, followed by 20 mL saline flush (0.9%). Field of view used a matrix of 512 × 256.

4.2.2 MRI Analysis

The diagnostic evaluation of both MRI studies was done by independent radiologists not involved in the study. The breast MRI studies of all patients were analyzed by an experienced breast imaging radiologist using both subjective evaluation of the contrast enhancement (visual qualitative analysis) as well as by standard image analysis software (quantitative analysis). The radiologist was blinded to the status of the studies (i.e. not knowing their chronological order). For each scanned slice of the breast, 4 images were taken including a pre-contrast image followed by 3 post-contrast images at 1 min, 2 min and 6 min. These 3 images represented the contrast uptake and washout by the breast tissue, and each selected slice was examined by stacking all 4 images. A total of 448 images were analyzed.

At analysis, three random slices of the left breast were selected for all patients by another investigator attending with the radiologist. The Multiphase Dynamic sequences were selected in all comparisons. The slices were selected to include a majority of fibroglandular
tissue. Anatomically similar slices - using both structural landmarks and anatomical site - were selected in the pre and post treatment MRI studies. The radiologist was asked to evaluate four parameters: 1) To confirm the anatomical agreement of each compared pair of slices, 2) To select a region of interest (ROI) of normal parenchymal breast tissue that showed the most recognized enhancement (ductal, nodal and vascular structures were excluded in the selection). 3) To determine the kinetic pattern of enhancement in that ROI, whether washout, plateau or progressive and then the same region was assessed for enhancement by the image analysis software. 4) To review the whole breast and explore other MRI sequences - including the subtracted sequences - of each study to determine which study was of greater diagnostic confidence, determined by the abundance of lesions with suspicious pattern of enhancement. For further confirmation, after finishing the blinded analysis, the radiologist was asked to unblind the studies and select the slice that appeared to include the most obvious enhanced background area in the pre-treatment study to compare it – using the image analysis- with the corresponding area in the post-treatment study.

Image analysis was performed using the e-film workstation 3.1 (Merge Healthcare, Milwaukee, USA). A region of interest (ROI) of 0.1 cm diameter was selected in all the images. The ROI was copied to all the 4 scans of each slice. The average number representing signal intensity was recorded and the relative enhancement (percentage of increase in signal intensity) was calculated as \((\text{SI}_c - \text{SI})/\text{SI} \times 100\), where \(\text{SI}\) and \(\text{SI}_c\) are the precontrast and the postcontrast signal intensities, respectively [14]. This was calculated for the 3 time points post-contrast (i.e. after 1, 2 and 6 minutes).
4.2.3 Statistical analysis

E-film image analysis data and the radiologist’s evaluation were recorded and percentage enhancement was calculated using Microsoft Office Excel 2003. The statistical tests were performed using PASW 18.00 for Windows (Release 18.0.0, SPSS Inc., IBM, Chicago, IL). The paired samples t-test and Wilcoxon’s signed-rank test were used to analyze the related continuous variables (the change of the relative enhancement in same patients between the pre and the post-treatment studies). P < 0.05 was considered statistically significant.

4.3 Results

Fourteen women completed the study while two women withdrew during their baseline MRI study (due to MRI claustrophobia and overweight). Relevant clinical characteristics of the study subjects are shown in Table 4-1. One patient had previous unilateral mastectomy for breast carcinoma. Two patients were on long-term GnRH-agonist treatment for endometriosis, rendering them in a menopausal state.

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SD</th>
<th>Number of Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years old)</td>
<td>60± 11</td>
<td></td>
</tr>
<tr>
<td>Weight (Ibs)</td>
<td>144± 26</td>
<td></td>
</tr>
<tr>
<td>Duration of HRT (years)</td>
<td>12± 10</td>
<td></td>
</tr>
<tr>
<td>Menopausal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post menopausal</td>
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<td>12</td>
</tr>
<tr>
<td>Premenopausal (on GnRh agonist)</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Type of HRT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Combined HRT</td>
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<td>11</td>
</tr>
<tr>
<td>Estrogen alone</td>
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<td>2</td>
</tr>
<tr>
<td>Androgen</td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

Table 4-1: Clinical Characteristics of Study Subjects

Following the baseline MRI study, additional work-up was recommended for the indeterminate areas of enhancement in four patients. These four patients underwent additional imaging studies and biopsy if necessary. Specifically, targeted breast sonography
was performed in all four cases, mammogram was performed in two patients, and ultrasound-guided core biopsy was performed in one patient. None of the patients was diagnosed with cancer during the follow-up period between 6-14 months. None of the patients had significant adverse effects and they all tolerated the high dose of letrozole well (the usual therapeutic dose is 2.5 mg/day) and any reported side effects were minimal and self-limiting. One patient had headaches during the 3 day treatment course and two had arthralgia during the first week following the letrozole intake.

Using the blinded image analysis of the breast MRI sections, overall the average breast enhancement of all patients in the post-treatment group was statistically lower than the average enhancement in the post-treatment studies (p=0.005) (Figure 4-1). The breast enhancement was reduced in ten of the fourteen patients after the treatment, and 7 patients showed significant reduction. When the average enhancement at each time point (i.e. SI1, SI2 and SI6) were independently evaluated, the reduction was yet not significant (SI1:SI0, SI2:SI0 and SI6:SI0 showed p-values of 0.32, 0.09, and 0.14 respectively).
Figure 4-1: Average Background Enhancement (blinded analysis): This shows the average of the relative contrast enhancement in all patients (3 breast slices of each patient) at baseline and after treatment with letrozole at the three time points after contrast injection. Error bars represent average SD of the enhancement in the 3 breast slices in all patients.

The un-blinded confirmatory analysis by the radiologist of the most enhanced lesion in the breast also showed a similar significant reduction of the average enhancement ($p=0.010$) (Figure 4-2). However, only six of the 14 patients showed significant reduction in their post-treatment scans. The individual time point comparison showed a non-significant reduction with $SI_1:SI_0$, $SI_2:SI_0$ and $SI_6:SI_0$ showing p-values of 0.31, 0.33 and 0.15 respectively.
The radiologist showed more confidence (i.e. fewer/less suspicious areas of breast enhancement) when interpreting the post treatment MRIs in 8 patients compared to the pre-treatment MRI studies. The radiologist judged an equal confidence in 2 patients and more confidence in 4 patients in the pre-treatment MRI.

4.4 Discussion

The breast is a dynamic and highly hormonally sensitive organ [252]. Clinically, breast tenderness is experienced by premenopausal women when estrogen and progesterone levels are elevated such as in the premenstrual phase, during pregnancy, with hormonal contraceptives or in postmenopausal women on hormone replacement therapy [253].
These symptoms could be attributed to composition and vascular changes due to elevations of the ovarian hormones. Furthermore, these hormonal variations might affect the appearance and behavior of breast tissue in different imaging techniques. For instance, the breast parenchyma enhances differently in MRI during the follicular (i.e. pre-ovulation) and luteal (post-ovulation) phases of the menstrual cycle [254-257]. The fat and water composition of the breast was also shown to be variable during the menstrual cycle [258, 259]. Furthermore, breast volume (evaluated by MRI) could increase in the luteal compared to the follicular phase [260]. Also, breast density in mammograms has been shown in some reports to be variable across the menstrual cycle [261].

These observations led to exploring the hypothesis that breast parenchyma might also show characteristic changes on breast MRI in response to anti-hormonal treatment. Although the long-term effect of selective estrogen receptor modulators (SERM) or estrogen deprivation by aromatase inhibitors on mammographic breast density have been studied [262] [263-265], there are only studies of the effect of SERMs on breast MRI. Oksa et al studied the effect of toremifene on breast MRI indices, and found a significant decrease in the maximum slope of enhancement after 3 menstrual cycles of treatment. [266]. Also, raloxifene was shown to significantly decrease breast MRI volume after 1 and 2 years of use [267]. One study has shown that breast cancer patients receiving tamoxifen had a lower rate of false positive benign enhancement and the MRI was able to detect all cancers (i.e. no false negative results)[268].

Despite being the gold standard in the adjuvant endocrine treatment of breast cancer [87, 269], there have been no studies, apart from ours, of the effect of aromatase inhibitors on breast MRI. AIs suppress aromatase, the rate-limiting enzyme catalyzing estrogen synthesis
from androgenic precursors. Unlike tamoxifen which functions mainly by blocking the estrogen receptors at specific sites, AIs reduce the production of estrogen made in peripheral tissues. This peripheral intracrine aromatization of androgens is the primary source of estrogen production in postmenopausal women and is responsible for all the estrogen exposure of the breast in menopausal women. In fact the increased expression of the aromatase gene was linked to several pathological structural changes in the breast [73]. Considering the evidence that postmenopausal women might have a higher expression levels of the aromatase [72, 74-76], the study group was selected mainly from postmenopausal women, who also represent the majority of high risk women.

The results of this study suggested an overall suppression of breast parenchymal enhancement on MRI by letrozole, and a slightly better subjective level of diagnostic confidence of the radiologist in image evaluation. These findings provide a preliminary indication that the acute administration of an aromatase inhibitor prior to MRI might help improve the technique specificity by reducing the intensity of non-specific parenchymal enhancement (Figure 4-3). This seems to be of importance in the light of the low specificity of MRI. In this study, four out of fourteen patients were called back after their baseline MRI for another imaging technique or biopsy, all of which confirmed the benign nature of suspicious areas of enhancement (i.e. specificity of 71% which agrees with previous reports).

Hypothetically, the decrease in the level of contrast enhancement of the breast tissue might improve detection of a small true enhancing cancerous lesion. This however needs to be independently investigated in patients with early breast cancer. On the other hand, the possibility of masking the enhancement of a malignant lesion by such protocol should be also
excluded prior to its wide clinical application. In Schnall, Orel at al’s study of tamoxifen, this possibility was excluded as mentioned above.

Figure 4-3: Breast enhancement in one subject pre and post treatment MRI studies: Panel A) shows the serial MRI images of one breast slice of one of the study subjects at baseline, the region of interest is circled. Image 1 is pre-contrast while images 2-4 are after contrast injection (1, 2 and 6 minutes). Panel B) follows the same area
The insert at the top right shows the change of the average breast enhancement of all the 3 breast slices evaluated in this subject.

The short-term-high-dose protocol used could be of value for a pre-screening diagnostic application instead of the regular regimens that suit therapeutic indications. The prospective nature of the study, the short duration between the two MRIs and the inclusion of healthy women not on breast cancer therapies that might affect the enhancement all support the above findings.

The limitations of our study include the small sample size. Nevertheless, the number of women studied was appropriate for a pilot trial for this new indication and protocol of the use of letrozole. As with all breast MRI, there is an element of complexity to visually differentiate various types of background breast tissue which could be fibrous, glandular, ductal, fatty or even atypical appearance of a lymph node or blood vessel. Each of these tissues could behave distinctly in response to the anti-hormonal treatment. This might explain the high standard deviations of the enhanced breast areas in the same patient (Figure 4-1). However, the radiologist was confidently able to exclude vascular, nodal and ductal enhanced areas in most breast sections. When uncertain, an extra breast slice was analyzed to increase the reliability of the findings.

4.5 Conclusion

In conclusion, this is a pilot prospective clinical trial presenting a new diagnostic indication for aromatase inhibitors in postmenopausal women. The study provides preliminary evidence of the usefulness of this protocol in suppressing the non-specific background breast enhancement which could result in improved specificity of the breast MRI. Further
randomized studies are needed for wider clinical application of this protocol for pre-screening and diagnostic purposes.
Chapter 5 Droplet-Scale Estrogen Assays in Breast Tissue, Blood, and Serum

The previous chapters emphasized the importance of the intracrine mechanism in the pathogenesis and consequently the management of various hormonally sensitive disorders significant for women’s health; examples discussed included menopausal breast cancer and endometriosis. Similar disorders such as fibroids, endometrial polyps, endometrial cancer and some types of ovarian cancer might have a similar association. This having been said, the need to evaluate the local tissue hormonal milieu is extremely important, since the clinical and research evidence show the irrelevancy of the circulatory levels of the hormones to the intracrine hormonal production process contained inside the tissues and cells.

In this chapter, I introduce an innovative method for measuring estrogen in micro-samples of breast tissue and other clinical samples. In spite of their importance, tissue concentrations of estrogens are not routinely measured because conventional techniques require large samples of biopsies for analysis. In response to this need, a digital microfluidic method was developed and applied to the extraction and quantification of estrogen in one-microliter samples of breast tissue homogenate, as well as in whole blood and serum. This method may be broadly applicable to conditions requiring frequent analysis of hormones in clinical samples (for example, infertility and cancer).

5.1 Introduction

In addition to its well-known role as a blood-borne hormone, estrogen is an important intracrine and paracrine messenger in many tissues including the breast [65, 66, 192, 270].
There is an essential need for measuring estrogen concentrations in breast tissue to identify women at risk for developing breast cancer or to monitor the effect of anti-estrogen breast cancer therapies such as aromatase inhibitors [271]. Unfortunately, local breast tissue estrogen concentrations are not routinely measured because existing methods require invasive biopsies of hundreds to thousands of milligrams of tissue [272, 273]. Such procedures are not performed in part because they require local anaesthesia and carry the risk of scarring or deformity. Moreover, prior to analysis, large tissue samples must be processed (including lysis, homogenization, extraction, purification, and resolubilization), which requires many hours of laboratory time [274, 275]. These procedures are ill-suited for routine testing. Although most problematic for tissue samples, many of these same limitations apply to blood and serum samples (for example, in applications related to monitoring low levels of hormones [175, 276-278] or in management of infertility).

In response to these challenges, a miniaturized, automated and integrated method was developed in this study for hormone analysis in one-microliter samples. The method relies on digital microfluidics (DMF), a technique in which sample and reagent droplets are moved across an open surface by applying electrical potentials to an array of electrodes [279]. This technique is particularly well suited to multistep sample processing, and, in this paper, I describe the application of DMF to sample clean-up and extraction of estradiol (the most biologically active form of estrogen) in breast tissue from postmenopausal breast cancer patients, as well as from samples of whole blood and serum.
5.2 Experimental

5.2.1 Study subjects

Breast tissue was obtained from apparently normal areas adjacent to breast cancer tumors during surgery in two postmenopausal breast cancer patients and kept at −80°C until analysis. Blood and serum samples were collected from a healthy female volunteer during 5 different reproductive cycles (mid luteal phase) and kept at −20°C until analysis. Human ethics approvals were obtained from Mount Sinai Hospital and the Ontario Tumor Bank Research Ethics Boards.

5.2.2 Chemicals and reagents

Dichloromethane (DCM) and 2,2,4 Trimethylpentane (Isooctane) 99.8% and HPLC-grade water were purchased from Sigma. Methyl alcohol (Methanol, HPLC grade) was from Fisher Scientific. Estradiol (17-ß) was purchased from Steraloids Inc. Estradiol ELISA kits were from ALPCO Diagnostics.

5.2.3 Cleanroom reagents and supplies

5.2.4 Device fabrication

Digital microfluidic devices were fabricated in the University of Toronto Emerging Communications Technology Institute (ECTI) clean room facility, using transparency photomasks printed at City Graphics. Glass wafers (Howard Glass Co. Inc.) were cleaned in piranha solution (a 3/1 v/v mixture of sulfuric acid/hydrogen peroxide) for 10 min, and coated with chromium (150 nm) by electron beam deposition (BOC Edwards). After rinsing (acetone, methanol, DI water) and baking on a hot plate (115°C, 5 min), substrates were primed by spin-coating HMDS (3000 rpm, 30 s) and then spin-coating Shipley S1811 photoresist (3000 rpm, 30 s). Substrates were baked on a hot plate (100 °C, 2 min) and exposed (35.5 mW/cm², 4 s) through a transparency photomask using a Karl Suss MA6 mask aligner. Then substrates were developed (MF321 developer, 3 min) and postbaked on a hot plate (100 °C, 1 min). After photolithography, exposed chromium was etched (CR-4, 2 min) and the remaining photoresist was stripped by sonicating in AZ300T (5 min).

After forming electrodes and cleaning in piranha solution (30 s), a photoresist wall was formed, using methods similar to those reported by Moon et al. [280]. Briefly, substrates were spin-coated with SU-8-25 (500 rpm, 5 s, then 1000 rpm, 30 s), baked on a hotplate (65°C, 5 min, then 95°C, 15 min), and then exposed to UV light (35.5 W/cm², 7 s). After baking (65°C, 1 min, then 95°C, 4 min), and developing in SU-8 developer, substrates were coated with 2 µm of parylene-C and 100 nm of Teflon-AF. Parylene-C was applied using a vapor deposition instrument (Specialty Coating Systems) and Teflon-AF was spin-coated (1% by weight in Fluorinert FC-40, 1000 rpm, 1 min) followed by baking on a hot plate (160°C, 10 min). The polymer coatings were removed from contact pads by gentle scraping with a scalpel to facilitate electrical contact for droplet actuation. In addition to patterned
devices, unpatterned indium-tin oxide (ITO) coated glass substrates (Delta Technologies Ltd) were coated with Teflon-AF using the conditions described above, to serve as the top plate on assembled devices (as described below).

### 5.2.5 Device Operation

The device design included three input reservoir electrodes (3.5 x 3.5 mm) for the raw sample, lysing solvent, and polar extraction solvent, respectively, and a fourth reservoir electrode for collection of the processed sample (Figure 5-1-A). Actuation electrodes (1.5 mm x 1.5 mm with a 40 µm inter-electrode gap) formed a path linking the input reservoirs, which passed through a fifth reservoir (delineated by a photoresist wall) containing non-polar extraction solvent. Devices were assembled with an unpatterned ITO–glass top plate and a patterned bottom plate separated by a spacer formed from one or two pieces of double-sided tape (90 or 180 µm thick). Thus, depending on the spacer thickness, reservoir volumes were ~1.1 or 2.2 µl, and unit droplets (covering a single actuation electrode) were ~200 or 400 nl. A single spacer was used to process standard solutions of estradiol, while a double spacer was used for blood, serum, and tissue. Droplets were sandwiched between the two plates and actuated by applying AC potentials (18 kHz, 100 V) between the top electrode (ground) and sequential electrodes on the bottom plate via the exposed contact pads. Droplet motion was monitored by a CCD camera mated to an imaging lens positioned over the top of the device.

The methods reported here required the manipulation of droplets of fluids that have a wide range of characteristics (surface tension, conductivity, etc.) by DMF. In developing the methods, it was found that almost all of the reagents required for the procedure, including methanol, acetone, dichloromethane, and aqueous buffers, were actutable. According to Chatterjee et al. [281] the only liquids that are not actutable by DMF are those with
negligible conductivity and/or dipole moment; in the current work, this was observed to be the case for isooctane. The non-actuable nature of isooctane was useful for estrogen extraction, as it facilitated manipulation of droplets of methanol inside of a (non-actuated) pool of isooctane.

5.2.6 Digital Microfluidic Estrogen Extraction

Two DMF-driven estrogen extraction techniques were developed; method one, used in most experiments, comprised four steps. First, an aliquot of whole blood, serum, breast tissue homogenate, or estradiol standard solution was positioned in the sample reservoir of a device. Standard solutions were used immediately, and blood, serum, or tissue homogenate samples were allowed to dry on the surface. The top plate was then affixed and the solvents (DCM/acetone 80:20 v/v as lysing solvent, methanol as polar extracting solvent, and isooctane as non-polar extracting solvent) were loaded. Second, a series of reservoir volumes (9 x 1.1 µl or 5 x 2.2 µl) of DCM/acetone was dispensed and driven by DMF drop-wise to the sample, each of which was allowed to incubate at room temperature until dry (~1 min per reservoir volume). Third, a reservoir volume of methanol (1.1 or 2.2 µl) was dispensed and driven by DMF to the dried lysate to dissolve the steroids. A unit droplet of the dissolved sample (200 or 400 nl) was dispensed and delivered by DMF to the isooctane reservoir and circulated within the pool for ~20 seconds prior to driving the droplet out of the isooctane and towards the collection reservoir. This process was repeated until the sample reservoir was empty of the methanol. Fourth, step three was repeated with successive reservoir volumes of methanol (for a total of 9 x 1.1 µl or 5 x 2.2 µl) to ensure the extraction of all of the free estradiol. Finally, all extractate droplets were pooled in the output reservoir and allowed to dry.
In method two, used to analyze percent recovery and experimental precision by ELISA, a standard solution of estradiol in methanol (1 µl) was positioned in the sample reservoir and the top plate was affixed (the lysis and polar solvent reservoirs remained empty). In each experiment, a single unit droplet (200 nl) of sample was dispensed, translated (and circulated) through isooctane, and delivered to the collection reservoir, all by DMF. The sample reservoir was then washed (manually) with methanol three times, and a fresh sample was positioned in the reservoir and the process was repeated (twice), such that 3 extractate droplets from replicate samples (~600 nl total volume) were pooled in the collection reservoir and allowed to dry.

For all experiments, after collecting and drying the extract, the devices were stored at –20°C. Immediately prior to analysis, each extract was resolubilized in an aliquot (30 µl) of methanol/DCM (2:1 v/v), which was then dispensed into a small centrifuge tube. The solvent was then evaporated and the dry extract was reconstituted in a medium specific for the desired analysis.
Figure 5-1: Digital Microfluidic (DMF) Device Design and Operation. (a) Schematic of the DMF device, which includes sample and solvent reservoirs and the liquid-liquid extraction zone (bounded by a photoresist “wall”). (b) A series of frames from a movie (1-8) illustrating the key steps in the DMF-based extraction of estrogen from a droplet of human blood (1 µl). As shown, samples are lysed, the estradiol is extracted into a polar solvent (methanol), unwanted constituents are extracted into a non-polar solvent (isooctane), and the extractate is delivered to a collection reservoir. Among the remarkable features of this technique is the ease with which the methanolic phase is controlled within the isooctane phase (frame 6) and then separated from it after liquid-liquid extraction (frame 7).

5.2.7 Mass Spectrometry

Mass spectrometry was used to evaluate the performance of the DMF clean-up process in samples of whole blood or serum (obtained at two different days of the reproductive cycle). In each experiment, a 5-µl sample was dried and extracted by DMF (method one, as above),
and the extractate was reconstituted in 50 µl of methanol containing formic acid (0.1% v/v). Control (non-extracted) samples were prepared by drying and reconstituting 5-µl aliquots of blood or serum in 50 µl of methanol/formic acid (0.1% v/v), sonicating (10 min), and passing through a syringe filter (nylon membrane, 0.2 µm pore diameter). Samples were injected by nanoelectrospray into an LTQ Mass Spectrometer (Thermo Scientific) operating in the negative mode at 250°C with a flow rate of 0.5 µl/min. Under these conditions, the highest magnitude peak observed for estradiol standards alone (not shown) was m/z 183, which could be explained by the retrocyclization structure shown in (Figure 5-2). Replicate spectra were obtained for DMF-extracted and control samples of both blood and serum.

Liquid chromatography and tandem mass spectrometry (LC-MS/MS) with selected reaction monitoring (SRM) was used to evaluate estradiol in extractates from standard solutions, breast tissue homogenate, and blood. Standard solutions (1 µl, 2 mg/ml in methanol) and blood (dried from 1 µl) were extracted by DMF (method one, as above) with no prior processing, while breast tissue (400 mg) was manually homogenized in DCM (1 ml), from which 1 µl samples were dried and processed similarly. In all cases, after extraction, samples were resuspended in 100 µl of methanol: water (80:20 v/v), 10 µl of which was injected onto an HPLC (HP-Agilent 1100 series LC) interfaced by electrospray to a QTRAP LC-MS/MS (Applied Biosystems). The samples were analyzed in negative mode with SRM, evaluating an ion transition of m/z 271/145 to identify and determine the abundance of estradiol. Operating parameters included 300 µl/min flow rate, 4200 V spray potential, 60 V collision energy and 400°C nebulizing temperature. A microbore (2.1 mm i.d. x 50 mm) Thermo Gold C18 (2.2 µm) column with isocratic elution via a mobile phase of methanol: water (80:20 v/v) was used for LC separation.
5.2.8 ELISA

An estradiol-specific ELISA (ALPCO Diagnostics) was used (i) to evaluate the recovery and precision of the DMF method, and (ii) to quantify estradiol in breast tissue samples. In these experiments, at least 3 replicate samples were evaluated for each condition by absorbance, measured at 450 nm with a µQuant microplate spectrophotometer (Bio-Tek, Instruments). The optical densities of samples and controls were compared with those of ELISA calibrator solutions using a standard curve to calculate estradiol concentrations as per the manufacturer’s instructions.

For application (i), analysis of recovery and precision, the samples comprised serial dilutions of estradiol standard in methanol. DMF-extracts of 3 x 1 µl samples were prepared using method two (as above), and, for comparison, (non-extracted) control samples were prepared using the same procedure, but in devices lacking isoctane. After extraction, samples were resuspended in a 50-µl mixture of estrogen-free serum (ALPCO Diagnostics) and methanol (4:1 v/v) and evaluated by ELISA (final concentrations 40, 1200, 3000, and 6000 pg/ml). Recovery percentages were calculated as the concentrations of extracted samples divided by those from non-extracted controls. For application (ii), 1-µl samples of breast tissue homogenate (60 mg) in DCM (0.15 ml) from a breast cancer patient (a different patient than the one evaluated by MS) were processed manually (as above) and extracted by DMF using method one. The extractate was reconstituted in a 50-µl mixture of estrogen-free serum/methanol mixture and was evaluated by ELISA.

5.3 Results

Figure 5-1-A depicts the device designed to adapt conventional techniques for estrogen extraction to the digital microfluidic format. As shown, an array of electrodes connects a
series of reservoirs containing the sample and reagents. The process of estrogen extraction from a sample of human blood is depicted in Figure 5-1-B. As shown, in typical assays, samples were lysed, the estradiol was extracted into a polar solvent (methanol), unwanted constituents were extracted into a non-polar solvent (isooctane), and the extractate was delivered to a collection reservoir. The device illustrated in Figure 5-1 could be used with breast tissue homogenate, whole blood, serum, and standard solutions.

Mass spectrometry was used to confirm that estradiol was extracted by the digital microfluidic method. Whole samples and DMF-extracted samples of blood and serum were obtained from a female volunteer at two different days of one reproductive cycle. As shown, the dominant estradiol fragment (m/z 183) was not detected in the spectra from the whole samples, but was the peak of highest intensity in those from DMF-extracted samples. In addition, the peaks of potential interfering compounds, tentatively identified as fragments of tyrosine [282] (m/z 178), DNA helicase [283] (m/z 677) and porphyrin [284] (m/z 715), were suppressed in the spectra of extracted samples, indicating that their concentrations had been substantially reduced relative to that of estradiol. These data highlight the importance of sample processing for this application. Estradiol can only be ionized (and thus detected by mass spectrometry) after the many interfering compounds are removed [285] (Figure 5-2).
Figure 5-2: Mass Spectra of Whole and DMF-Extracted Samples. Representative spectra generated from (a) blood and (b) serum obtained from a female volunteer at different days of the menstrual cycle. The insets show that the estrogen fragment at m/z 183 is detected in extracted samples but not in whole samples. In addition, several peaks of potential interferants, tentatively identified as fragments of tyrosine (m/z 178) DNA helicase (m/z 677), and porphyrin (m/z 715) are absent in the extracted samples, suggesting that their concentrations have been substantially reduced.

To test whether estradiol can be quantified in samples extracted by the digital microfluidic method, liquid chromatography and tandem mass spectrometry (LC-MS/MS)
with selected reaction monitoring (SRM) [286] was used to evaluate estradiol extracted by DMF from standard solutions (Figure 5-3-A), from breast tissue homogenate from a postmenopausal breast cancer patient (Figure 5-3-B) and from whole blood from a female volunteer (Figure 5-3-C). As shown, estradiol was detected with high signal-to-noise ratio (S/N) at retention time \( \sim 3.2 \) min for all cases.

![Figure 5-3: LC-MS/MS Analysis of DMF-Extracted Samples. Chromatograms generated by LC-MS/MS with selective reaction monitoring (SRM) from 1 µl samples of (a) estradiol standard solution (2 mg/ml), (b) breast tissue homogenate from a postmenopausal patient with breast cancer, and (c) whole blood. The estradiol-specific ion pair evaluated for SRM was m/z 271/145.](image)

A commercial ELISA test was used to evaluate the percent recovery of the digital microfluidic technique and to evaluate the variance in the measurements. As shown in Figure 5-4, the DMF-based recoveries were high, ranging from 86-119% and the coefficients of variation (CVs) were low, ranging from 7-10%. A similar method was then applied to analyzing extracts from breast tissue homogenate from a postmenopausal breast cancer patient. Replicate analyses of 1 µl samples of breast tissue homogenate solution (requiring \( \sim 20 \) min processing by DMF) yielded an amount of 522 pg estradiol per mg tissue (with a CV of 1%). These data verify that the new method is capable of quantitative analyses of estrogen in tiny amounts of breast tissue and other clinical samples.
Figure 5-4: Extraction Efficiency Analysis by ELISA. Estradiol standards before and after extraction were evaluated by ELISA. As shown, extraction efficiencies from standard solutions ranged from 86 to 119% with a CVs ranging from 7 to 10%.

5.4 Discussion

Estrogen and other steroid hormones are fundamental for growth and reproduction, and disturbances in their physiological levels can be associated with a multitude of clinical disorders, including hormone-sensitive cancers (for example, breast, endometrial and prostate cancers), infertility, and pregnancy complications such as intrauterine growth restriction [287-291]. Moreover, hormonal therapeutics have been used for decades as anti-cancer medications, contraceptives, hormone replacement therapy, and fertility drugs [292-295]. Thus, there are a wide range of clinical conditions that require frequent monitoring of these hormones in tissue or blood for accurate diagnosis and treatment.

In this study I report the extraction and quantification of estradiol in 1 µl volume samples. This sample size is 1000-4000 times smaller than that required for conventional
methods of extraction and quantification of steroids including extraction followed by immunoassays or mass spectrometry [272, 273, 296-299]. As shown in Figure 5-5, this reduction is substantial. The method could be applied to routine screening of breast estrogen concentrations in micro aspirates as a potential marker of cancer risk, or blood estrogen in finger pricks to monitor hormone levels in infertility patients. In addition to the advantages that come with smaller samples, automation of the digital microfluidic method allows considerably less time- and labor-intensive assays relative to conventional processing techniques. Specifically, the conventional 5 to 6 hour hormone extraction techniques (including various liquid-liquid extraction and solid phase extraction based protocols) that require extensive pipetting, centrifugation and drying but could be replaced with the 10 to 20-min digital microfluidic process described here.
The method described here is powered by digital microfluidics [279, 300, 301], a technology similar to but distinct from microchannel-based fluidics. Although microchannels are well suited for many applications (for example, electrophoresis, in vitro culture and analysis of cells), microchannel-based fluidics would likely be a poor match for the application described here. Indeed, in few reports [302-304] of microchannel-powered methods for liquid-liquid extraction (representing only one of the series of steps required for
estrogen processing from clinical samples), the techniques have been inherently limited by the challenge of separating and collecting one liquid phase from the other after they have come into contact. In contrast, this step is straightforward in the method I have reported here (Fig. 1B, frame 7). The precise control over different reagents [281], phases [305] and volumes [306] afforded by digital microfluidics makes it a good match for this application.

Finally, I note that sample clean-up, extraction, and recovery are necessary steps for estrogen measurement in tissue, whole blood and plasma; however, there are some immunoassay kits intended for detection of estrogen and other steroids in non-extracted samples of serum. The utility of these tests has been contested because of cross reactivity with other steroid hormones, and consensus is building that sample clean-up is a prerequisite for accurate quantification of steroids, even in serum [276, 278, 307-309]. Thus, integrated sample cleanup methods like the one described here may prove useful for a wide range of clinically relevant applications in many different sample types.

### 5.5 Digital Microfluidics for Extraction of Multiple Steroid Hormones: Preliminary Data

#### 5.5.1 Objective

To evaluate the primary applicability of the above detailed DMF method in extracting multiple sex steroid hormones of clinical significance.

#### 5.5.2 Methods

Estradiol (17-beta), Estrone, Testosterone and Androstenedione, deuterated Testosterone and deuterated androstenedione, were purchased from Steraloids Inc. (Newport, RI).
Progesterone, Estriol, and 16-alpha-hydroxy estrone were purchased from Sigma (Oakville, ON). Deuterated progesterone was purchased from ICDN isotopes (Quebec, Canada).

The same device design as described above and the same extraction method detailed for estradiol have been used. Two groups of hormones were studied. The first group included a mixture of Progesterone (P), Androstenedione (A) and Testosterone (T). The second included a mixture four estrogens including (Estrone (E1), Estradiol (E2), Estriol (E3) and 16-alpha-hydroxy-estrone (16-alpha-OH-E1).

Five microliter droplets of each mixture were extracted in these experiments. The detection and efficiency of extraction of the DMF was evaluated for a methanolic standard mix of progesterone, testosterone and androstenedione using nanoelectrospray into an LTQ Mass Spectrometer (Thermo Scientific, Waltman, MA) operating in the positive mode at 350°C with a flow rate of 0.5 µL/min. The efficiency of extraction was calculated based on intensity relative to that of the deuterated internal standard of each analyte. Both of MS1 and MS2 were applied for confirmation of analytes identity by comparing it to non extracted standards.

The detection of the DMF extracted four estrogenic hormones in a methanolic standard mix was evaluated by a NanoLC (Eksigent)/LTQ Mass Spectrometer operated in the negative mode at 260°C. The LC method included multiple reaction monitoring method for the four hormones as following (E1 m/z 269/145, E2 m/z 255/237, E3 m/z 287/269 and 16-alpha-OH-E1 m/z 285/213).

5.5.3 Results

The following figures demonstrate the capability of the developed DMF method in the simultaneous extraction of multiple sex steroid hormones.
Figure 5-6 presents mass spectra of the three hormones (Progesterone, Testosterone and Androstenedione) simultaneously after extraction using the DMF device. The extracted droplet was analyzed using mass spectrometry. The parent ions (m/z 315, 289 and 287 for Progesterone, Testosterone and Androstenedione respectively) were identified using MS1. MS2 showed the corresponding daughter ions resulting from the fragmentation of the original molecules and that are similar to the pattern of the non-extracted standards.

The DMF extraction efficiency is shown in Figure 5-7. The average recovery ranged between 77 - 92% with a CV% that ranged between 2-14%.

Figure 5-8 shows the total-ion count (TIC) chromatogram that shows the extraction of all the 4 estrogens extracted by the DMF method. The chromatogram peaks represent the 16 alpha hydroxyl estrone (m/z 285), Estriol (m/z 287), Estradiol (m/z 271) and Estrone (m/z 269). The retention time ranged between 13 and 16 minutes for all the four hormones. All the MS2 scans showed the same batch of daughter ions as those in the non-extracted reference standards.
Figure 5-6: Mass spectra (MS1) of DMF extracted of 5μL droplet of a standard mix of three steroid hormones including progesterone (P), testosterone (T) and androstenedione (A).

Figure 5-7: Plots of Recovery (%) representing the efficiency of extraction by DMF for each of progesterone, testosterone and androstenedione. The efficiency of extraction was evaluated by mass spectrometry using the deuterated internal standards for each analyte.

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Figure 5-8: LC-MS/MS analysis of DMF-extracted 5µL droplet mixture of multiple hormones showing total-ion count (TIC) chromatograms generated by LC-MS/MS with multiple reaction monitoring. The chromatogram peaks show the extracted hormones including 16 alpha hydroxyl estrone (m/z 285), Estriol (m/z 287), Estradiol (m/z 271) and Estrone (m/z 269).

5.6 Conclusion

This is a novel digital microfluidic method for the extraction and quantification of estrogen in microliter samples of various clinical samples including breast tissue, whole blood and serum. This method is valuable for clinical disorders conditions requiring frequent or minimally invasive analysis of steroid hormones such as in breast cancer and infertility. The method can be also applied to extract multiple steroid hormones simultaneously from microliter samples.
Chapter 6 Double-Blinded Randomized Clinical Trial of Aromatase Inhibitors and Hormone Therapy in Postmenopausal Women: Design and Protocol of the LETROZOLE-HT study.

6.1 Introduction

In this chapter, I present the design and protocol of the LETROZOLE-HT clinical trial I have developed based on the knowledge and work presented in the previous chapters of this thesis. The initial hypothesis of the protocol of this trial and method development started in 2007, approved by Health Canada and institutional ethics board in 2008 and funded by the Canadian Breast Cancer Foundation in 2009. Actual patient enrollment commenced in February 2010.

6.2 Hypothesis

Aromatase inhibitors can be added to low dose estrogenic hormone therapy without reducing the therapeutic benefit of either. The combination could reduce the risks and side effects associated with each individual drug.

Specifically, one year of AI therapy could reduce mammographic breast density, a surrogate biomarker of breast cancer risk, in menopausal women taking HRT compared to women taking HT alone.

Compared to HRT alone, an AI added to HRT will result in:

- Lower levels of local breast tissue estrogens and higher levels of androgens.
• No difference in the control of menopausal symptoms.
• No difference in bone mineral density and bone turn-over markers.
• No difference in blood pressure and lipid profiles

6.3 **Significance of the Study**

If the hypothesis is correct, it will provide evidence that:

1. Adding AIs to HRT could offset the breast cancer risk reported in the WHI study with HRT regimens that contain progestin, and an AI and HRT combination could be a useful and well tolerated option for the primary prevention of breast cancer in menopausal women who are taking HRT for the management of menopausal symptoms. The evidence obtained from this study could lead to future phase III long-term trials to examine the impact of this combination on the incidence of breast cancer.

2. A combination of AI plus estrogen therapy can be further investigated in other groups including those with a high genetic breast cancer risk. In fact, there is growing evidence that estrogen deprivation therapy could be effective in this genetically predisposed group. For instance, an increase in aromatase gene expression occurs upon knockdown of the BRCA1 gene in adipose tissue cells [179]. Also, breast cancer risk in BRCA1/2 mutation carriers increases with an increase in MBD [208] and the use of GnRh-agonists were able to reduce mammographic breast density in BRCA1 mutation carriers [217].
6.4 Study Objectives

6.4.1 Objective #1:

Determine the effectiveness of the aromatase inhibitor letrozole in reducing biomarkers of breast cancer risk in healthy postmenopausal women taking HRT.

Outcome Measures of Effectiveness:

- Primary: Quantitative changes in mammographic breast density (MBD)
- Secondary: Local breast tissue estrogens and androgens.

6.4.2 Objective #2:

Determine the tolerability and adverse effects of the AI in the presence of estrogen and progestin hormone therapy in these women.

Outcome Measures of Safety and Compliance:

Primary: Bone mineral density.

Secondary:

1. Levels of biochemical markers of bone turnover.
2. Symptoms of menopause (vasomotor, psychological, genitourinary and somato-skeletal symptoms) measured by the Menopause Rating Scale.
3. Blood pressure, C-reactive protein and lipid profile.

6.5 Study Design

A Phase II, Prospective, Randomized, Double-Blind, Placebo Control, Interventional study with three arms: an active treatment arm of women who will receive AI and standard low dose HRT for 12 months and 2 control arms of women who will receive placebo and standard dose HRT or placebo and half dose HRT for the same time period.
**Randomization and the two placebo arms:** According to ASCO: “Placebo controls are appropriate for breast cancer risk reduction trials since no intervention has been demonstrated to have a favorable impact on net health or survival” [105, 310].

Adding a second control placebo arm who will receive the half dose HT is important for the following reasons:

- If MBD is significantly reduced by adding AIs to the standard dose of HT but not with reducing the HRT to the ultra-low dose, this will substantiate the evidence that controlling the concentrations of the local breast estrogens by using AIs is more crucial for modifying breast density and the risk to breast cancer than just reducing estrogen blood levels by lowering the dose of HRT.

- If women in the AIs plus the standard HRT dose get a similar control of their menopausal hypoestrogenic symptoms compared to women who receive the standard dose plus a placebo and a better control of these symptoms than women who have only their HT dose lowered, this will further support the hypothesis and previous retrospective data of the good tolerability profile of AIs in women receiving standard HRT doses.

### 6.6 Inclusion Criteria

- Postmenopausal, defined by any of these criteria: a) Cessation of menstrual bleeding of 12 months or more and serum FSH level in the postmenopausal range (>20 IU/L).
- Already have been taking HT for a minimal duration of 3 months prior to the study.
- At elevated risk of breast cancer due to:
1. A 5 years invasive breast cancer risk equal to or more than 1.67% based on the Gail model (Online NCI Breast Cancer Assessment tool are used to calculate the risk)[198] (Figure 6-1).

And/or

2. Mammographic percent breast density of 50% or more in most recent mammogram.

And/or

3. Using combined hormone replacement therapy for more than 5 years.

Figure 6-1: Screenshot of the Breast Cancer Risk Assessment Tool available at the National Cancer Institute website. Having a risk score of more than 1.67% is one of the indicators of increased breast cancer risk in the Letrozole-HT trial.
6.7 Exclusion Criteria

- Prior hysterectomy: Only women who have an intact uterus will be included in this study.
- Present or past diagnosis of breast cancer including ductal carcinoma in situ (DCIS), or malignancy within the past 5 years except cured basal cell or squamous cell skin cancer or cervical carcinoma in situ.
- Diagnoses as carrier of one of the breast cancer susceptibility high-penetrance genetic mutations (BRCA1/2, PTEN and TP53’)[311].
- Osteoporosis (not osteopenia) or history of pathological/fragility fractures.
- History of coronary heart diseases and stroke.
- Mental health status that jeopardizes the patient or the integrity of the data obtained.
- Concurrent or prior use within the past 3 months of gonadotrophin releasing hormone analogues, SERMs, phytoestrogens, other AIs, androgens and dopamine agonists.

6.8 Discontinuation Criteria

In both arms, treatment continues for one year in the absence of: Diagnosis of breast cancer including (DCIS), any primary or secondary cancers, pathological or fragility fractures, any major health problem that affects the well-being of the patient and allergic reactions to study medications.

6.9 Methodology

6.9.1 Outline

Study subjects are randomized to one of three treatment arms:
Arm I: Patients receive standard dose HRT plus letrozole every other day for 1 year.

Arm II: Patients receive standard dose HRT plus placebo (identical to letrozole) every other day for 1 year.

Arm III: Patients receive half dose HRT identical to the standard dose HRT plus placebo (identical to letrozole) every other day for 1 year.

6.9.2 Drugs Formulation and Dosage

Letrozole (Femara™, Novartis Pharmaceuticals, Dorval, Canada) will be given in a dose of one tablet 2.5 mg/day orally. Women in the HRT arms are switched to a standard dose of HT comprising Estrace (17β-estradiol) 1 mg or 0.5 mg once per day and Micronor (norethindrone) 0.35 mg (Janssen-Ortho Inc., Toronto, Canada) 2 days on and 2 days off.

6.9.3 Ethics

This study has been approved by Health Canada and a NOL obtained (Protocol# RFCLET1, December 2007) as well as by the Mount Sinai Hospital REB (MSH-07-0015-A, February, 2008). A signed informed consent is obtained prior to participation in the study.

6.9.4 Timetable

Total study duration: 36 months; Starting date: January, 2010; Anticipated finishing date: December 2012; Anticipated accrual dates: January, 2010 to December, 2010; Post treatment follow up period: 2 months for each study subject. Outcome measures assessed at baseline, 6 months, and 12 months. Clinical Visits Timetable is shown in Figure 6-2.
6.10 Outcome Measures

6.10.1 Mammographic breast density (MBD)

MBD was chosen as primary outcome measure for the following reasons:

1. Breast density is one of the strongest risk factors for breast cancer [205] with women with the highest MBD having a 4 to 6 fold increase in breast cancer risk compared to women with the lowest MBD [207]. Dense breasts are associated with an increase in proliferative lesions such as hyperplasia, atypia and carcinoma in situ [209].

2. High breast density tends to decrease the sensitivity of mammograms for cancer detection [212] and a reduction of breast density should make mammograms more
sensitive for earlier detection [213]. Reduction of MBD was associated with a reduction of breast cancer risk [210, 211].

Therefore, recognizing or developing factors that modify breast density is a key approach in breast cancer prevention [214].

6.10.1.1 MBD-Methods

MBD is measured at baseline and after 12 months of treatment using subjective analysis by experienced breast imaging radiologist in addition to measuring quantitative percentage changes in Total Integrated Pixel Intensity (TIPI) evaluated by image analysis software (Image Quant) and (ImageJ) as detailed in 0. All mammograms are performed at The Marvelle Koffler Breast Centre in MSH. This facility equipped with three state-of-the-art full-field digital mammography systems (Senographe Essential FFDM, GE Medical Systems, Wis, USA). All mammography equipment is regularly inspected as a part of ongoing quality control of the Ontario Breast Cancer Screening Program. High quality digital mammograms are obtained in all women before and after the treatment using similar settings. In addition, this mammography unit provides an automatic exposure control (AEC) system that automatically selects optimal radiation exposure for each breast tissue type.

6.10.2 Local Breast Tissue Sex Steroid Hormones

In general, breast cancer risk reduction studies should adopt measuring local breast tissue estrogens, rather than the circulatory levels of estrogens, to more precisely estimate the actual estrogenic exposure of the breast. This is particularly essential in our study, since all our subjects are receiving exogenous HRT and their blood hormones are not expected to be changed either by letrozole or the placebo. In addition, there have been many studies
showing no correlation of the circulatory levels of the sex hormones (commonly termed as endogenous sex hormones) with the mammographic breast density [298, 312, 313] and therefore, serum estrogen measurements should not be used to identify women at high breast cancer risk [314].

Breast tissue Estradiol, Estrone, Androstenedione, and Testosterone will be measured in needle biopsy samples (16 G needle). The digital microfluidic method outlined in Chapter 5 is used to quantify the hormones coupled with a highly sensitive high performance liquid chromatography (HPLC) - Mass Spectrometer (LTQ–Linear IT-Thermo Scientific, Waltman, MA). LC/MS/MS with a Multiple Reaction Monitoring (MRM) method was used successfully to measure estrogen in volumes as small as 1 microliter.

Initial trials to use the microneedle biopsy in a small group of patients (7 patients) showed that the procedure is extremely well tolerated and pain was absent or minimal. The tissue yield from the needle biopsy seems to be adequate for quantification compared to samples used in our preliminary studies. To standardize the quality and quantity of tissues obtained before and after the treatment, the same location at 10’oclock, 5 cm from the areola in the same breast will be used. Tissue will be weighed on the digital microfluidic chip before extraction.

6.10.3 Bone Mineral Density and Markers of Bone Turnover

Participants undergo a dual x-ray absorptiometry (DEXA) scan at baseline and after 12 months of the treatment on the same machine at the UHN Osteoporosis Clinic. Bone mineral density is traditionally used to monitor response to anti-osteoporotic treatments at the clinical and research levels. Additionally, early indicators of bone loss include increase of the bone collagen degradation products, as a result of osteoclast activation. Bone turnover markers
coupled with the bone densitometry provide more specific markers for the prediction of risk of fractures and monitoring of various pro and anti-osteoporotic therapies [315, 316]. In addition to BMD, the baseline and 6 months levels of biochemical bone turnover markers including telopeptide cross-links (X-links), serum Osteoclin (OC) and bone specific alkaline phosphatase (bAP) will be measured.

6.10.4 Menopausal Symptoms

The Menopause Rating Scale (MRS) is a widely used validated scale composed of 11 questions scored from 0 to 4, and includes common menopausal and hypoestrogenic symptoms (vasomotor, psychological, genito-urinary and somato-skeletal symptoms) [317]. This scale will be used to evaluate the menopausal hypoestrogenic symptoms in the 3 arms of the study at baseline, 6 months and one year of the treatment (Figure 6-3).
Menopause Rating Scale (MRS)

Which of the following symptoms apply to you at this time? Please, mark the appropriate box for each symptom. For symptoms that do not apply, please mark ‘none’.

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>none</th>
<th>mild</th>
<th>moderate</th>
<th>severe</th>
<th>very severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Hot flushes, sweating (episodes of sweating)</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>2. Heart discomfort (unusual awareness of heart beat, heart skipping, heart racing, tightness)</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>3. Sleep problems (difficulty in falling asleep, difficulty in sleeping through, waking up early)</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>4. Depressive mood (feeling down, sad, on the verge of tears, lack of drive, mood swings)</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>5. Irritability (feeling nervous, inner tension, feeling aggressive)</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>6. Anxiety (inner restlessness, feeling panioky)</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>7. Physical and mental exhaustion (general decrease in performance, impaired memory, decrease in concentration, forgetfulness)</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>8. Sexual problems (change in sexual desire, in sexual activity and satisfaction)</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>9. Bladder problems (difficulty in urinating, increased need to urinate, bladder incontinence)</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>10. Dryness of vagina (sensation of dryness or burning in the vagina, difficulty with sexual intercourse)</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>11. Joint and muscular discomfort (pain in the joints, rheumatoid complaints)</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
</tr>
</tbody>
</table>

Score = 0 1 2 3 4

Figure 6-3: Menopause Rating Scale. Study participants answer the questionnaire at baseline, 3, 6 and 12 months after the treatment.

6.10.5 Blood Pressure and Lipid Profile

The effect of the combined protocol on indices of cardiovascular risk will be examined including serial blood pressure measurements, c-reactive protein and the lipid profile (total cholesterol, HDL-cholesterol, triglycerides after a 12 hour fast).
6.11 Statistical Analysis

6.11.1 Sample Size Considerations:

*Main efficacy outcome:* In the pilot study detailed in 0, MBD was measured using the total integrated pixel intensity (TIPI). Change in MBD was quantified by the change in TIPI expressed as a percent of the baseline TIPI. The hypothesis here is that the change in MBD as measured by percent TIPI change from baseline will be greater in Arm I than in Arm II and also greater in Arm I than in Arm III. Using the standard deviation of 24 obtained in the pilot study, 65 patients per study arm (195 in total) are needed to have 90% power to detect a difference in change for either of the two comparisons as small as 15 percentage points, given the Whitney-Mann U-test as the analysis method and testing each of the hypotheses at the 2.5% level to maintain an overall 5% false positive error rate. (The study will have 79% power to detect a difference of 13 percentage points.) To account for potential drop-out during follow-up of 10%, 73 patients per arm will be enrolled (219 in total).

*Main safety outcome:* Our main safety hypothesis is that after a year of treatment, bone density will be maintained at the same level in Arms I and II. Since equivalence is being hypothesized, an equivalence test using the confidence interval approach is performed. Assuming a standard deviation of 0.12 [264, 318] and equivalence in the population, with 65 patients per arm, an 80% power can be declared to the two groups equivalent using a 90% confidence interval for the difference between groups, when defining the equivalence limits as plus or minus 0.062 g/cm2. This means if the true mean is 0.82 g/cm2 the equivalence limits are ±7% of the true mean. The same holds for the comparison between Arms I and III, but as these are secondary hypotheses, type I error is not controlled for.
6.11.2 Statistical Analysis of Outcome Measures:

Descriptive summaries will be generated for all study variables at baseline and follow-up. Special care will be taken to provide summaries of the data useful for future sample size calculations, including baseline and follow-up means and standard deviations as well as change over time statistics.

Main efficacy outcome: The primary outcome will be calculated as the change in total integrated pixel intensity (TIPI) from baseline to 12 months, expressed as a percentage of the baseline value. It will be compared between arms (Arm I versus II, Arm I versus III) using the non-parametric Mann–Whitney U-test to eliminate undue influence of the potentially large outliers common in image data.

Secondary efficacy outcomes: For local estrogen and androgens in the breast the main outcomes are the changes from baseline to 12 months. Arms I and II will be compared using analysis of covariance (ANCOVA) to correct for the baseline value. Arms I and III will be compared in the same way.

Main safety outcome: For BMD the main outcome is the confidence interval for the difference in mean BMD at 12 months between Arms I and II, corrected for the baseline BMD level. This will be obtained from an ANCOVA model with baseline BMD level as covariate. The confidence limits will be compared to the equivalence limits ±0.062 g/cm to decide if the arms are equivalent. The difference between Arms I and III will be summarized and compared in the same way.

Secondary safety outcomes: For the Menopause Rating Scale the scale total will be calculated by adding all the answers. For the secondary safety outcomes (Menopause Rating Scale total and subscales, the markers of bone turnover, blood pressure and blood lipids) the main
outcomes are the confidence intervals for the difference in the 12 month level between Arms I and II, corrected for the baseline levels. These confidence intervals will be obtained from ANCOVA models with the baseline values as covariates. The difference between Arms I and III will be compared likewise. The confidence intervals will be useful for informal hypothesis testing and will provide useful estimates of effect sizes. Although the power may be low for some tests, they can raise cautionary flags if extreme results are obtained.

6.12 Recruitment

Recruitment is currently ongoing through:

- Menopause clinics: Direct approach of postmenopausal women on hormone therapy who are eligible to participate.
- Website: Online web-based resource providing information in a dedicated website [www.togetherwepreventbreastcancer.com](http://www.togetherwepreventbreastcancer.com) (Figure 6-4).
- Information brochures and posters: posted and distributed at various clinics that serve menopausal women.
Figure 6-4: Letrozole-HT clinical trial website. Sections include information for patients about the study design, study investigations, background and research as well as study team.
Chapter 7 Summary and Future Directions

7.1 Summary of Achievements

A summary of the work accomplished in this thesis is described below:

1. A new protocol of hormone replacement therapy coupled with aromatase inhibitors is presented in Chapter 3. This protocol is aimed to be used for the management of menopausal symptoms in postmenopausal women, while reducing their risk of breast cancer by suppressing the breast local estrogen. Primary efficacy of the protocol in reducing mammographic breast density – a biomarker of breast cancer risk - was demonstrated in a retrospective cohort study with a matched control group. Women on the co-therapy gained a significant reduction of their mammographic breast density compared to women who used hormone replacement therapy alone. Adding the aromatase inhibitors to the hormone therapy did not compromise their effectiveness in alleviating the menopausal symptoms and patients tolerated the co-therapy for several years with a good compliance. Further evaluation of this protocol is intended in a randomized double-blinded clinical trial that investigates its effect on a number of effectiveness and safety indices including mammographic breast density, local estrogen levels in the breast, bone mineral density, bone resorption markers and some cardiovascular markers (blood pressure, lipid profile and C - reactive protein). The protocol of this clinical trial is presented in Chapter 6.

2. A new protocol using aromatase inhibitors in postmenopausal women for the acute inhibition of local breast estrogens for breast diagnostics is presented in Chapter 4. The protocol involves using a high-dose short-term regimen of aromatase inhibitors...
prior to screening or diagnostic breast imaging techniques. The primary effectiveness of this method has been evaluated in a prospective pilot phase II clinical trial, using breast MRI as the model technique. There was a significant suppression of the non-specific breast parenchymal enhancement and improvement of the diagnostic confidence of the radiologist in the post-treatment breast MRI compared to the baseline MRI. This could be translated into an improved specificity of the breast MRI and less unnecessary call-backs or invasive procedures for patients. The study is the first investigation of the effect of aromatase inhibitors on the breast MRI.

3. A novel technique for minimally invasive estrogen assays was developed, Chapter 5. Primarily, this technique enables convenient monitoring of estrogen in the breast to measure the response to aromatase inhibitors. Current conventional assays are impractical as they require large volumes of tissue obtained by invasive procedures. The new technique is based on a “Lab-on-Chip” technology known as “Digital Microfluidics”. The digital microfluidics allows manipulation of liquid droplets freely on an array of microelectrodes. I adapted this method for complex extraction steps of estrogen from raw samples including breast tissue, blood and serum. This was coupled with an off-chip quantification using liquid-chromatography mass spectrometry (LC-MS) or ELISA. A preliminary study has also shown the validity of this method in extracting multiple steroid hormones simultaneously including androgens, progesterone and different types of estrogen and its metabolites. This microassay could have extended applications in women’s health such as in monitoring sex hormones in infertility, evaluating the risk of breast cancer and
measuring the response of anti-oestrogen therapies in breast cancer, endometriosis and fibroids.

4. The use of aromatase inhibitors in the treatment of severe postmenopausal endometriosis is discussed in the Appendix. The protocol has been reported in few studies previously, but is not yet widely applied in clinical management of endometriosis. A case report study is presented demonstrating the effectiveness of peripheral estrogen suppression in relieving endometriosis related pelvic pain that did not respond to surgical menopause or other treatment options. Using add-back estrogen therapy did not return the endometriosis related symptoms in that patient.

7.2 Future Directions

The work presented in this thesis encompasses several studies that demonstrate the clinical significance of extra-ovarian peripherally synthesized estrogen and how its monitoring and modulation could provide key therapeutic and diagnostic answers for many questions in our current clinical practice.

This work can be expanded in multiple directions:

1. The combined use of aromatase inhibitors and hormone therapy is a promising protocol that could reduce the HRT-associated breast cancer risk and enable symptomatic postmenopausal women to use HRT safely for longer durations. At the same time, as a chemopreventative option, it is expected to attain better patient compliance compared to using aromatase inhibitors alone for women at elevated risk for breast cancer. Following the retrospective study presented in Chapter 3, a randomized double-blinded phase II clinical trial has been recently initiated to confirm the effectiveness and safety of this protocol in a larger number of patients,
Chapter 6. The completion of this trial is the first step towards establishment of sufficient evidence for wide clinical application. Subsequently, a phase III clinical trial for a longer duration should be pursued to investigate the incidence of breast cancer as the main outcome measure while bone fractures and cardiac events should be evaluated for long-term safety.

2. The work in Chapter 4 can be further developed in several investigations. One is to confirm the effectiveness of aromatase inhibitors on breast MRI specificity in a larger population of patients in a randomized study design. This could include trying higher and lower doses of AIs. Theoretically, the findings of the described study suggest a better sensitivity by unmasking small cancerous lesions that otherwise could be missed amongst the enhanced benign parenchyma. This needs to be verified in breast cancer patients diagnosed with early stages of cancer, by randomizing them to either receiving AIs or placebo prior to breast MRI. On the other hand, the potential application of acute aromatase inhibition prior to the screening mammograms to reduce breast density is another interesting route as its benefits could be of wider application.

3. The digital microfluidics hormone extraction technique presented in Chapter 5 could be the most potentially expandable work in this thesis. The technique is a malleable tool that can be translated into an unlimited number of applications. A wide clinical validation of the method using a multitude of clinical samples is the first step towards establishment of technique usefulness and identifying potential obstacles. Indeed, a study by this objective has been recently initiated to evaluate sex steroid hormones in large number of blood samples, breast tissue obtained by micro-needle biopsy and
breast cyst aspirates. Additionally, application of the assay in epidemiological studies to evaluate local estrogens and androgens as potential biomarkers of breast cancer is an important pathway. Women with different clinical and risk profiles could be evaluated for their breast hormonal levels.

On the other hand, further technical validation of the method to improve the practicality of the device design and operation is essential to achieve maximal efficiency of the extraction procedure. Full automation of the device is also under development by the current team. Building a hybrid device that contains both an extraction digital microfluidics platform and a separation platform using microchannels will allow direct introduction of the extracted-separated sample to the mass spectrometry. As well, combining the extraction and detection using on-chip ELISA and on chip detection electrodes can make an ideal device for point-of-care applications. The utility of the digital microfluidics in the quantification of other hormones and hormone groups including glycoproteins such as FSH and LH could be also investigated.

It should be noted, however, that this technique needs several development steps before being transferred to the clinic. Challenges are multiple, including the non-standardized quantification methods using mass spectrometry, full device automation and development of a friendly chip-world interface that could be used easily in a clinical setting.

4. The use of AIs in suppressing the local estrogenic activity of the endometriotic tissues is a new direction in treating endometriosis. Few case-report studies including the one presented in the appendix and prospective studies with small number of patients are
currently available. No phase III clinical trial of significance was attempted yet. Other closely related disorders include fibroid and endometrial polyps. Both are very common benign pathologies of significant estrogenic dependency. The increased local aromatase and estrogenic activity has been demonstrated in several small scale studies. AIs have been also attempted for their treatment. More studies are needed to confirm the effectiveness of this treatment.
Appendix I: Aromatase Inhibitors in the Treatment of Severe Postmenopausal Endometriosis

In this appendix, I present a case of a menopausal woman who presented with endometriosis and severe pelvic pain that did not respond to hysterectomy and bilateral salpingo-oophorectomy. The patient was eventually treated by using aromatase inhibitors while a concomitant treatment with estrogen relieved her hot flashes without pain reactivation. The case is another example supporting the role of the intracrine mechanism in the development and maintenance of hormonally sensitive gynecologic disorders such as endometriosis. Inhibition of the local estrogen synthesis process in the endometriotic lesions seems to be essential for disease inactivation.

Introduction

Endometriosis is defined as the presence of endometrial tissue outside the endometrial cavity. It is estimated that endometriosis affects approximately 10% of the general female population and up to 82% of women with chronic pelvic pain [319]. Endometriosis is typically a disease of the child-bearing period and it is less common after menopause. However, there have been several reports of severe cases of postmenopausal endometriosis [320, 321]. The objective of this case report is to demonstrate that the use of aromatase inhibitors resulted in improvement of severe endometriosis-related pelvic pain that persisted after hysterectomy and bilateral salpingo-oophorectomy.
Case

A middle-aged woman with a long history of severe pelvic endometriosis was referred for the management of persistent pelvic pain, bladder, and bowel symptoms. The patient had undergone a hysterectomy and unilateral oophorectomy for uterine fibroids and menorrhagia several years ago. During the surgery she was diagnosed with endometriosis. She had two subsequent laparoscopic surgeries for the management of her pelvic pain. She also had a two-year history of cyclic rectal bleeding, which was further investigated by three colonoscopic procedures and CT scans. These investigations determined the presence of a rectosigmoid stricture and a pelvic mass. An open explorative surgery revealed extensive endometriosis throughout the pelvis. The endometriotic tissue had infiltrated all the layers of the sigmoid colon, forming a central focus of transmural endometriosis as well as other foci in the terminal ileum and in the vaginal vault. Accordingly, she had an anterior bowel resection, small bowel resection, removal of the remaining ovary with its endometriotic cyst, and excision of a vaginal vault fibrotic mass. A few months later, she developed urinary symptoms including frequency and dysuria and recurrence of the pelvic pain. Cystoscopy showed an endometriotic nodule in the posterior wall of the bladder. The patient had a menopausal serum FSH level (82 IU/L) and was not taking estrogen replacement.

The patient was given the aromatase inhibitor, exemestane, 25 mg daily for two weeks with no improvement of her pain. A different aromatase inhibitor was then tried. Surprisingly, a marked and rapid relief of symptoms occurred when letrozole was given at a dose of 2.5 mg three times per week for one month. After four months of letrozole treatment, the patient continued to be free of pain and other symptoms, but complained of hot flashes. Low dose estrogen (micronized estradiol, 0.5 mg daily) was then added with improvement of hot
flashes and no recurrence of pain over the following four months of follow-up during concomitant use with letrozole.

Discussion

Chronic pelvic pain is the most frequent presentation of endometriosis. It can be manifested in one or more forms including abdominal pain, dysmenorrhea, low back pain, dyspareunia, dysuria and dyschezia. The overall clinical syndrome varies with the involvement of multiple pelvic organs [319] but can be severe enough to impair quality of life [322]. The severity of pain is not correlated with the stage of endometriosis [323]. However, severe pain is found when endometriosis is deeply infiltrating specific organs such as the association of severe dyspareunia to deep endometriosis in the uterosacral ligament [324].

Different treatments have been attempted in the management of endometriosis with variable outcomes. GnRH-agonists together with hormone therapy add back have been shown to relieve pelvic pain associated with endometriosis for up to 10 years [325, 326]. Conservative laparoscopic surgery [327] and non-classic treatments such as presacral neurectomy and spinal cord stimulation showed significant, although usually temporary, relief of the pelvic pain associated with endometriosis [328, 329].

Hysterectomy with bilateral salpingo-oophorectomy remains, in the majority of cases, the definitive solution for severe endometriosis in women who have completed their families or passed the child-bearing period [330]. Unfortunately, the patient in the present study had rapid return of relentless pain after surgical ovarian ablation. This resistance to ovarian hormonal deprivation is believed to be a reflection of independent production of estrogen in endometriotic tissues which showed signs of local estrogen bioactivity[331]. Consequently, lesional bio-formation of estrogen seems to be more critical than circulating estrogen in
maintaining active endometriosis especially with the substantial evidence of increased aromatase activity in the endometriotic foci [134, 139].

Aromatase is the rate-limiting enzyme for estrogens synthesis from androgens. Aromatase inhibitors have been suggested as the next generation of drugs for the management of endometriosis[139]. Letrozole and anastrozole are triazole derivatives that are reversible, competitive aromatase inhibitors and, at doses of 1-5 mg/day, inhibit estrogen levels by 97% to >99% [82]. Exemestane is a steroidal, irreversible inhibitor that binds to the active site of the aromatase enzyme and inactivates it effectively at a dose of 25 mg/day [81].

In this case, exemestane was expected to attain symptom relief by blocking local endometriotic production of estrogen. Exemestane was chosen, because of its potential bone-sparing effects compared to the non-steroidal aromatase inhibitors [332]. The difference in response of the patient to the two drugs may reflect their different potencies. Letrozole is believed to be more potent in attaining estrogen suppression than exemestane or anastrozole [333, 334]. Additionally, letrozole was the only drug that demonstrated significant clinical effectiveness over tamoxifen in the adjuvant therapy of breast cancer and to show improvement in survival as a first line treatment [334]. Therefore, the differences in potency and in the mechanism of action between the two drugs might explain the preferential response this patient showed to letrozole. It is possible that the patient may have experienced improvement with a course of exemestane treatment longer than two weeks. However, we should consider that the time to reach the steady state plasma level is shorter in case of exemestane (7 days) than letrozole (60 days) [333].
Conclusion

From the response of this patient, and from other studies [145, 335], aromatase inhibitors could represent an effective and potential therapeutic option in the management of endometriosis-associated pain in postmenopausal women and in other resistant cases that do not respond to conventional medical or surgical therapy. However, aromatase inhibitors seem to differ in their clinical outcome. Letrozole might be superior to exemestane in relieving the endometriotic pain, an assumption that needs to be proven by controlled studies.
References


177. Amos Pines DWS, Martin Birkhäuser WHI and breast cancer: a response to a recent publication from the WHI. Climacteric 2006, 9(4):244 - 244


263. Eilertsen AL, Karssemeijer N, Skaane P, Qvigstad E, Sandset PM: Differential impact of conventional and low-dose oral hormone therapy, tibolone and


