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## Clinical and Genetic Predictors of Aromatase Inhibitor-Induced Arthralgia

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Graduate Program in Physiology and Pharmacology  
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## Abstract

Aromatase inhibitors (AIs) are the most commonly used first-line endocrine treatment for postmenopausal women with estrogen receptor-positive breast cancer. Significant adverse drug reactions are associated with AIs, the most common being arthralgia. We hypothesized that a comprehensive assessment of pharmacogenetic and clinical variables that affect AI tolerability could improve AI selection and treatment for breast cancer patients. We recruited 196 patients diagnosed with breast cancer initiating AI therapy at the London Regional Cancer Program. Patients completed questionnaires regarding arthralgia symptoms and provided blood samples at baseline, 2 months and 6 months after AI initiation. Plasma letrozole drug concentration was measured by liquid chromatography tandem-mass spectrometry (LC-MS/MS). DNA was extracted and used for pharmacogenetic analysis. More than half of patients had increases in pain and stiffness in their hands, shoulders, and arms, hips and knees. A LC-MS/MS analysis demonstrated that plasma concentrations of letrozole were negatively associated with body mass index ( $P = 0.0003$ ) and positively associated with age ( $P = 0.0430$ ). *CYP2A6* genotype was significantly associated with letrozole levels ( $P < 0.0001$ ), and increased plasma letrozole levels were observed in patients with *CYP2A6* reduced-function genotypes. However, letrozole concentration level and *CYP2A6* genotype were not significantly associated with a change in pain score from baseline. Further pharmacogenetic investigations revealed that four SNPs within the estrogen synthesis and metabolism pathway, namely, *CYP19A1* (rs4775936) and *ESR1* (rs9322336, rs2234693, rs930799), were associated with the development of arthralgia. *CYP19A1* (rs4775936) was also a significant predictor of discontinuation of drug. Finally, we found that a SNP

within the vitamin D pathway *CYP27B1* (rs4646536) was associated with an increase in pain in the hands, arms, and shoulders. Patients who had a vitamin D level of at least 50ng/ml were found to be four times less likely to develop AI arthralgia. Understanding the impact of genes and drug levels on AI-induced arthralgia will help clinicians better manage AI therapy. This work will facilitate personalized medicine for women with breast cancer and advance understanding of endocrine biology.

## Keywords

Breast cancer, aromatase inhibitors, arthralgia, pharmacogenetics, adverse drug reactions, endocrine therapy, single nucleotide polymorphisms, estrogen, vitamin D

## Co-Authorship Statement

### Chapter One:

Borrie AE, Kim RB. 2016. Molecular basis of aromatase inhibitor associated arthralgia: known and potential candidate genes and associated biomarkers. *Expert Opin Drug Metab Toxicol*,13(2):149-156

AEB wrote the manuscript. RBK provided feedback on the manuscript and wrote the expert opinion section (expert opinion section was omitted from this thesis). Both authors approved the final version of the manuscript.

### Chapter Two:

Borrie AE, Rose RV, Choi YH, Perera FE, Read N, Sexton T, Lock M, Vandenberg TA, Hahn K, Dinniwell R, Younus J, Logan D, Potvin K, Yaremko B, Yu E, Lenehan J, Welch S, Tyndale RF, Teft WA, and Kim RB. Letrozole concentration is associated with CYP2A6 variation but not with arthralgia in patients with breast cancer. *Breast Cancer Research and Treatment, Epub Aug. 9, 2018*

AEB, WAT, and RBK designed the research study. AEB participated in patient enrolment and data acquisition. AEB, RVR, and YHC conducted the statistical analysis. AEB and RBK wrote the manuscript. All authors provided feedback on the manuscript for important intellectual content. All authors approved the final version of the manuscript.

### Chapter Three:

Borrie AE, Rose RV, Choi YH, Perera FE, Read N, Sexton T, Lock M, Vandenberg TA, Hahn K, Dinniwell R, Younus J, Logan D, Potvin K, Yaremko B, Yu E, Lenehan J, Welch S, Teft WA, and Kim RB. Genetic and clinical predictors of aromatase inhibitor drug arthralgia in breast cancer patients. *Manuscript in preparation.*

AEB, WAT, and RBK designed the research study. AEB participated in patient enrolment and data acquisition. AEB, RVR, and YHC conducted the statistical analysis. AEB and RBK wrote the manuscript. All authors provided feedback on the manuscript for important intellectual content. All authors approved the final version of the manuscript.

### Chapter Four:

Borrie AE, Rose RV, Choi YH, Perera FE, Read N, Sexton T, Lock M, Vandenberg TA, Hahn K, Dinniwell R, Younus J, Logan D, Potvin K, Yaremko B, Yu E, Lenehan J, Welch S, Teft WA, and Kim RB. Prospective analysis of vitamin D levels and vitamin D receptor polymorphisms in postmenopausal women on aromatase inhibitors. *Manuscript in preparation.*

AEB, WAT, and RBK designed the research study. AEB participated in patient enrolment and data acquisition. AEB, RVR, and YHC conducted the statistical analysis. AEB and RBK wrote the manuscript. All authors provided feedback on the manuscript for important intellectual content. All authors approved the final version of the manuscript.

# Dedication

To my grandparents,

Grace, Bill, Helen, and John

## Acknowledgments

This thesis is the product of many people who have guided me on my graduate school journey and made the experience of doing my PhD so enjoyable and meaningful. The lab I have worked in is comprised of a hilarious, kind-hearted, and helpful group of individuals who have been such a pleasure to work alongside. Thank you to all of my wonderful lab members, Jody Murray, Brandi Povitz, Sara Mansell, Cameron Ross, Heidi Liao, Bradley Linton, Brittany Digou, David Sheshelidze, Robin Legan, Crystal Engelage, and Maureen Trinnear. I am thankful to my lovely graduate student and postdoctoral fellow friends who have been with me on this adventure: Cheyenne McLean, Michelle Kim, Mandy Li, Laura Russell, Aze Wilson, Ahmed Almousa, Samantha Medwid, and Theo Wigle. I'd like to extend a special thanks to Markus Gulilat for hustling with me in the desk beside mine all these years. I am also deeply grateful to my dear friend, colleague, and statistics wizard, Rhiannon Rose.

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## Abbreviations

ADR: Adverse drug reaction

AI: Aromatase inhibitor

AUSCAN: Australian/Canadian Osteoarthritis Hand Index

BMI: Body mass index

ELISA: Enzyme-linked immunosorbent assay

ER: Estrogen receptor

ER $\alpha$ : Estrogen receptor alpha

ERE: Estrogen response element

GWAS: Genome-wide association study

HER2: Human epidermal growth factor receptor 2

IL-1: Interleukin-1

IL-6: Interleukin-6

LC-MS/MS: Liquid chromatography-mass spectrometry

NF- $\kappa$ B: Nuclear factor kappa B

PR: Progesterone receptor

SNP: Single nucleotide polymorphism

TNF- $\alpha$ : Tumor necrosis factor-alpha

WOMAC: Western Ontario and McMaster Osteoarthritis Index



# 1 INTRODUCTION TO THE PATHOGENESIS AND PHARMACOLOGICAL TREATMENT OF BREAST CANCER

Section 1.6 to 1.10 reproduced from: Borrie AE, Kim RB. 2016. Molecular basis of aromatase inhibitor associated arthralgia: known and potential candidate genes and associated biomarkers. *Expert Opin Drug Metab Toxicol* 13(2):149-156

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## 1.1 Introduction

Breast cancer is the most common cancer among Canadian women and second only to lung cancer as a leading cause of death from cancer in Canadian women [1]. Breast cancer is a heterogeneous group of diseases with subtypes that are characterized by different clinical, molecular, genetic, histological, and prognostic characteristics [2]. Several different types of neoplasms arise from the cells in the breast. Most breast cancers are adenocarcinomas, which are tumours that originate in a gland cell. The most common adenocarcinomas of the breast are ductal carcinoma, which begins in the milk ducts, and lobular carcinoma, which starts in the lobules. Both men and women have breast tissue but women have a much larger amount and a much higher risk for breast cancer; less than 1% of all breast cancers occur in men [1].

## 1.2 Characterization of breast cancer

When a patient with breast cancer is first diagnosed, their disease is described by stage, grade, and molecular subtype. Stage describes the anatomical extent of the tumour within the body and is typically expressed as a number on a scale of 0 through IV. Stage is based on the TNM (tumour, node, metastasis) system describing the size and extent of the primary of the tumour, whether the cancer has spread to the regional lymph nodes, and whether the cancer has spread to other areas of the body (metastasis). Grade is a score based on visual assessment of cell morphology and architecture, and indicates how quickly the cells are dividing and if the organization and morphology of the cells are abnormal [3]. Typically, less differentiated and higher grade tumours are more biologically aggressive.

Breast cancer is also characterized by immunohistochemical and molecular subtypes, and these subtypes have distinct pathological features as well as clinical implications. Traditionally, breast cancers were classified as tumours that expressed the estrogen receptor (ER-positive breast cancer) and those that did not (ER-negative breast cancer) [4]. Early studies found that tumours in which the ER and progesterone receptor (PR) were present were distinct from the more aggressive ER/PR-negative tumours that had a less favourable prognosis. Human epidermal growth factor receptor 2 (HER2) is a protein found to be overexpressed in approximately 15-30% of breast cancers and is associated with more aggressive disease and poorer prognosis [5, 6]. ER, PR and HER2 can all be measured by immunohistochemistry [7]. The four major molecular subtypes first identified by Perou and colleagues are luminal A, luminal B, HER2-type, and basal-like [8, 9]. Luminal A tumours are characterized by higher expression of ER (ER-positive), normal expression of HER2 (HER2-negative), low histological grade, and are associated with a favourable prognosis. Luminal A tumours have low levels of the protein Ki-67, a cellular marker for proliferation. Approximately 50-70% of all breast cancers are luminal A tumours, making it the most common subtype [10]. Luminal B tumours make up approximately 10-20% of breast cancers and this subtype is characterized by high expression of ER, high levels of Ki-67, normal or high expression of HER2, as well as intermediate histological grade and larger tumour size. Luminal B tumours tend to have a relatively good prognosis, though not as favourable as luminal A tumours [10]. HER2-type tumours are characterized by intermediate to high histological grade, are ER-negative, predominately HER2-positive, and account for 15-20% of breast cancers. HER2-type breast cancer has a poorer prognosis than the luminal subtypes. However,

treatment with HER2 targeted therapies such as trastuzumab (Herceptin) may improve outcomes [10, 11]. Basal-like tumours, which make up approximately 10-15% of all breast cancers, tend to be ER, PR, and HER2-negative, and therefore, they are also known as triple negative breast cancer. Basal-like tumours are the most aggressive form of breast cancer and have the poorest prognosis [10]. These four subtypes were identified based on gene expression patterns in tumor samples from patients with locally advanced breast cancer and were confirmed using microarray expression profiling in a separate patient cohort [8, 12]. Perou *et al.* also identified a fifth subtype, called normal-like, which resembled the features of luminal A. However, this subtype was later hypothesized to be a technical artifact thought to arise from contamination of samples with normal mammary cells, and is not widely accepted as an official subtype [13]. Heterogeneous expression of the estrogen, progesterone, and HER2 receptors has been observed among different patients with breast cancer, as well as between matched samples from primary tumours and their metastases. Patient treatment is planned based on factors such as tumour size, grade, and molecular subtype. Table 1.1 summarizes the four major types of breast cancer, their features, and their indicated treatments [14-16].

**Table 1.1 Summary of breast tumour molecular subtypes**

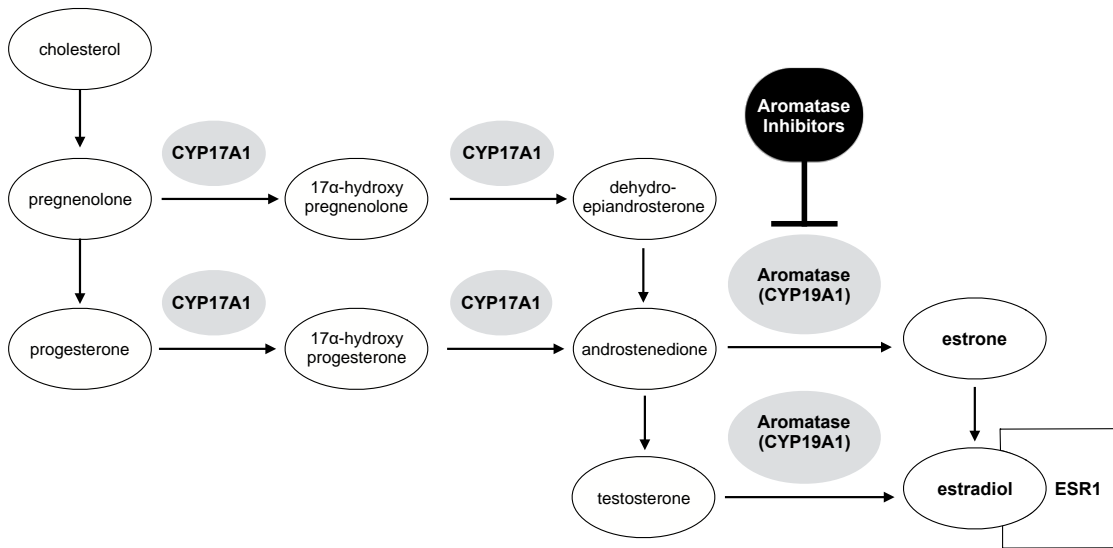
<b>Molecular Subtype</b>	<b>Luminal A</b>	<b>Luminal B</b>	<b>HER2-type</b>	<b>Basal-like</b>
ER status	+	+	-	-
PR status	+	+	-	-
HER2 status	-	+/-	+	-
Grade	I/II	II/III	II/III	III
Prevalence	50-70%	10-20%	15-20%	10-15%
Prognosis	Good	Intermediate/Poor	Poor	Poor
Treatment	Endocrine therapy		Trastuzumab	Chemotherapy

## 1.3 Estrogen

Estrogen is an important hormone and signalling molecule best known for its role in female reproduction, but is also involved in male reproduction as well as playing a significant function in the immune, neuroendocrine, skeletal, and vascular systems of both males and females [17]. Estrogen acts as a mediator in multiple physiological pathways and has been implicated in the development and progression of diseases such as cancer, cardiovascular disease, osteoporosis, obesity, and endometriosis [18]. It is well established that estrogen is an essential mediator in the development of breast tissue in females during puberty and pregnancy [19, 20]. Epithelial cells in the breast respond to estrogen signalling through its receptor, estrogen receptor alpha ( $ER\alpha$ ), and progesterone signalling through PR. Estrogen stimulates the development of tubules into the ductal system of the breasts, the growth of stromal tissue, and the accumulation of adipose tissue. Estrogen is a pivotal driver of ductal growth and elongation during breast development [21, 22].

Estrogen also plays an important role in breast cancer; there is considerable evidence linking estrogen exposure with an increased risk of breast cancer. While no clear mechanism through which estrogen induces breast cancer has been found, one possible pathway is that of estrogen binding to  $ER\alpha$  and inducing breast cell proliferation through its direct and indirect actions on the enhanced production of growth factors [23]. A longer duration of estrogen exposure, through early menarche and late menopause, is known to lead to an increased risk of developing breast cancer [24].

There are two major types of estrogen in postmenopausal women – estrone and estradiol. Aromatase is an enzyme transcribed from the *CYP19A1* gene that catalyzes estrogen biosynthesis through the conversion of testosterone to estradiol and androstenedione to estrone (Figure 1.1) [25, 26]. While estrogen is produced in the ovaries and adipose tissue in premenopausal women, among postmenopausal women, adipose tissue is considered to be the major source of estrogen synthesis[27]. Aromatase is expressed across multiple human tissues including the ovaries, testes, adipose tissue, brain, muscle, skin fibroblasts and osteoblasts of bone[28]. Therefore, use of aromatase inhibitors (AIs) results in reduced formation of estrogens, thus lowering the amount of circulating estrogens and thereby reduce ER-dependent cellular proliferation [29].



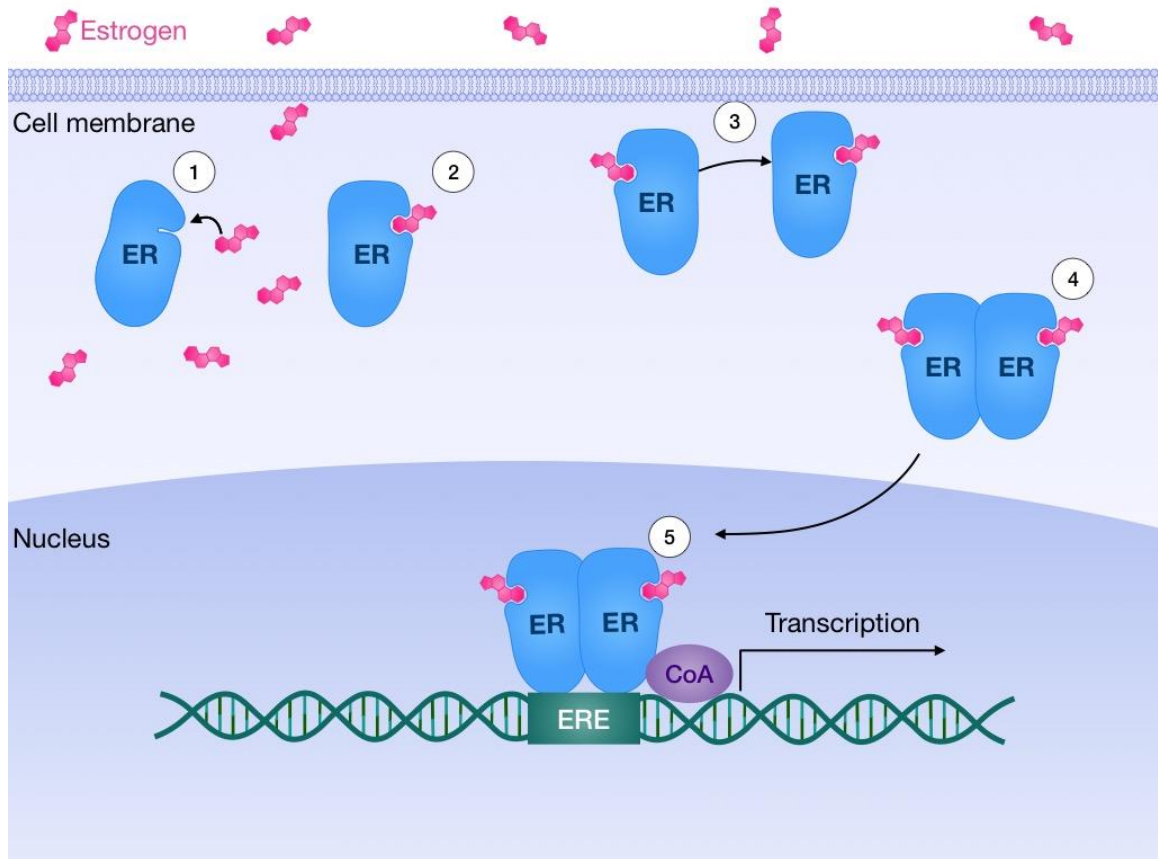
**Figure 1.1 Estrogen synthesis and interaction with aromatase inhibitors**



## 1.4 Estrogen receptor

Estrogenic effects largely occur as a consequence of the activation of the two forms of the ER, ER $\alpha$  and ER $\beta$ , upon binding with estrogen [30]. When a tumour is classified as ER+ or ER-, it is the ER $\alpha$  that is present. ER $\alpha$ , encoded by the *ESR1* gene, is activated by estrogen binding [31], resulting in increased cell proliferation. Thus, expression of ER $\alpha$  has been proposed as a possible mechanism for the initiation of breast cancer. The ER $\beta$  is encoded by the *ESR2* gene, but its role is less well understood [32]. Both ER $\alpha$  and ER $\beta$  are widely expressed across many tissues. Figure 1.2 illustrates how estrogen acts as a ligand to initiate estrogen receptor signaling, which regulates the expression of multiple target genes [17].

Though estrogen effects are primarily mediated by ER $\alpha$  and ER $\beta$ , a G protein estrogen receptor, (GPER) also mediates estrogen action in both normal and malignant cells [33]. GPER, also known as GPR30, is localized on the cell membrane and the endoplasmic reticulum. It is expressed in a variety of tissues including the breast and the blood vessel endothelium, and is involved in regulating breast development and vasodilation [34, 35]. Until 2005, GPER was considered an orphan receptor, however recent studies have shown that its' stimulation may play an important role in cell proliferation and cancer cell invasion [36]. Currently there are no large clinical studies evaluating GPER expression in ER-positive breast cancer, though this could be an area that is addressed in the future.



**Figure 1.2 Signalling of the estrogen receptor in breast cancer**

1) Estrogen binds with high affinity and 2) changes the shape of the receptor. 3) Specific sites are activated, and in this state, the receptors can combine to form dimers. 4) The dimers translocate into the nucleus and 5) bind to discrete DNA sequences called estrogen response elements (ERE). Co-activators are recruited, and this results in RNA polymerase.

## 1.5 Breast cancer in postmenopausal women

Approximately 80% of breast cancers occur in postmenopausal women. While menopause is not a risk factor for breast cancer, the risk of developing cancer increases with age [37]. Obesity is also associated with a significantly elevated risk of breast cancer [38] and a poor prognosis among postmenopausal breast cancer patients [39, 40]. In postmenopausal women, estrogen is no longer produced by the ovaries but is still synthesized in many tissues, with adipose tissue becoming the main source of estrogen. Other estrogen-producing tissues include skin, muscle, and healthy and malignant breast tissue [26]. Increased fat tissue leads to higher estrogen levels, which can increase breast cancer risk [24].

## 1.6 Breast Cancer Treatment

Breast cancer treatment is prescribed by an oncologist and is based on the molecular subtype, size, node status, and grade of the tumour, as well as the age, sex, and menopausal status of the patient [41]. Surgery and radiation therapy are localized regional treatments used to target the cancer at the tumour site. Surgery involves the removal of the cancer tissues and a small amount of healthy tissue surrounding the tumour, while radiation therapy ablates cancer cells using high-energy radiation. Breast cancer may also be treated with systematic treatments that include chemotherapy, endocrine therapy, and biological therapy [41].

### 1.6.1 Endocrine therapy

Over a century ago, the discovery that ovarian ablation may precipitate tumour regression in premenopausal women marked the beginning of endocrine-based treatment of breast cancer [42]. The approval of tamoxifen, a selective estrogen receptor modulator, for breast cancer in 1977 led to a marked improvement in survival for ER-positive breast cancer. The next advance in endocrine therapy came with the aromatase inhibitors (AIs), letrozole, anastrozole, and exemestane, whose chemical structures are depicted in Figure 1.3. These therapies improved 10-year survival rates and decreased the risk of disease relapse in postmenopausal women with breast cancer over treatment with tamoxifen [43, 44]. Today, endocrine-based therapy is prescribed to women with ER/PR-positive breast cancer based on their menopausal status and cancer relapse risk. Endocrine therapy slows or stops cancer growth by disrupting the signalling of ER $\alpha$  in the case of tamoxifen, or by reducing the available estrogen to cancer cells in the case of AIs. Tamoxifen is primarily used to treat pre-menopausal women, although it can also be used to treat post-menopausal women, and AIs are used to treat post-menopausal women. Because AIs interfere with the conversion of androgens into estrogens in peripheral tissues (skin, muscle, fat, and benign and malignant breast tissue), they are only capable of lowering estrogen production outside of the ovaries and are therefore only prescribed to post-menopausal women [26, 45].

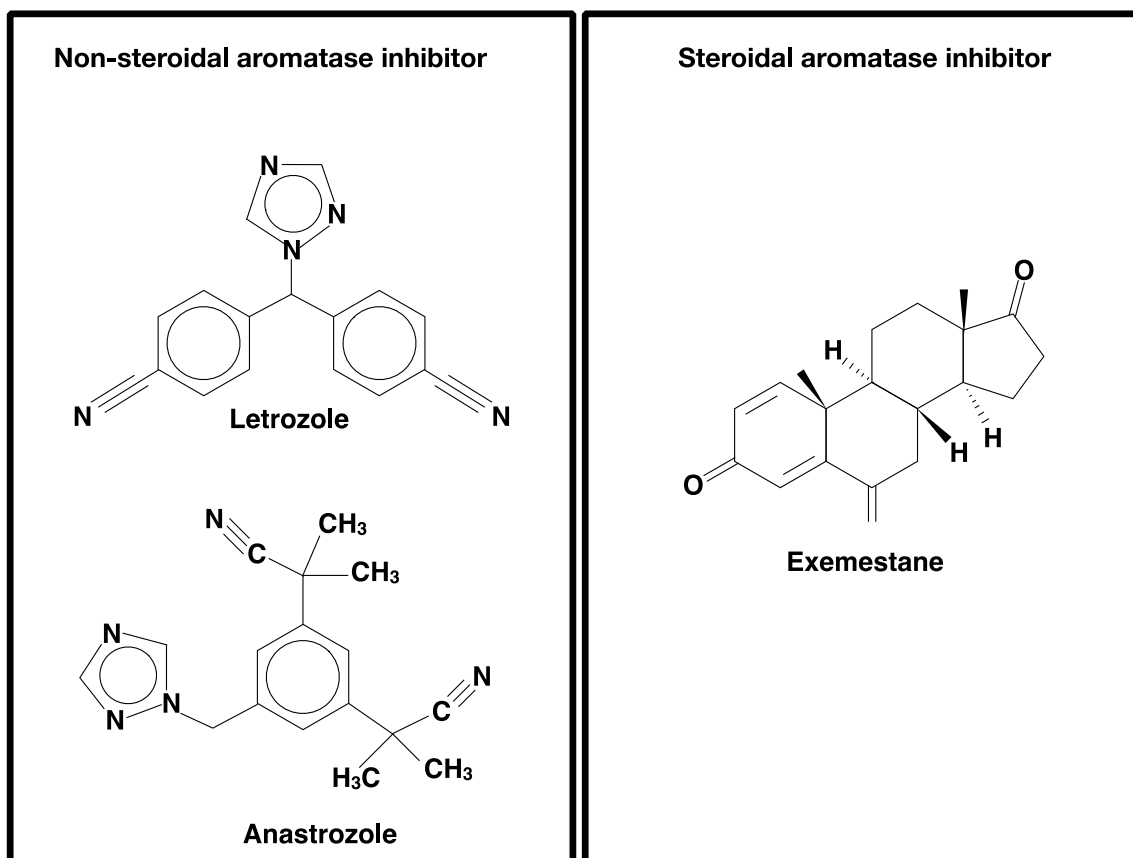


Figure 1.3 Chemical structures of the aromatase inhibitors

## 1.6.2 Aromatase inhibitors

Currently, letrozole, anastrozole, and exemestane, are the most commonly prescribed AIs for the treatment of women with ER+ breast cancer. Lowering estrogen causes the tumour growth to be reduced, and limits disease progression and recurrence [46-48]. AIs block the activity of aromatase by binding to the enzyme either reversibly, as in the case of letrozole and anastrozole, or irreversibly, as in the case of exemestane. Letrozole and anastrozole are known to competitively bind to the AI substrate-binding site, prevent binding of androgens, thus limiting the catalytic conversion of androgens to estrogen. Exemestane binds irreversibly to the AI active site to inactivate the enzyme commonly referred to as “suicide inhibition” [49]. All three AIs potently reduce estrogen below the level that can be detected using most of the current clinical assays [50]. Letrozole inhibits approximately 99% of estrogen biosynthesis, while anastrozole has an inhibition rate of 97%, and exemestane inhibits 98% of estrogen biosynthesis [51]. It should be noted that AIs are typically prescribed for 5 years, usually after adjuvant chemotherapy, surgery, and/or radiation, although it can be prescribed as a monotherapy for early stages of breast cancer. AIs may also be prescribed for 2-3 years following 2-3 years of tamoxifen therapy [46-48]. While aromatase inhibitors greatly reduce estrogen levels, dihydrotestosterone, total testosterone, androstenedione, DHEA, or free androgen index levels are not affected [52].

### 1.6.2.1 Letrozole

Letrozole, also known by the trade name Femara, is a non-steroidal aromatase inhibitor that binds reversibly to the aromatase enzyme and blocks the conversion of androgen to estrogen via competitive inhibition. It is prescribed as a standard oral dose of 2.5mg daily for five years, and its absorption is not affected by food [53]. It has a terminal half-life of 48h and steady-state plasma concentration is reached in 2-6 weeks [53] although some studies have shown that it may take up to 8 weeks to reach steady-state concentration [54]. Maximal estrogen suppression of greater than 90% is reached within 14 days of the drug start date [55].

### 1.6.2.2 Anastrozole

Anastrozole, also known as Arimidex, is a non-steroidal aromatase inhibitor, which like letrozole, reversibly binds to the aromatase enzyme and blocks estrogen production through competitive inhibition [56]. Anastrozole has a standard daily oral dose of 1mg for a total treatment duration of five years. The terminal half-life of anastrozole is 46 hours, and plasma concentrations reach steady-state levels at approximately 7 days [56]. Maximal estrogen suppression of greater than 90% is achieved within 2-4 days of daily 1mg dosage [57].

### 1.6.2.3 Exemestane

Exemestane, also known by the brand name Aromasin, is a steroidal aromatase inhibitor structurally related to androstenedione, and it binds to the active site of aromatase enzyme irreversibly, resulting in inactivation by suicide inhibition. Exemestane is given at a daily oral dose of 25mg for five years, has a terminal half-life of 24 hours, and reaches steady-state concentration level by 7 days [58, 59].

### 1.6.3 Adverse drug reactions associated with AIs

Meta-analysis and cross-trial comparisons of the three AIs have shown that they have similar adverse drug reaction (ADR) profiles across the drug class [60, 61]. The most common ADRs are arthralgia and joint symptoms, which affect up to 50% of patients [62, 63]. Significantly, up to 30% of patients on AIs will discontinue their use due to ADRs, and 75% report the cause of discontinuation is arthralgia [64]. Other common ADRs include hot flashes and night sweats, fatigue, loss of bone mineral density, loss of sex drive, and vaginal dryness or itching. Less frequent ADRs include thinning of the hair, increased blood pressure, increased cholesterol, and cognitive effects such as mood swings and depression. Due to the androgenic structure of exemestane, it is known to give rise to hormonal effects apart from the decrease in estrogen production and is sometimes associated with weight gain and acne [58]. Letrozole and anastrozole have a non-steroidal molecular structure and therefore do not have the same androgenic, progestogenic, or estrogenic ADRs as exemestane [65].



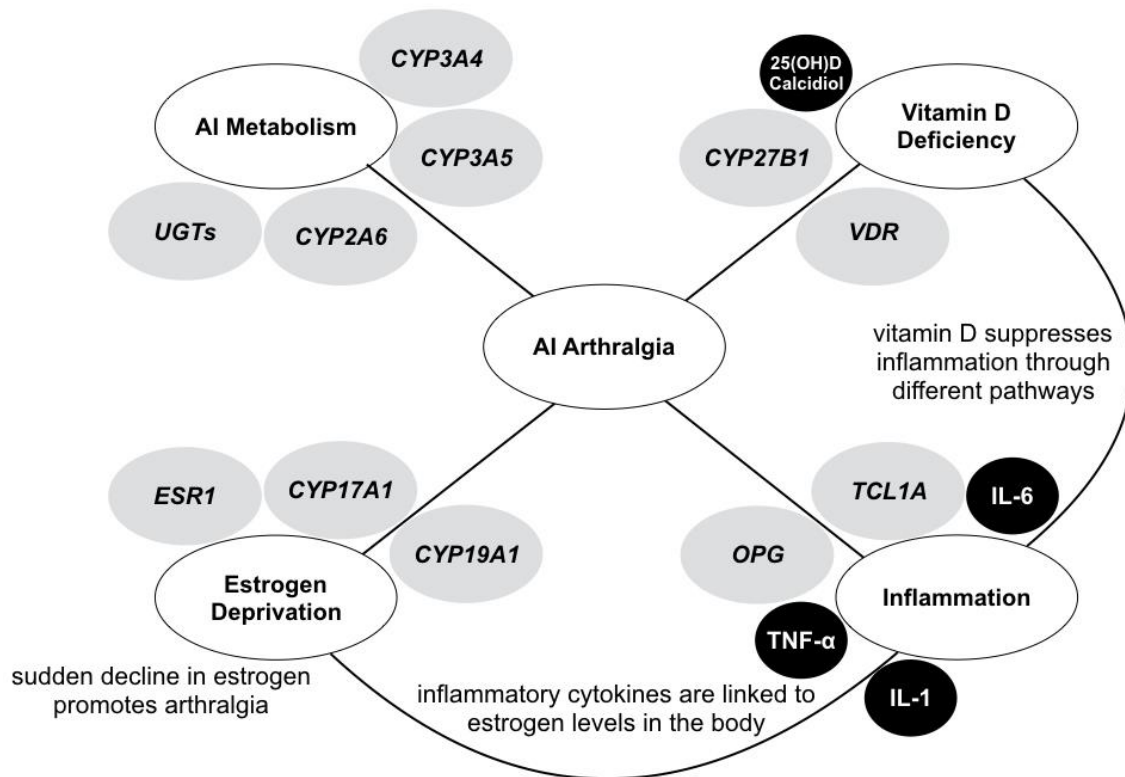
#### 1.6.4 Aromatase inhibitor induced arthralgia

AIs are effective for treating breast cancer, but they may have unwanted musculoskeletal effects, including joint pain (arthralgia) and muscle pain (myalgia) [62, 66, 67]. This adverse effect is often referred to as AI-associated musculoskeletal syndrome. Arthralgia and myalgia are widely regarded as the most common and pervasive adverse drug reactions reported by patients treated with AIs. AI-induced arthralgia presents in patients with joint pain, typically affecting the wrists, hands, shoulders, hips and knees [62]. AI-induced arthralgia often compromises quality of life and lead to non-adherence of the drug. Other less common adverse drug reactions include thinning of the bones or osteoporosis, vaginal dryness, vaginal bleeding, and loss of libido [68-70]. Patients who experience AI-induced arthralgia find that these symptoms typically appear within the first month of drug initiation and worsen in the second and third months of drug use [71]. AI-induced arthralgia is resistant to treatment with NSAIDs, opioids, and other pain medications [72].

It is difficult to pinpoint the exact prevalence of AI-induced arthralgia for a variety of reasons. Within the literature that describes AI-induced arthralgia, there is not a consistent definition relating to severity or types of pain. Furthermore, the largest clinical trials of AIs were not designed to capture and examine arthralgia. In a 5-year study that compared anastrozole to tamoxifen, the prevalence of arthralgia in breast cancer patients on anastrozole was 35% [73]. A large randomized study of more than 4,000 patients, which compared patients on exemestane to patients on tamoxifen, reported that the prevalence of arthralgia was only 5% in patients taking exemestane [46]. However,

another similarly powered study of exemestane in post-menopausal women found that 30% of patients had arthralgia [74]. The rates of arthralgia vary greatly when measured by patient self-reporting. However, studies that were specifically designed to measure the prevalence of AI-induced arthralgia, found the rates are consistently higher, and closer to 50% of patients. One study of 200 women on AIs utilized a 25-item questionnaire designed to assess joint symptoms and found that 47% of women developed AI-related joint pain and 44% developed AI-related stiffness [62].

Currently, the mechanism that underlies AI-induced arthralgia is largely unknown, although there is a growing body of literature that suggests there may be multiple intersecting mechanisms. Patient variability in AI metabolism and drug levels, the rate of estrogen decline during AI treatment, vitamin D deficiency, and increased muscle and joint inflammation have all been associated with arthralgia (Table 1.2). Links between these factors suggest an interplay of complex mechanisms (Figure 1.4).



**Figure 1.4 An overview of proposed mechanisms for AI arthralgia**

Associated genes are depicted in grey. Associated biomarkers are depicted in black.

**Table 1.2 Gene variants involved in AI arthralgia**

<b>Select variants and variant effects within estrogen metabolism genes</b>				
<b>ESR1</b>				
rs2234693	upstream SNP	<i>In vivo</i>	Currently unknown	[85]
rs9340799	intronic SNP	<i>In vivo</i>	Currently unknown	[85]
rs9322336	intronic SNP	<i>In vivo</i>	Currently unknown	[86]
<b>CYP17A1</b>				
rs4919686	a SNP	<i>In vivo</i>	Currently unknown	[79]
rs4919683	a SNP	<i>In vivo</i>	Currently unknown	[79]
rs4919687	a SNP	<i>In vivo</i>	Currently unknown	[79]
rs3781287	a SNP	<i>In vivo</i>	Currently unknown	[79]
rs10786712	a SNP	<i>In vivo</i>	Currently unknown	[79]
rs6163	synonymous SNP	<i>In vivo</i>	Currently unknown	[79]
rs743572	a SNP	<i>In vivo</i>	Currently unknown	[79]
<b>CYP19A1</b>				
rs60271534	repeat polymorphism (intron 4)	<i>In vivo</i>	Currently unknown	[81]
Haplotype M_3_5	haplotype of 14 SNPs	<i>In vivo</i>	Currently unknown	[82]
rs4775936	intronic SNP	<i>In vivo</i>	Currently unknown	[79]
<b>Select variants and variant effects within genes which regulate inflammation</b>				
<b>OPG</b>				
rs2073618	a SNP	<i>In vivo</i>	Currently unknown	[78, 83]
<b>TCLIA</b>				
rs11849538	a functional SNP	<i>In vivo and in vitro studies</i>	Creates an estrogen response element	[92, 93]
<b>Select variants and variant effects within aromatase inhibitor metabolism genes</b>				
<b>CYP2A6</b>				
CYP2A6*2 (rs1801272)	a SNP	<i>In vivo</i>	results in less than 40% CYP2A6 activity	[99]
CYP2A6*4	a whole deletion of gene	<i>In vivo</i>	results in no CYP2A6 activity	[99]
CYP2A6*9 (rs28399433)	a SNP	<i>In vivo</i>	results in 40-50% CYP2A6 activity	[99]
CYP2A6*12	a translocation	<i>In vivo</i>	results in 40-50% CYP2A6 activity	[99]
<b>CYP3A4</b>				
CYP3A4*22 (rs35599367)	a SNP	<i>In vivo and in vitro studies</i>	associated with low CYP3A4 activity	[104]
<b>CYP3A5</b>				
CYP3A5 *3 (rs35599367)	a SNP	<i>In vivo and in vitro studies</i>	associated with lower clearance of drugs metabolized by the gene product of CYP3A5	[105]
<b>UGT2B17</b>				
Deletion	a whole deletion of gene	<i>In vivo and in vitro studies</i>	decreases 17-hydroexemestane glucuronidation process by 14 times, effecting both metabolism and excretion of the active metabolite	[106]
<b>Select variants and variant effects within Vitamin D metabolism/pathway genes</b>				
<b>VDR</b>				
rs11568820	a SNP in a cdx-2-binding site in the VDR promoter and can modulate gene expression levels	<i>In vivo and in vitro studies</i>	in vitro study that rs11568820 alleles have different transcriptional activities	[79]
<b>CYP27B1</b>				
rs4646536	SNP in intronic region	<i>In vivo</i>	Currently unknown	[79]
rs10877012	SNP in promoter region	<i>In vivo</i>	Currently unknown	[79]

## 1.7 Estrogen deprivation and metabolism

As early as 1925, loss of estrogen in women was noted to link with arthralgia [75]. There is also evidence to suggest that the sudden decline in estrogen levels, rather than simply a low estrogen state, stimulates arthralgia. In a large study of over 2000 women, 41% of peri-menopausal women presented with increased joint pain and stiffness, while only 25% of pre-menopausal women and 29% of post-menopausal women had these same symptoms [76]. Furthermore, the Women's Health Initiative found that estrogen replacement therapy improves arthralgia and joint health in post-menopausal women [77]. A study of 420 post-menopausal breast cancer patients, all on AIs, found that mean estradiol levels were significantly lower in patients with AI arthralgia than in patients without AI arthralgia [78].

### 1.7.1 Estrogen synthesis (*CYP17A1* and *CYP19A1*)

*CYP17A1* (also known as  $17\alpha$ -hydroxylase) and *CYP19A1* (also known as aromatase) are the key enzymes that both play pivotal roles in estrogen synthesis. Specifically, *CYP17A1* adds a hydroxyl group to both pregnenolone and progesterone, and then further converts them to androstenedione. Aromatase catalyzes the final step of converting androstenedione to estrone (Figure 1.1).

A clinical study of postmenopausal women with breast cancer on AIs found an association between AI arthralgia and several single nucleotide polymorphisms (SNPs) in *CYP17A1* (rs4919686, rs4919683, rs4919687, rs3781287, rs10786712, rs6163, rs743572) [79].

*CYP19A1* is highly polymorphic, with more than 88 known SNPs, including at least 4 non-synonymous SNPs [80]. Variation in the *CYP19A1* gene, specifically a tetranucleotide repeat polymorphism in intron 4 (rs60271534), has been shown to alter levels of estrone and estradiol in patients on AI therapy. In the same study, patients who carry at least one 8-repeat allele had a lower risk of developing AI arthralgia [81]. Another study which focused on haplotypes within *CYP19A1* found that AI arthralgia was significantly and strongly associated with haplotype M\_3\_5, which contained the following 14 SNPs: rs12148604, rs4646, rs10046, rs700519, rs4324076, rs700518, rs3759811, rs727479, rs4775936, rs10459592, rs767199, rs10519297, rs1062033, rs2008691, rs1008805, and rs17523527 [82]. However, a subsequent study of 154 patients on AIs failed to replicate the association with AI arthralgia when they tested 6 SNPs from haplotype M\_3\_5 (rs10046, rs700519, rs700518, rs727479, rs4775936, rs10459592) [83]. When Garcia-Giralt *et al.* tested two of the SNPs included in haplotype M\_3\_5 (rs4775936 and rs1062033) in a study of 343 postmenopausal women on AIs, they did not find that either SNP alone predicted AI arthralgia. However, one of the haplotype M\_3\_5 SNPs, rs4775936, was associated with worsening pain [79].

### 1.7.2 Estrogen receptor 1 (*ESR1*)

Estrogen receptor alpha ( $ER\alpha$ ) is encoded by *ESR1* and is an important member of a nuclear hormone receptor superfamily.  $ER\alpha$  is responsible for mediating the effects of estrogens and because it is expressed in ER-positive breast tumours and drives proliferation of breast cancer cells, thus it is the primary target of endocrine therapies [84]. A clinical study of patients on AI therapy found that two SNPs in the *ESR1* gene

(rs2234693 and rs9340799) were associated with AI arthralgia [85]. rs2234693 is a SNP located upstream of *ESR1* and is also known as the -397T>C variation. rs9340799 is located in an intronic region of *ESR1*. A large study of patients on letrozole and exemestane demonstrated an association between *ESR1* SNP rs9322336 and discontinuation of AI therapy due to musculoskeletal toxicity; however, this effect was only present for the patients on exemestane [86]. Further replication and functional validation of these 3 SNPs are needed.

## 1.8 Potential role of Inflammation in AI-arthralgia

Arthralgia is often caused by inflammation within the muscles and joints, and inflammatory cytokines are linked to estrogen levels in the body. Specifically, higher levels of estrogen suppress inflammatory cytokine production, while lower estrogen levels increase their production [87]. Specific inflammatory cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 (IL-1) have been detected in peri-menopausal women and found at higher concentrations in the synovial fluid of joints, which may contribute to inflammation and pain [88]. As AIs suddenly and markedly deplete estrogen in treated patients, this rapid lowering of estrogen may be part of the mechanism of AI-induced arthralgia.

However, the evidence to support a direct effect of lower estrogen levels to arthralgia is somewhat conflicting. A recent study of 30 cases with AI arthralgia and 22 controls without AI arthralgia measured changes in serum concentrations of inflammatory molecules, including IL-1, interleukin-6 (IL-6), and TNF- $\alpha$  [89]. Contrary to previous reports, this study found no statistically significant differences in serum concentrations

for any of the inflammatory biomarker between cases and controls. In fact, for 6 of 36 factors, baseline measurements of serum concentration levels were significantly lower in cases than controls. One of the limitations of this study was that inflammatory molecules were measured systemically, whereas inflammation may be occurring locally at the joints. A study that used magnetic resonance imaging was able to demonstrate tenosynovial and articular changes in patients who developed AI arthralgia in their hands and wrists, suggesting active inflammation in the actual joints or tissues around the joint [90].

### 1.8.1 Osteoprotegerin and RANKL in AI arthralgia

Osteoprotegerin is a cytokine receptor and a member of the TNF receptor superfamily. Recent studies have found an association between a SNP (rs2073618) in osteoprotegerin (*OPG*) gene and AI arthralgia [78, 83]. OPG is a receptor for a ligand called receptor activator of nuclear factor kappa-B ligand (RANKL). When OPG binds to RANKL, it prevents nuclear factor kappa B (NF- $\kappa$ B) activation, which is a key transcription factor for immune-related genes, and a known regulator of inflammatory response [91].

### 1.8.2 T-cell leukemia 1A (*TCL1A*)

Variation in the *TCL1A* gene was identified to be associated with AI-arthralgia in a genome-wide association study (GWAS) that included 293 cases with AI-arthralgia and 585 controls. Four SNPs close to *TCL1A* were found to be the most significantly associated with AI arthralgia ( $P = 2.23E-06$  to  $6.67E-07$ ) [92]. The same research group conducted functional validation studies and found that one SNP in particular



(rs11849538) created an estrogen response element (ERE). This finding is significant because the ERE demonstrated estrogen receptor binding and increased estrogen induction of the *TCLIA* gene for the variant genotype. These SNPs were also found to regulate the expression of cytokines and cytokine receptors in an estrogen-dependent manner [93]. These findings link not only inflammation pathways but also estrogen signaling to AI-arthritis.

## 1.9 Pharmacogenetics of aromatase inhibitors

Cytochrome P450 (CYP) enzymes are known to be important to the metabolism of endogenous as well as xenobiotic compounds. Certain CYP enzymes, including the aromatase CYP19A1, have a limited spectrum of endogenous substrates such as androgen. However, other members of the CYP family of enzymes, including those in the CYP2 and 3 families are involved in the metabolism of xenobiotics. We know CYP3A4 and CYP2A6 have been shown to be responsible for metabolizing the currently available AIs. Although all patients on AIs take the same daily dose of each of their prescribed drugs (2.5mg for letrozole, 1mg for anastrozole, and 25mg for exemestane), there is significant interpatient variability in circulating drug concentration [94-99]. A study that examined the drug plasma levels of patients on letrozole found that there was more than a 12-fold variation in drug concentration when corrected for time of the patient's last dose [99]. Similarly, there is a greater than 10-fold variability in plasma concentration of anastrozole and exemestane [97]. Thus it is likely variation in the expression and function of CYP3A4 or CYP2A6 likely impact attained AI levels, and thereby increase the risk of AI-induced arthralgia. Interestingly, switching from one aromatase inhibitor to another in

some cases seem to resolve AI arthralgia. One study of patients on anastrozole with AI arthralgia demonstrated that 71.5% of patients who switched to letrozole were able to tolerate therapy on the second AI [100]. This establishes that patients may experience AI arthralgia with one AI, but not another, indicating that inter-patient differences in metabolism may play a role, since as noted previously, all of the current AIs are potent inhibitors of their target, CYP19A1. Therefore, some of the variation in attained AI levels may relate to pharmacogenetic variation [94-97].

### 1.9.1 Letrozole pharmacogenetics

Letrozole is metabolized to its pharmacologically inactive secondary metabolite, carbinol or 4,4-methanol-bisbenzotrile, by the enzyme CYP2A6 [94, 95]. CYP2A6 is a highly polymorphic enzyme, however, most SNPs that have been detected are extremely rare, thus unlikely to account for the commonly observed arthralgia during letrozole use. However, there are several relatively commonly occurring genetic variations in *CYP2A6*. Such variations include *CYP2A6*\*2, a SNP that results in less than 40% activity, *CYP2A6*\*4, a deletion of the gene resulting in no activity, *CYP2A6*\*9, a SNP that results in 40-50% activity, and *CYP2A6*\*12, a translocation which results in 40-50% activity [101-103]. Letrozole plasma levels were significantly linked to whether a patient was a normal, intermediate, or slow metabolizer of letrozole in relation to their *CYP2A6* genotype. Intermediate and slow metabolizers of *CYP2A6* consistently had the highest levels of letrozole [99].

### 1.9.2 Anastrozole pharmacogenetics

Anastrozole is predominantly oxidized in the liver to hydroxyanastrozole by CYP3A4 and CYP3A5 [96, 97]. For anastrozole, previous studies have shown a range of plasma concentration levels of approximately 10ng/ml to 120ng/ml [97]. *CYP3A4\*22* is a relatively recently identified SNP associated with low CYP3A4 activity [104]. For CYP3A5, 80% of Caucasians are homozygous for the complete loss of function allele *CYP3A5\*3* [105]. Accordingly, a subset of patients would be predicted to more rapidly metabolize drugs such as anastrozole. Note that Phase II enzymes are also involved in the further conversion of hydroxyanastrozole to its glucuronide form through the action of UGT1A4 and, less commonly, by UGT2B7 and UGT1A3 [97].

### 1.9.3 Exemestane pharmacogenetics

Exemestane is metabolized by two main pathways – it is reduced to its biologically active metabolite, 17-hydroexemestane by CYP4A11, CYP1A1, and CYP1A2, and it is oxidized to 6-hydroxymethylexemestane by CYP3A4, CYP3A5, CYP2C8 [96]. 17-hydroexemestane is further metabolized by glucuronidation to exemestane-17-O-glucuronide predominantly by UGT2B17. In vitro studies have shown that an entire gene deletion polymorphism of *UGT2B17* decreases 17-hydroexemestane glucuronidation processes, affecting both metabolism and excretion of the active metabolite [106]. A recent paper validated this in humans by demonstrating that a *UGT2B17* gene deletion polymorphism significantly alters 17-hydroexemestane pharmacokinetic profile [107].

## 1.10 Vitamin D deficiency in AI-arthralgia

Vitamin D also appears to play a role in the development of AI-induced arthralgia. Vitamin D deficiency and insufficiency are common in post-menopausal women initiating adjuvant AI. Several studies have demonstrated that patients who have insufficient or deficient levels of vitamin D are more likely to experience arthralgia during AI therapy. One study found that patients on AIs with musculoskeletal symptoms were more likely to have deficient vitamin D baseline levels when compared to asymptomatic patients [108]. Another study found that vitamin D levels were closely related to the intensity of the arthralgia, with the most severely affected patients having the lowest vitamin D levels [109]. A double-blind placebo-controlled randomized was performed with 60 women who were taking anastrozole to establish whether vitamin D supplementation could improve the symptoms AI-induced arthralgia. The study found that weekly high dose vitamin D significantly improved AI-induced arthralgia [110]. Like estrogen, vitamin D has been linked with inflammation signaling pathways, which suggests a potential mechanistic role of vitamin D in AI-induced arthralgia [111]. However, more research is needed to more fully clarify how vitamin D related factors interact with AIs to in terms of joint pain.

### 1.10.1 The role of *CYP27B1* in vitamin D metabolism

*CYP27B1* encodes 1- $\alpha$ -hydroxylase that catalyzes the hydroxylation of 25(OH)D or calcidiol to the bioactive form 1,25(OH)<sub>2</sub>D or calcitriol. Estrogen is known to increase the activity of 1- $\alpha$ -hydroxylase, thereby increasing the conversion of calcidiol to calcitriol [72]. Hormone replacement therapy in postmenopausal women has been found to

increase circulating levels of calcitriol and is known to support musculoskeletal health [112]. An AI-induced decrease in estrogen levels may result in reduced activation of 1- $\alpha$  hydroxylase and therefore a reduction in circulating calcitriol levels. Therefore it is possible that decreased calcitriol may be contributing to AI arthralgia.

The potential role of calcitriol in AI arthralgia is supported in a study that identified two SNPs in *CYP27B1* (rs4646536 and rs10877012) which were associated with AI arthralgia and one SNP in *CYP27B1* associated with discontinuation of therapy (rs4646536) [79]. Interestingly, in this same study, the authors reported an interaction between *CYP27B1* and *CYP17A1*. Patients who carried risk alleles for both genes had the worst clinical response, with the greatest degree of increased arthralgia at 3 months and 1 year of follow-up on AIs [79].

### 1.10.2 Vitamin D receptor (*VDR*)

The vitamin D receptor (*VDR*) or calcitriol receptor, encoded by the *VDR* gene, binds to calcitriol and mediates its biological activity. *VDR* is widely expressed in the human body and is responsible for regulating the gene expression of a variety of genes involved in inflammation, mineral homeostasis, and skeletal health, as well as renal and cardiovascular protection [113]. Estrogen is known to increase the activity of vitamin D receptor [72]. One SNP in *VDR* (rs11568820) has been shown to be significantly associated with AI arthralgia [79]. The SNP rs11568820 is located in a cdx-2-binding site of the *VDR* promoter. In vitro studies have shown that the presence of this SNP effect *VDR* transcriptional activity and thereby modulate gene expression levels, and of

potential functional relevance to AI arthralgia [114]. This Cdx-2 SNP has also been previously associated with an increased risk of cancer, particularly ovarian cancer [115].

## 1.11 Specific Aims and Hypotheses

### 1.11.1 Specific Aim 1

**Document and quantify adverse drug reactions in patients with ER-positive breast cancer on AIs.** Based on the allele frequency of the genetic variants to be examined, we recruited 200 patients from the London Regional Cancer Program. Clinical variables and adverse drug reaction history were recorded.

Inclusion criteria for enrollment into the study included:

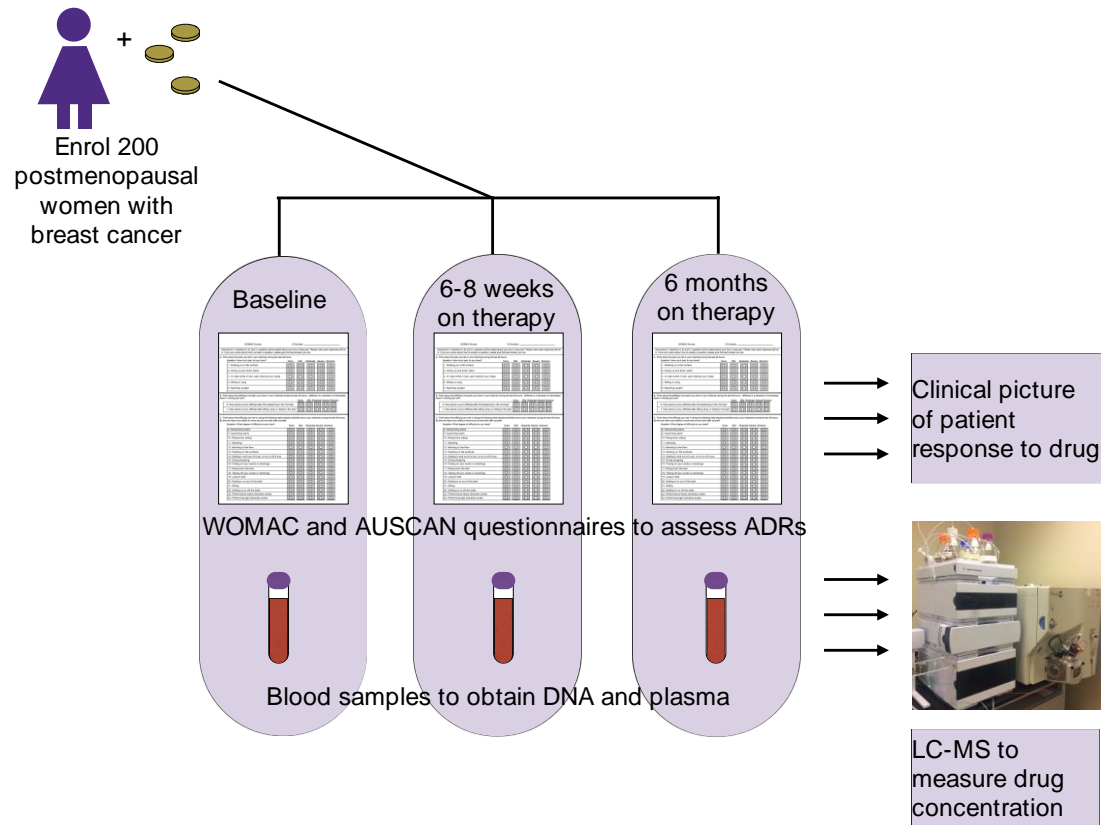
- 1) Postmenopausal women with a diagnosis of stage I to stage III estrogen-receptor positive breast cancer.
- 2) Patients who had completed their initial treatment of surgery, radiation therapy, IV chemotherapy regimens or tamoxifen therapy.
- 3) Patients who had been prescribed aromatase inhibitor treatment (letrozole, anastrozole, exemestane) but had not yet started their medication.

Exclusion criteria for enrollment into the study included:

- 1) Patients who were unable to understand written or spoken English.
- 2) Patients who had a psychiatric history or disability that could affect their ability to give informed consent.

Patients recommended for initiation of AI therapy were identified by their treating

oncologist and were approached by our research team at their clinic appointments. Once informed consent was obtained, the patients were asked to complete two validated questionnaires regarding musculoskeletal side effects. The questionnaires were administered to patients on three separate occasions; prior to AI therapy initiation, approximately 6 weeks post-initiation and 6 months post-initiation. The administration of questionnaires coincided with the patient's clinic visits to avoid additional appointments for the patients. An overview of the study is shown in Figure 1.5.



**Figure 1.5 Overview of study design**



The first questionnaire was the Australian/Canadian Osteoarthritis Hand Index (AUSCAN), version 3.1, which is a 15-item questionnaire that assesses pain, stiffness, and physical functioning of the hands. The AUSCAN is a reliable measure of clinical outcomes and has validated age and gender-specific normative values [98, 116]. The questionnaire contains three subscales (pain, stiffness, and physical functioning); each is scored as the sum of the items on the subscale. An example of the AUSCAN questionnaire is attached as Appendix C. The second questionnaire that was administered was the Western Ontario and McMaster Osteoarthritis Index (WOMAC), version 3.1. This clinical tool assesses pain, stiffness, and physical functioning in the lower extremities, knees, and hips. The questionnaire has 24 items and validated age- and gender-specific normative values are available [117]. The WOMAC consists of three subscales (pain, stiffness, and physical functioning), each scored as the sum of the items on that subscale. An example of the WOMAC questionnaire is attached as Appendix D.

An important element of this aim was to evaluate whether there are changes in the occurrence of adverse drug reactions over time. In order to test whether AI-induced arthralgia increase over time, I administered the AUSCAN and WOMAC questionnaires and measured changes over 6 months. AI-induced arthralgia symptoms have been reported to typically appear in the first 6 months of treatment. The first symptoms tend to appear within the first month of drug initiation, and intensifying in the second and third months of drug use [71]. Because AI-induced arthralgia have not been known to emerge or be reported for the first time after the 6 month point, we chose our patient follow-up times to be 6 weeks and 6 months. Oncologists at the LRCP typically see patients in

clinic at 6 weeks and 6 months following initiation of therapy, and I collected blood samples and administered two questionnaires at patients' regular appointments.

### 1.11.2 Specific Aim 2

**Measure drug levels of AIs and vitamin D levels in breast cancer patients to determine the range of AI levels in response to standard doses.** By measuring drug concentration levels of patients on AIs, we were able to determine whether some patients were outside the therapeutic range, either sub-therapeutic which may affect efficacy or a higher than normal drug concentration level which may cause toxicity. Blood samples were obtained for drug level analysis by liquid chromatography/mass spectrometry and vitamin D level analysis at 6 weeks and 6 months post-initiation of therapy. Drug concentration level analysis of the aromatase inhibitors was achieved by using liquid chromatography/mass spectrometry, as described in Chapter 4.

### 1.11.3 Specific Aim 3

**Determine the genotype of relevant genes in the metabolic pathway of AIs including *CYP3A4*, *CYP3A5*, and *CYP2A6*. Determine the genotype of other potential arthralgia biomarkers, including the estrogen synthesis pathway (*ESR1*, *CYP19A1*), the inflammation pathway (*OPG*, *RANKL*, *TLC1A*), and the vitamin D pathway (*VDR* and *CYP27B1*).** Blood samples were obtained for genotyping by TaqMan SNP assays. Statistical modeling on our patient cohort was used to determine important genetic predictors of AI efficacy and adverse drug reaction risk.

A thorough review of the current literature has revealed which genes are known to contribute to the metabolism of AIs. The gene product of *CYP2A6* metabolizes letrozole

to its pharmacologically inactive secondary metabolite called carbinol or 4,4-methanol-bisbenzotrile [94, 95]. There are more than 50 different types of variation in *CYP2A6*, however most SNPs that have been detected are extremely rare, and may not affect the activity of *CYP2A6*. Therefore, I genotyped patients using the 4 most common star-alleles found within our target population. These included *CYP2A6*\*2, a SNP that results in less than 40% activity, *CYP2A6*\*4, a deletion of the gene resulting in no activity, *CYP2A6*\*9, a SNP that results in 40-50% activity, and *CYP2A6*\*12, a translocation which results in 40-50% activity [101-103]. As described in Chapters 2, 5, and 6, SNPs from estrogen synthesis pathway (*ESR1*, *CYP17A1*, *CYP19A1*), the inflammation pathway (*OPG*, *RANKL*, *TLC1A*), and the vitamin D pathway (*VDR* and *CYP27B1*) were chosen based on previous literature in this field.

#### 1.11.4 Hypotheses

The variability within genes and pharmacokinetics of the AIs in patients with breast cancer explains much of the differences in efficacy and adverse drug reactions between individuals. We hypothesized that polymorphisms in *CYP2A6* and *CYP3A4/5* are associated with altered plasma drug levels and adverse drug reactions. Variation in other arthralgia biomarker genes (*ESR1*, *CYP19A1*, *OPG*, *TLC1A*, *RANKL*, *VDR*, and *CYP27B1*) may explain arthralgia through additional pathways. Understanding the variability can also support the prediction of optimal drug levels, thereby improving efficacy and safety of outcomes in breast cancer patients.

## 1.12 Outline of thesis

The analyses in this thesis have been done on a population of 200 postmenopausal women with breast cancer who were initiated on two different aromatase inhibitors, letrozole and anastrozole. We did not have any patients enrolled on exemestane at our site at the London Regional Cancer Program. Chapter 2 focuses on a sub-analysis of the 126 patients that were on letrozole. We selected these patients for a sub-analysis of the total group to measure letrozole drug levels and genotypes related specifically to letrozole metabolism. Because there were only 70 patients on anastrozole and no patients on exemestane, we did not measure drug levels in these patients. Chapters 3 and 4 focus on the full population of patients on AIs with the patients on letrozole and anastrozole grouped together. Chapter 3 examines SNPs in the estrogen and inflammation pathways and their association with AI induced arthralgia. Chapter 4 investigates the effect of vitamin D on AI induced arthralgia, including both vitamin D levels and SNPs within the vitamin D pathway.

## 1.13 Conclusion

Breast cancer is both the most common cancer and one of the leading causes of death in women. Approximately 80% of breast cancer in postmenopausal women is ER-positive, indicating that these tumours respond to estrogen, which promotes cancer cell proliferation. AIs are the most commonly used first-line endocrine treatment for postmenopausal women with ER-positive breast cancer, but AI activity varies widely among patients. AIs, including letrozole, anastrozole, and exemestane, have been found to deplete estrogen levels, reducing tumour growth and limiting disease progression and

recurrence. Significant ADRs are associated with AIs, the most common being arthralgia. Currently, the therapeutic response and occurrence of adverse effects with AIs for ER-positive breast cancers is largely unpredictable. Candidate genes and molecular biomarkers associated with AI arthralgia offer valuable insight into the complex mechanism of the adverse drug reaction. Additional research is needed to replicate and validate candidate genes and biomarkers to develop utility in a clinical setting to prevent the development of AI arthralgia. Understanding endocrine-based treatments and their associated ADRs will help clinicians better manage AI therapy and deliver better care to patients with breast cancer.

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## 2 LETROZOLE CONCENTRATION IS ASSOCIATED WITH CYP2A6 VARIATION BUT NOT WITH ARTHRALGIA IN PATIENTS WITH BREAST CANCER

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## 2.1 Introduction

Aromatase inhibitors (AIs) are the most commonly used first-line endocrine treatment for postmenopausal women with estrogen receptor-positive (ER-positive) breast cancer [1]. Several adverse drug reactions (ADRs) are associated with AIs with the most common being arthralgia; specifically, bilateral arthritic joint pain affecting the hands, elbows, shoulders, hips, and knees [2]. AI-induced arthralgia affects up to 50% of patients and 20-30% of patients discontinue the medication due to intolerable symptoms [3]. Although the etiology is currently unknown, several underlying mechanisms have been proposed, including AI metabolism and pharmacogenetic factors [4].

Letrozole is one of three third-generation selective AIs and is prescribed as a standard dose of 2.5mg daily for five years. The terminal half-life of letrozole is 48 hours, and steady-state plasma concentration is reached in two to six weeks [5]. Letrozole is metabolized primarily by cytochrome P450 2A6 (CYP2A6), an enzyme encoded by the *CYP2A6* gene, which is expressed mainly in the liver [6-8]. There are over 50 types of *CYP2A6* genetic alterations which effect its' enzymatic activity. However, many of these variants are rare, with minor allele frequencies of less than one percent. *CYP2A6*\*1 represents the wild-type, or reference allele, and indicates normal CYP2A6 function. The variant alleles most commonly found in Caucasian populations are *CYP2A6*\*2, *CYP2A6*\*4, *CYP2A6*\*9, and *CYP2A6*\*12 [9]. *CYP2A6*\*2 is a single nucleotide polymorphism (SNP) that results in less than 40% enzyme activity. *CYP2A6*\*4 represents a deletion of the gene resulting in no activity [10]. *CYP2A6*\*9 is a SNP within the TATA box of the *CYP2A6* promoter region that results in a 50-60% reduction in activity [11].

*CYP2A6\*12* is a translocation which is caused by a crossover between the *CYP2A6* and *CYP2A7* genes, resulting in a hybrid allele, and 40-50% activity [12].

Despite being prescribed as a standard dose, plasma concentrations of letrozole vary greatly, ranging from 25ng/mL to 350ng/mL [7]. Several clinical factors have been found to be associated with letrozole plasma concentration. Circulating concentrations of letrozole are found to be negatively correlated with BMI and positively correlated with age [7]. Furthermore, variation in *CYP2A6* explains a large degree of the variability in steady-state letrozole concentrations in patients with breast cancer [7] and healthy postmenopausal women [13].

Though AI-induced arthralgia is widely recognized as the most common adverse reaction for breast cancer patients taking letrozole, there is little consensus on how best to measure these symptoms. A study by Swenson *et al.* compared the responsiveness of six validated self-report questionnaires of musculoskeletal symptoms and two performance-based tests of physical function during treatment with AIs [14]. They found that the instruments with the greatest sensitivity to changes over the first six months of AI use were the Australian/Canadian Osteoarthritis hand index (AUSCAN) and the Western Ontario and McMaster Osteoarthritis Index (WOMAC) [15]. Importantly, the physical function subscales of the AUSCAN and WOMAC were the most sensitive to change and able to effectively translate the measures of pain and stiffness to the impact on a patient's life.

While studies measuring letrozole concentration have established an association between genotype and drug concentration, there is a lack of prospective studies that specifically

examined the effect of plasma concentration and *CYP2A6* genotype on the development of letrozole-related arthralgia. We hypothesized that genetic polymorphisms in *CYP2A6* were associated with altered letrozole plasma levels and arthralgia. In this study, we prospectively examined the association of WOMAC and AUSCAN scores with circulating levels of letrozole and *CYP2A6* genotypes in postmenopausal women initiated on letrozole therapy.

## 2.2 Materials and Methods

### 2.2.1 Study population

Postmenopausal breast cancer patients (n = 126) diagnosed with stages I-III ER-positive breast cancer initiating letrozole therapy were enrolled at the London Regional Cancer Program in London Ontario between April 2015 – December 2017. All study participants provided written informed consent. This study was approved by the Research Ethics Board at the University of Western Ontario.

### 2.2.2 Sample collection and storage

Blood samples were obtained from each patient at three time points: prior to letrozole therapy initiation, approximately two months post-initiation, and six months post-initiation on 2.5mg of letrozole. The date and time of blood collection and last letrozole dose were recorded and used to calculate the time (in hours) since the last dose. Plasma was isolated from venous blood and stored at -80°C until analysis.



### 2.2.3 Letrozole measurement

Steady-state plasma letrozole concentration was measured by liquid chromatography-mass spectrometry (LC-MS/MS) using methods as described previously [16]. Letrozole and the internal standard, letrozole-D4, were obtained from Toronto Research Chemicals (Toronto, Ontario, Canada). Plasma samples (150  $\mu$ L) were prepared with 10  $\mu$ L of internal standard and underwent solid phase extraction in BondElut C18 96-well plates (Agilent Technologies, Santa Clara, California, USA). Upon injection into the liquid chromatograph (Agilent 1200) the molecules were separated on a ZORBAX Eclipse XDB-C18 column using a gradient elution with acetonitrile in 0.1% acetic acid. Standard curves and quality control samples were prepared in drug-free plasma. The Thermo TSQ Vantage mass spectrometer with heated electrospray ionization source was set in positive mode for detection of letrozole and letrozole-D4 with transitions of 286.1  $\rightarrow$  217 m/z and 290.1  $\rightarrow$  221 m/z respectively.

### 2.2.4 Genotyping

DNA was extracted from whole EDTA blood using either the MagNA Pure Compact instrument (Roche, Laval, Quebec, Canada) or the MagMax<sup>TM</sup> Express instrument (Thermo Fisher Scientific, Waltham, Massachusetts, United States). DNA samples were stored at -20°C until analysis. The following TaqMan<sup>®</sup> allelic discrimination assays (Applied Biosystems, Carlsbad, CA) were used for genotyping: *CYP2A6*\*2 (rs1801272, assay ID: C\_\_27861808\_60), *CYP2A6*\*9 (rs28399433, assay ID: C\_\_30634332\_10), *CYP2A6*\*12 (rs4803380, assay ID: C\_\_32350075\_20). A TaqMan<sup>®</sup> gene copy number assay (Applied Biosystems, Carlsbad, CA) was utilized to detect gene deletion (\*4) and

duplication (assay ID: Hs07545274\_cn). Hardy-Weinberg equilibrium was assessed for all genotypes using the chi-square goodness-of-fit test. Individuals with one copy of the *CYP2A6*\*9 decreased-function allele were categorized as intermediate metabolizers; those with two copies of the *CYP2A6*\*9 allele or one or two copies of loss-of-function alleles (*CYP2A6*\*2, \*4, and \*12) were categorized as slow metabolizers as described previously [7].

### 2.2.5 Clinical data collection

Patient demographics including age, height, weight, ethnicity, and medication history were documented at the time of the first blood draw, and the letrozole start date was recorded. Cancer stage, as well as ER, progesterone receptor (PR), and human epidermal growth factor 2 (HER2) status were collected from the patients' medical chart.

### 2.2.6 Instruments

Patients were asked to complete two musculoskeletal questionnaires, the AUSCAN version 3.1 and the WOMAC version 3.1, which were previously validated as sensitive measures for AI-induced arthralgia [14]. The AUSCAN is a 15-item questionnaire assessing pain, stiffness, and physical function in the upper body extremities, with possible scores ranging from 0-20 for pain, 0-4 for stiffness, and 0-36 for physical function [15]. The WOMAC is a 24-item questionnaire that assesses pain, stiffness, and physical function in the lower extremities, with possible scores ranging from 0-20 for pain, 0-8 for stiffness, and 0-68 for physical function [15]. For both instruments and their subscales, higher scores represent worse symptoms. Both questionnaires were completed

by participants at the time of each of the three blood draws: prior to letrozole therapy initiation, at two months post-initiation, and six months post-initiation.

### 2.2.7 Statistical analysis

All statistical analyses were performed using R (version 3.31) [17] and GraphPad Prism (version 6.0, San Diego, CA) statistical software. All tests were two-sided and were considered statistically significant at  $P \leq 0.05$ . A student's t-test was performed to determine whether letrozole plasma concentration differed between patients who were normal and reduced metabolizing patients. A linear mixed effect model was used to assess the association between letrozole plasma concentration, genotype, and time since last dose while adjusting for BMI and age. The same linear mixed effects model was used for modelling AUSCAN and WOMAC scores repeatedly measured over time. We compared adjusted mean questionnaire scores at each visit to estimate the effect of time by visit while adjusting for age, BMI, and genotype. Multiple linear regression analyses were performed to assess the effects estimate of age, BMI, and letrozole concentration on the outcome of change in AUSCAN and WOMAC scores.  $P$  values for these estimates were adjusted for multiple testing using the Holm-Bonferroni method.

## 2.3 Results

### 2.3.1 Patient demographics and medication history

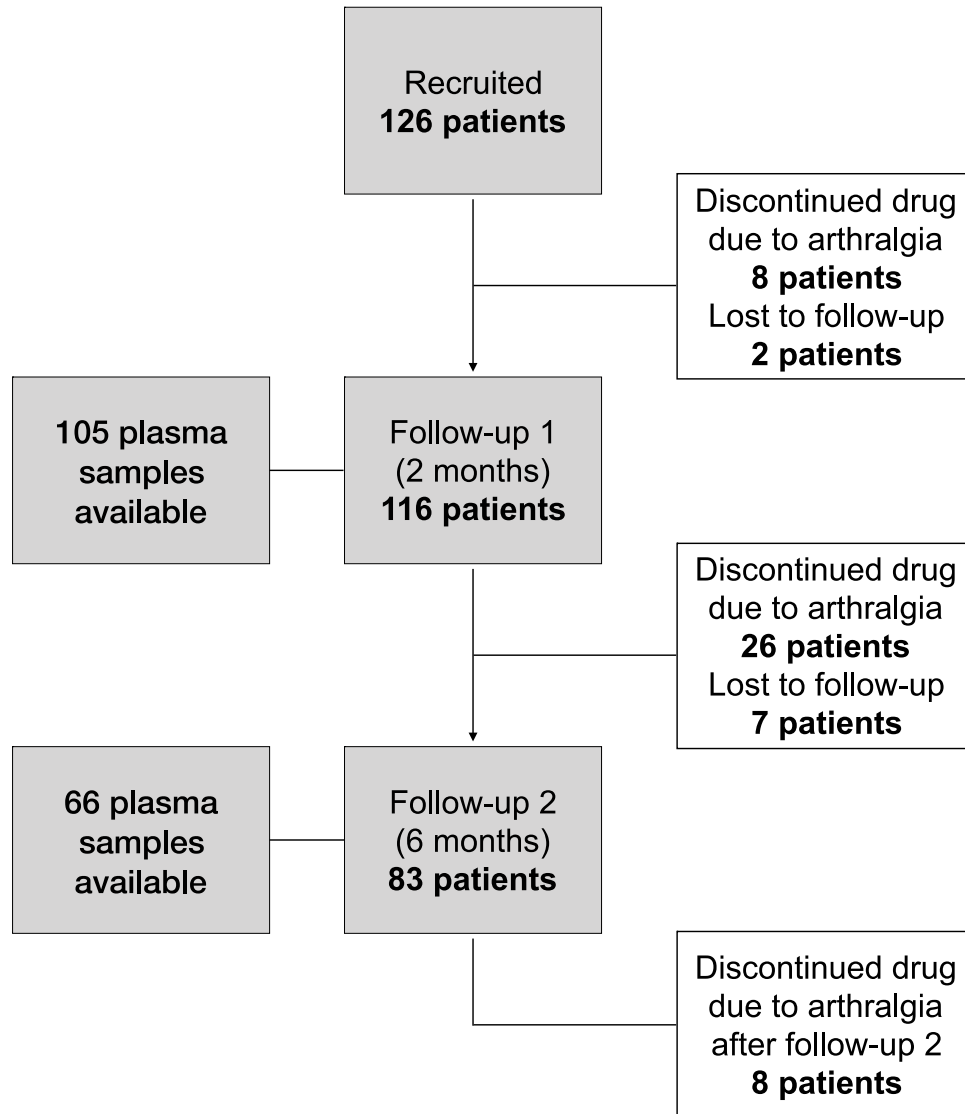
We analyzed clinical variables and letrozole concentration data from 126 patients with participant characteristics summarized in Table 2.1. Of these, 116 (92.1%) completed at least two months of treatment and returned for follow-up visit 1, and 83 (65.9%)

completed the six-month follow-up 2 visit (Figure 2.1). The majority of patients were Caucasian (92.4%). Patients were not on any CYP2A6 inhibiting drugs (methoxsalen, selegiline, tranylcypromine, ketoconazole) [18]. All patients were initiated on the standard dose of 2.5mg of letrozole, with no adjustments in the dose.

**Table 2.1 Patient demographics (n = 126)**

Patient characteristic	
Age (Median (Min, Max))	65.5 (31, 88)
BMI (Median (Min, Max))	29.3 (20.1, 52.6)
Race/Ethnicity ( <i>N</i> (%))	
Caucasian	118 (92.4%)
Asian	2 (1.6%)
Other	6 (4.8%)
ER status ( <i>N</i> (%))	
Positive	126 (100%)
Negative	0
PR status ( <i>N</i> (%))	
Positive	106 (84.1%)
Negative	20 (15.9%)
HER2 status ( <i>N</i> (%))	
Positive	16 (12.6%)
Negative	110 (87.3%)
Stage of cancer ( <i>N</i> (%))	
I (A/B)	38 (30.2%)
II (A/B)	68 (54.0%)
III (A/B/C)	20 (15.8%)
IV	0

*BMI* body mass index; *ER* estrogen receptor; *PR* progesterone receptor; *HER2* human epidermal growth factor receptor 2



**Figure 2.1** Flow chart for patient inclusion

### 2.3.2 Letrozole plasma concentrations

Plasma samples from the first and second follow-up visits were available from 105 and 66 enrolled patients, respectively. We observed a 16-fold variation in letrozole concentration level, with a median of 90.1 ng/mL and a range of 15.1 – 247.4 ng/mL. Time since last dose in hours was calculated for each follow-up visit. All patients had taken their daily dose of letrozole within the past 24 hours of blood draw and patients who self-reported non-compliance with their medication (n = 2) were excluded in the final analysis. The time since last dose was statistically significant ( $P = 0.01$ ) when age, BMI, and genotype were corrected for, however the effect estimate of time since last dose was minimal (effect estimate = -0.84 [95%CI, -1.48 to -0.18]), meaning that for each hour of time, letrozole concentration decreased 0.84 ng/mL. This indicates that time since last dose has very little impact on letrozole concentration and is consistent with the known long half-life (48 hours) of letrozole, demonstrating that patients were at a steady-state concentration.

### 2.3.3 Effect of genotype and clinical variables on letrozole concentration

*CYP2A6* genotype information is summarized in Table 2.2. Patients were classified as normal (n = 92; \*1/\*1), intermediate (n = 23; \*1/\*9), or slow (n = 11; \*9/\*9, \*1/\*2, \*1/\*4, \*1/\*12) metabolizers of letrozole based on their genotype as previously characterized [7]. Accordingly, 34 (27.0%) of patients were determined to have *CYP2A6* reduced-function genotypes. We did not detect any gene duplication of *CYP2A6* in this patient population. Normal metabolizers had significantly lower plasma letrozole

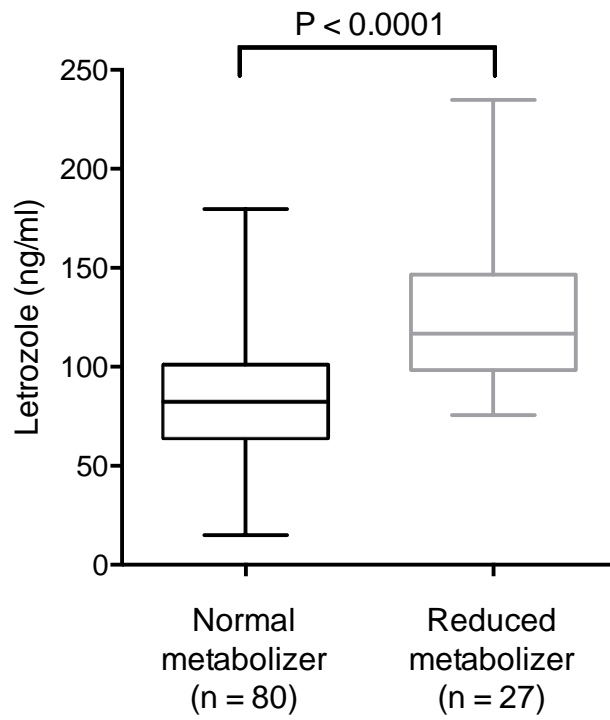
concentrations (median, 82.53 (min, max; 15.10, 179.70) ng/mL) than reduced metabolizers (median, 116.80 (min, max; 75.56, 247.40) ng/mL) ( $P < 0.0001$ ) (Figure 2.2). Letrozole concentration was significantly associated with both BMI (effect estimate = -1.92, [95% CI, -2.91 to -0.92],  $P = 0.0001$ ) and age (effect estimate = 0.96 [95% CI, 0.04 to 1.88,  $P = 0.04$ ) (Figure 2.3). When taking repeated visits into account, as well as adjusting for age, BMI, and time since last dose, the reduced metabolizer genotype remained significantly associated with letrozole concentration; the letrozole concentration level for those with reduced metabolizer genotypes was 36.55 ng/mL higher on average than that for those with normal metabolizer genotypes ([95% CI, 22.63 to 50.47],  $P < 0.0001$ ).



**Table 2.2 SNP information and genotyping quality control (n = 126)**

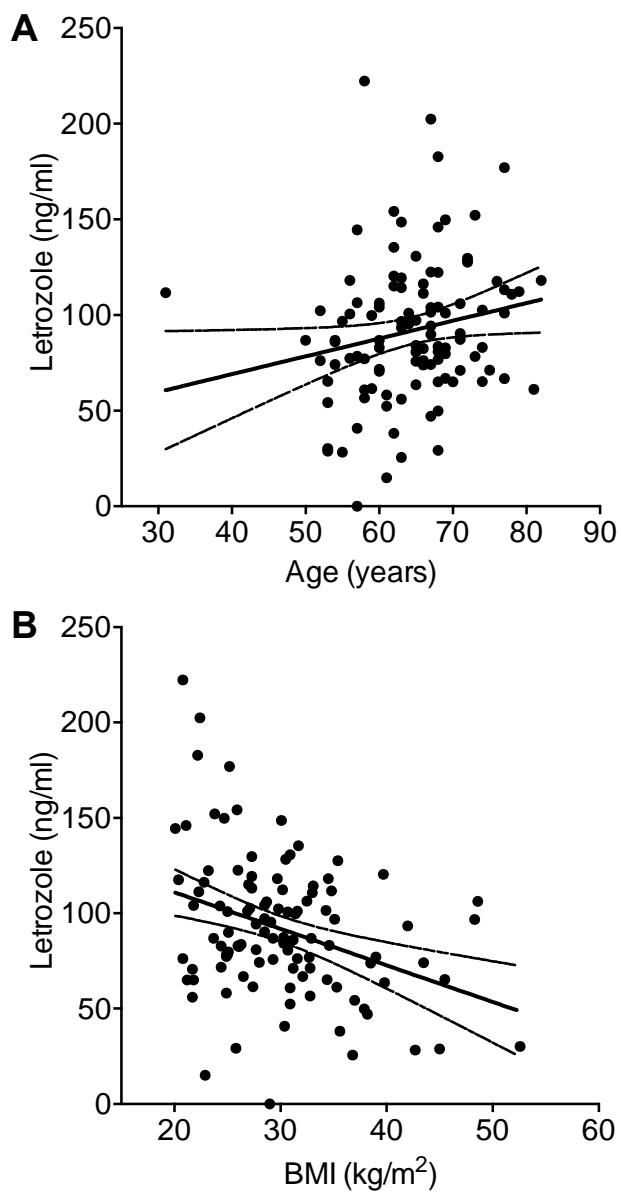
Gene	Common allele name	rs ID	Allelic change	Call rate	MAF in this cohort	HWE <i>P</i> value
<i>CYP2A6</i>	*2	rs1801272	1799T>A	1.0	0.03	0.01
<i>CYP2A6</i>	*4	N/A	gene deletion	1.0	0.004	0.89
<i>CYP2A6</i>	*9	rs28399433	-48T>G	1.0	0.09	0.96
<i>CYP2A6</i>	*12	rs4803380	translocation	1.0	0.008	0.92

*SNP* single nucleotide polymorphism; *MAF* minor allele frequency; HWE Hardy–Weinberg equilibrium



**Figure 2.2 Plasma letrozole concentration significantly associated with *CYP2A6* genetic variation**

Statistical test: student's t-test



**Figure 2.3 Influence of age and BMI on letrozole concentration**

Letrozole concentration was positively associated with (a) age ( $R^2 = 0.040$ ,  $P = 0.043$ ) and inversely associated with (b) BMI ( $R^2 = 0.1181$ ,  $P = 0.0003$ ). *Solid line* linear regression line; *dotted lines* 95% confidence intervals. Statistical test: Linear mixed effects model.

### 2.3.4 Measurement of arthralgia symptoms

For patients who completed follow-up visit 1, 64 of 116 (55.2%) experienced increases in their AUSCAN score, and 62 of 116 (53.4%) experienced increases in their WOMAC score compared to baseline. For patients who completed a second follow-up visit, 44 of the 83 (53.0%) experienced score increases on the AUSCAN, and 42 of the 83 (50.6%) experienced score increases on the WOMAC compared to baseline. Mean AUSCAN and WOMAC scores at each follow-up visit and the effect estimate of time by visit (adjusted by age, BMI, and genotype) using a mixed effects regression model are summarized in Table 2.3. We observed positive effect estimate of time on mean scores; mean scores increased from baseline to the first and second follow-up visits for the WOMAC for each measure (pain, stiffness, physical functioning and total score). We observed positive effect estimate of time between mean scores from baseline to the second follow-up for each AUSCAN measure (pain, stiffness, physical functioning and total score) and for pain and total score for the first follow-up visit. All of the visit effects were positive for each WOMAC and AUSCAN measure comparing visits 1 and 2 to the baseline, indicating that pain, stiffness, and difficulty with physical function increased over the follow-up periods. Furthermore, 42 of 126 patients (33.3%) discontinued their letrozole due to intolerable arthralgia. The mean time to discontinuation for patients who stopped using the medication was 140 days (SD = 108 days) with a median time to discontinuation of 92 days. Patients who discontinued letrozole before the first or second follow-up were excluded from the analysis and did not provide follow-up samples or questionnaires. Specifically, eight patients discontinued letrozole due to arthralgia between baseline and follow-up visit 1. A sub-analysis of the patients who discontinued

between follow-up visit 1 and follow-up visit 2 due to arthralgia ( $n = 26$ ) and the patients who continued on letrozole for at least 6 months and completed their second follow-up visit ( $n = 83$ ) revealed that the group that discontinued had a significantly higher change in AUSCAN scores at follow-up visit 1 ( $P = 0.027$ ). Letrozole level, age, BMI, and WOMAC score were not significantly different between the two groups.

**Table 2.3 Adjust mean questionnaire scores at three time points**

	Baseline (n = 126)	Follow-up visit 1 (n = 116)			
	Mean	Mean	Estimate	95% CI	Sig.
<b>AUSCAN</b>					
Pain	2.34	3.31	0.97	0.29 to 1.66	*
Stiffness	0.72	0.88	0.16	-0.02 to 0.34	*
Physical functioning	4.55	5.15	0.60	-0.39 to 1.58	*
Total score	7.61	9.36	1.75	0.12 to 3.37	*
<b>WOMAC</b>					
Pain	2.95	3.69	0.74	0.13 to 1.36	*
Stiffness	1.66	2.03	0.37	0.06 to 0.68	*
Physical functioning	9.29	11.66	2.37	0.73 to 4.01	*
Total score	13.90	17.4	3.50	1.18 to 5.81	*

(Table 2.3 continued)

	Follow-up visit 2 (n = 82)			
	Mean	Estimate	95% CI	Sig.
<b>AUSCAN</b>				
Pain	4.16	1.82	1.02 to 2.62	*
Stiffness	1.12	0.40	0.19 to 0.61	*
Physical functioning	6.88	2.33	1.19 to 3.48	*
Total score	12.24	4.62	2.73 to 6.52	*
<b>WOMAC</b>				
Pain	4.04	1.09	0.38 to 1.81	*
Stiffness	2.22	0.56	0.20 to 0.92	*
Physical functioning	12.94	3.64	1.73 to 5.56	*
Total score	19.20	5.32	2.61 to 8.03	*

*AUSCAN* Australian/Canadian Osteoarthritis Hand Index; *WOMAC* Western Ontario and McMaster Osteoarthritis Index; *CI* confidence interval *Sig.* Significant. Statistical test: mixed effects regression model. All scores and effect estimates are corrected for age, BMI, and genotype. Significance indicates that the 95% confidence interval for the effect estimate does not cross zero.

### 2.3.5 Letrozole concentration does not affect WOMAC and AUSCAN scores

Simple linear regression analyses showed that a change in AUSCAN scores was not significantly associated with steady-state letrozole concentration at follow-up visit 1 (n = 105) or follow-up visit 2 (n = 66). Changes in WOMAC scores at follow-up visit 1 (n = 105) and follow-up visit 2 (n = 66) suggested a very weak negative association with letrozole concentration. However, when *P* values were corrected for multiple testing using the Holm-Bonferroni method, the change in WOMAC score was not found to be associated with letrozole concentration, age or BMI (Table 2.4)

**Table 2.4 Influence of clinical factors on change in WOMAC and AUSCAN scores**

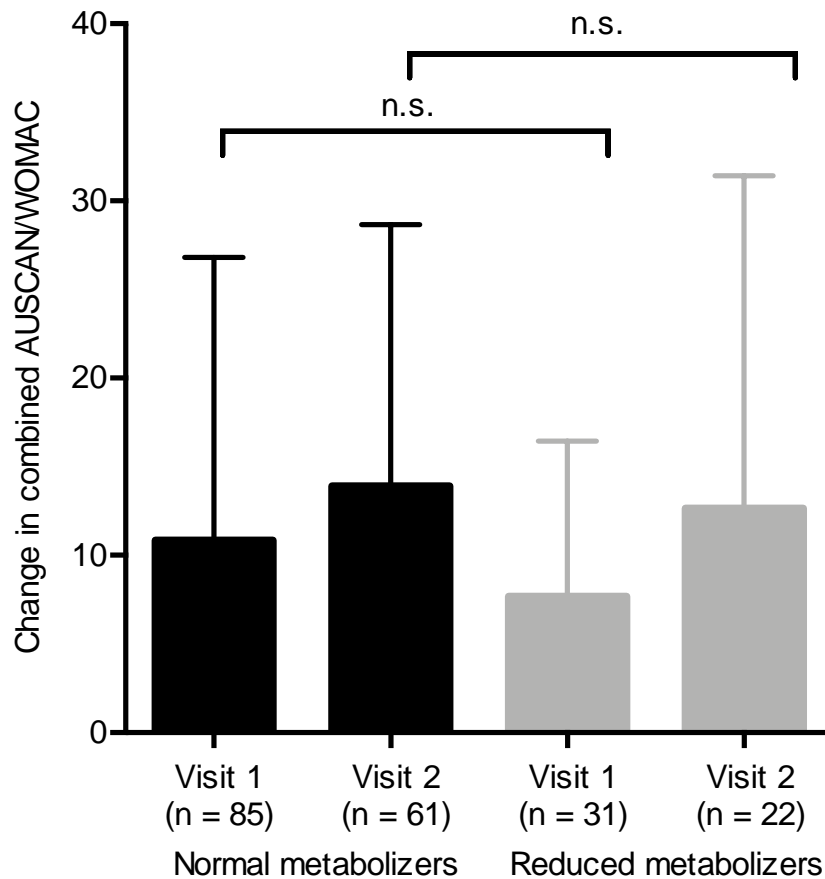
Outcome	Predictor variable	Effect estimate	95% CI	P value	Adjusted P value
Change in WOMAC (Follow-up visit 1)	Age	-0.132	-0.260 to -0.003	<b>0.045</b>	0.135
	BMI	0.077	-0.030 to 0.184	0.156	0.282
	[Letrozole]	-0.445	-1.041 to 0.151	0.141	0.282
Change in WOMAC (Follow-up visit 2)	Age	-0.100	-0.203 to 0.005	0.062	0.124
	BMI	0.083	-0.020 to 0.187	0.115	0.124
	<b>[Letrozole]</b>	-0.857	-1.608 to -0.105	<b>0.026</b>	0.078
Change in AUSCAN (Follow-up visit 1)	Age	-0.079	-0.258 to 0.100	0.383	1.000
	BMI	0.024	-0.124 to 0.172	0.744	1.000
	[Letrozole]	-0.331	-1.159 to 0.498	0.43	1.000
Change in AUSCAN (Follow-up visit 2)	Age	-0.118	-0.286 to 0.049	0.162	0.324
	BMI	-0.080	-0.229 to 0.068	0.283	0.324
	[Letrozole]	-0.883	-1.964 to 0.199	0.108	0.324

*AUSCAN* Australian/Canadian Osteoarthritis Hand Index; *WOMAC* Western Ontario and McMaster Osteoarthritis Index; *CI* confidence interval; Statistic test: Linear regression. *P* value was adjusted using the Holm–Bonferroni method



### 2.3.6 *CYP2A6* genotype does not predict arthralgia symptoms

There was no difference in combined pain scores between reduced metabolizers relative to those in normal metabolizers at the first follow-up visit ( $n = 116$ ,  $P = 0.30$ ) and second follow-up visit ( $n = 83$ ,  $P = 0.76$ ) time points (Figure 2.4) using two-tailed unpaired *t*-tests. A linear random intercept model was utilized to account for repeated measures and adjust for age and BMI, failed to detect an association between increased WOMAC and AUSCAN scores for follow-up visit 1 (effect estimate = -0.39 [95% CI, -5.70 to 4.92],  $P = 0.886$ ) or follow-up visit 2 (effect estimate = -0.87 [95% CI, -7.04 to 5.29],  $P = 0.78$ ).



**Figure 2.4 No association was found between change in pain score and *CYP2A6* genotype**

No association was found between change in pain score and *CYP2A6* activity the first follow-up visit ( $n = 116$ ,  $R^2 = 0.009$ ,  $P = 0.2981$ ) or second follow-up visit ( $n = 83$ ,  $R^2 = 0.001$ ,  $P = 0.7530$ ). Statistical test: linear random intercept model was utilized to account for repeated measures and adjust for age and BMI

## 2.4 Discussion

This is the first study to use the AUSCAN and WOMAC assessment tools to measure the effect of letrozole concentration and *CYP2A6* genotype on letrozole-induced arthralgia in patients with ER-positive breast cancer. In this prospective cohort of 126 patients, we confirmed that there was a significant association between letrozole concentration and *CYP2A6* genotype. However, changes in AUSCAN and WOMAC scores were not associated with letrozole concentration. Furthermore, the *CYP2A6* genotype was not significantly associated with a change in arthralgia symptoms. Thus, these results suggest that systemic letrozole concentrations and *CYP2A6* genotype are not clinically meaningful predictors of AI-induced arthralgia.

Thirty-four patients (27%) in this study had a genotype that is associated with decreased *CYP2A6* function. Patients carrying at least one of the *CYP2A6* reduced-function genotypes (\*2, \*4, \*9, or \*12) had significantly higher steady-state letrozole concentrations than patients lacking these variations. This finding is consistent with previous studies [7, 13]. We observed a 16-fold variation in the plasma concentration of letrozole level, which was significantly associated with *CYP2A6* genotype, age, and BMI. The *CYP2A6* genotype accounted for 17% of the explained variability, while BMI and age accounted for 12% and 4%, respectively.

The AUSCAN and WOMAC are validated self-report tools that effectively assess pain, stiffness and physical function changes, and can detect early letrozole-induced arthralgia symptoms. In this cohort, we found that mean scores on each subscale of the AUSCAN and WOMAC increased over the duration of letrozole treatment, indicating

worsening pain, stiffness, and physical function. Of note, the most substantial increase in AUSCAN and WOMAC scores occurred between baseline and follow-up visit 1, indicating arthralgia symptoms do not appear to increase linearly over time. Patients with an increase in AUSCAN score at follow-up visit 1 were more likely to be in the group that discontinued their medication by follow-up visit 2. However, individual factors such as stage of cancer and risk of recurrence may influence patient motivation for continuing on therapy despite AI-induced arthralgia. Further research is needed to determine whether clinicians could utilize these tools to predict which patients may discontinue letrozole therapy due to intolerable arthralgia. Clinicians may be able to better measure arthralgia symptoms by asking patients to complete AUSCAN and WOMAC questionnaires at regular intervals throughout the course of letrozole therapy, either through paper questionnaires or by recording the data in an electronic mobile phone application. These measures have been adapted and validated for mobile phone use to electronically capture the pain, stiffness, and physical function data, as well as recording date and time of assessment [19, 20]. Data can then be sent electronically to their physician where it can be stored in their electronic health record and evaluated, better informing clinicians of their patients' response to medication and potential for discontinuation.

An advantage of our study is that we collected data prospectively, and blood samples were taken at the same time as the AUSCAN and WOMAC were administered. The AUSCAN and WOMAC request that patients comment on the severity of their most recent symptoms from the past 48 hours [15]. Because letrozole has a long half-life [5] and time since last dose was recorded, letrozole levels in compliant patients were assumed to be in steady state. Our measurements of symptoms and drug levels were

robust, and therefore method inaccuracy is unlikely to explain an absence of an observed association. A larger study may confirm our findings with a greater degree of certainty.

A limitation of this study was the difficulty in collecting plasma samples or questionnaires from patients who discontinued their drug between the baseline and follow-up visits. Thus, for those patients, it is difficult to determine whether arthralgia was associated with letrozole plasma concentration. We were not able to analyze data from patients who discontinued letrozole before follow-up visit 1 as letrozole concentration, and AUSCAN and WOMAC scores were not collected. However, we did perform a sub-group analysis on the patients who discontinued their letrozole between follow-up visit 1 and follow-up visit 2. We found that those who discontinued had a significantly higher mean change in AUSCAN scores at follow-up visit 1 compared to patients who remained on letrozole therapy for the full 6 months. This provides further evidence that AUSCAN and WOMAC scores should be investigated as potentially predictive clinical assessment tools.

There are likely other clinical characteristics and genetic factors contributing to changes in pain, stiffness, and physical function scores, and the probability of patients discontinuing letrozole treatment due to arthralgia. A prospective study of 135 female patients with no prior pain who were on aromatase inhibitors investigated a number of different clinical, biological, environmental, and genetic risk factors for the development of arthralgia [21]. The authors found that patient anxiety may be a predictor of the development of AI-induced arthralgia [21]. As we did not measure anxiety, depression, or other psychological factors, further investigation is needed to replicate these findings. Additional SNPs in estrogen and inflammation pathways have been found to be

associated with AI-induced arthralgia in candidate gene and genome-wide analyses [4, 22]. However, these genetic findings remain to be replicated in prospective studies in order to determine if they are reliable predictors of AI-induced arthralgia.

Overall, this study indicates that *CYP2A6* genotype, age, and BMI are predictors of letrozole concentration. Although we did not observe an association between letrozole levels and arthralgia symptoms, we did find that the AUSCAN and WOMAC instruments are very useful measures of AI-induced pain, stiffness, and changes in physical function. These measures may be applied in clinical practice to monitor AI adverse effects. They could also be used to further investigate AI-induced arthralgia mechanisms as well as therapeutic interventions to reduce arthralgia.

## 2.5 References

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### 3 GENETIC AND CLINICAL PREDICTORS OF AROMATASE INHIBITOR ARTHRALGIA IN BREAST CANCER PATIENTS

### 3.1 Introduction

Breast cancer is the most common cancer in females worldwide, as well as the leading cause of cancer death in women [1]. As many as 80% of breast cancers are estrogen receptor (ER)-positive, meaning that the tumour grows in response to estrogen [2]. Anti-estrogen therapy includes tamoxifen for pre-menopausal women and aggressive tumours. Aromatase inhibitors (AIs) are well established as the most effective first-line endocrine treatment for postmenopausal women with ER-positive breast cancer and the most commonly used treatment in North America [3]. Aromatase inhibitors work through their inhibition of the aromatase enzyme, which is encoded by the *CYP19A1* gene [4]. Aromatase catalyzes the final step in the estrogen production pathway [5]. By inhibiting aromatase, these drugs reduce the amount of estrogen to nearly undetectable levels. All three aromatase inhibitors, letrozole, anastrozole, and exemestane have nearly identical effectiveness in reducing estrogen levels, but also have similar adverse drug reaction (ADR) profiles. The clinical impact of AIs varies widely among patients and up to 50% of patients experience ADRs, with the most common being arthralgia. These reactions adversely impact the quality of life for patients, leading to compromised compliance and contribute to early discontinuation [6]. The mechanism of the ADRs is largely unknown.

A number of different scales and measures have been employed to evaluate the intensity of the pain and stiffness that a patient with AI-induced arthralgia experiences, as well as the effect on the patient's quality of life. A prospective, longitudinal study by Swenson *et al.* [7] examined the responsiveness of standardized self-reported measures of AI-induced arthralgia, comparing six different questionnaires administered to 122

patients assessed at baseline, and at one, three, and six months post-initiation of therapy. They concluded that the Australian/Canadian Osteoarthritis Hand Index (AUSCAN), the Western Ontario and McMaster Osteoarthritis Index (WOMAC) had the greatest sensitivity and responsiveness in detecting and measuring AI-induced arthralgia.

Previous pharmacogenomic studies have attempted to identify biomarkers that predict drug response by investigating the association of ADRs with genetic variation in patients. Polymorphisms within genes in the estrogen pathway (*CYP19A1*, *ESR1*) and genes associated with inflammation (*OPG*, *TCLAI*, *RANKL*) have been shown to be associated with AI arthralgia [8]. Although several candidate SNPs have been identified, there are no established clinical practice guidelines currently, so a personalized medicine approach cannot be implemented within a clinical setting. Replication and validation of these biomarkers are an essential next step to advance these discoveries into implementation in patient care. We conducted a prospective study of patients treated with AIs and measured their arthralgia through the course of their treatment using the validated AUSCAN and WOMAC questionnaires.

## 3.2 Methods

### 3.2.1 Study design and participants

Female patients ( $n = 196$ ) who were prescribed AI therapy were recruited from the London Regional Cancer Program, London, Ontario, Canada between April 2015 and December 2017. All study participants provided written informed consent. The study was approved by the Research Ethics Board at the University of Western Ontario.

### 3.2.2 Demographics and medication history

Demographic information including gender, age, height, weight, and ethnicity of the participants was recorded at the patient's initial clinic visit. The type of AI prescribed, dose, date of initiation, and concomitant medication history were also recorded. At each follow-up visit, participants were asked whether they were still on AI treatment, and if not, the reason they had stopped and the date of their last dose. The cancer stage, as well as ER, progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) status were collected from the patients' medical chart.

### 3.2.3 Sample collection and storage

Blood samples were obtained from each patient at three time points: prior to AI therapy initiation, approximately two months' post-initiation, and six months' post-initiation on 2.5mg of letrozole or 1mg of anastrozole. Blood samples were immediately stored at 4°C before DNA extraction.

### 3.2.4 Arthralgia assessment

Participants were assessed at three time points: A baseline assessment was completed prior to initiation on AI therapy, at approximately two months post initiation and again at six months post initiation. The AUSCAN, version 3.1 and the WOMAC, version 3.1 questionnaires were administered during clinic visits where blood samples were obtained. The AUSCAN is a 15-item questionnaire that assesses pain, stiffness, and physical

functioning of the hands. The WOMAC is a 24-item questionnaire that evaluates pain, stiffness, and physical functioning in the lower extremities, knees, and hips.

### 3.2.5 Genotyping

Patient DNA was extracted from whole blood in EDTA tubes using either the MagNA Pure Compact instrument (Roche, Laval, Quebec, Canada) or the MagMax<sup>TM</sup> Express instrument (Thermo Fisher Scientific, Waltham, Massachusetts, United States). DNA samples were stored at -20°C until analysis. TaqMan<sup>®</sup> allelic discrimination assays (Applied Biosystems, Carlsbad, CA) were performed for the following SNPs: *CYP19A1* (rs4775936, assay ID: C\_\_11301451\_10); *ESR1* (rs9322336, assay ID: C\_\_29568677\_10; rs2234693, assay ID: C\_\_3163590\_10; and rs9340799, assay ID: C\_\_3163591\_10); *OPG* (rs2073618, assay ID: C\_\_1971047\_1\_); *RANKL* (rs7984870, assay ID: C\_\_29811035\_20); and *TCLIA* (rs11849538, assay ID: C\_\_1927667\_10). The Hardy-Weinberg equilibrium was assessed for all genotypes using Chi-square goodness-of-fit test.

### 3.2.6 Statistical analyses

All statistical analyses were performed using R (version 3.31)[9] and GraphPad Prism (version 6.0, San Diego, CA) statistical software. All tests were two-sided and were considered statistically significant at  $P \leq 0.05$ . Logistical regression was performed on each SNP using the development of arthralgia and discontinuation of drug as the outcome measure, correcting for the clinical variables of age and BMI. The time from initiation to discontinuation of AI therapy was compared among the three genotype groups of

*CYP19A1* using the log-rank test. For patients who did not discontinue treatment, the date of the last follow-up inquiry confirming that they were on AI treatment was recorded. Cox regression analysis was used to test for an independent contribution of the treatment variable. We report the odds ratio (OR) and the corresponding *P* value for each covariate. The odds ratio can be interpreted as the relative risk for development of AI arthralgia and discontinuation of AI therapy.

### 3.3 Results

#### 3.3.1 Patient demographics

The patient ( $n = 196$ ) characteristics are summarized in Table 3.1. Our study population was primarily of Caucasian ethnicity (93.9%, self reported). The median age was 65, and all but one patient were 50 years of age or older, and had proceeded through menopause. One 31-year-old patient had become menopausal surgically with a total abdominal hysterectomy with bilateral salpingo-oophorectomy. All patients were initiated on either the standard dose of 2.5mg of letrozole or 1mg of anastrozole with no adjustments in the dose. Although exemestane was also available as a therapeutic option, none of the patients enrolled in our study were initiated on exemestane. Of the 196 patients, 186 patients (94.9%) completed at least two months of treatment and returned for a follow-up visit and 138 patients (70.4%) completed at least six months of AI therapy and returned to the clinic for a second follow-up visit.

### 3.3.2 AI-induced arthralgia and early discontinuation of AI treatment

We measured arthralgia in our population using the AUSCAN and WOMAC questionnaires. Of the 196 patients, 104 (53.1%) reported joint pain or stiffness after initiation on an AI. The mean scores on all measures of both the AUSCAN and WOMAC increased significantly at each follow-up visit (Table 3.2). Of note, there was a positive association between BMI and both AUSCAN ( $P = 0.001$ , estimate = 0.370 [95% CI, 0.160 to 0.581]) and WOMAC ( $P < 0.0001$  estimate = 0.872 [95% CI, 0.561 to 1.183]) scores at baseline before AI initiation, adjusting for age. However, baseline AUSCAN ( $P = 0.123$ ) and WOMAC ( $P = 0.490$ ) scores did not predict the development of AI-induced arthralgia, after adjusting for age and BMI. Fifty-five patients (28.1%) discontinued AI therapy due to intolerable arthralgia, with a median time to discontinuation of 92 days. The mean time to discontinuation was 148 days (SE = 16.68). Two additional patients discontinued AI therapy for reasons other than arthralgia, including hair loss and mood changes.

**Table 3.1 Patient characteristics**

	Letrozole (n = 125)	Anastrozole (n = 71)	Total (n = 196)
Age (Median (Min, Max))	65 (31, 88)	65 (50, 84)	65 (31, 88)
BMI (Median (Min, Max))	30.1 (20.1, 52.6)	28.9 (18.6, 45.3)	29.2 (18.6, 52.6)
Race/Ethnicity ( <i>N</i> (%))			
Caucasian	117 (93.6%)	67 (94.3%)	184 (93.9%)
Asian	2 (1.6%)	1 (1.4%)	3 (1.5%)
Other	6 (4.8%)	3 (4.3%)	9 (4.6%)
ER status ( <i>N</i> (%))			
Positive	125 (100%)	71 (100%)	196 (100%)
Negative	0	0	0
PR status ( <i>N</i> (%))			
Positive	105 (84.0%)	60 (84.5%)	165 (84.2%)
Negative	20 (16.0%)	11 (15.5%)	31 (15.8%)
HER2 status ( <i>N</i> (%))			
Positive	16 (12.8%)	10 (14.1%)	26 (13.3%)
Negative	109 (87.2%)	61 (85.9%)	170 (86.7%)
Stage of cancer ( <i>N</i> (%))			
I (A/B)	38 (30.4%)	34 (47.9%)	72 (36.7%)
II (A/B)	67 (53.6%)	33 (46.5%)	100 (51.0%)
III (A/B/C)	20 (16.0%)	4 (5.6%)	24 (12.2%)
IV	0	0	0
Prior chemotherapy ( <i>N</i> (%))			
Yes	65 (52.0%)	25 (35.2%)	90 (45.9%)
No	60 (48%)	46 (64.8%)	106 (54.1%)
Prior taxane use ( <i>N</i> (%))			
Yes	60 (48.0%)	21 (29.6%)	81 (41.3%)
No	65 (52.0%)	50 (70.4%)	115 (58.7%)

*BMI* body mass index; *ER* estrogen receptor; *PR* progesterone receptor; *HER2* human epidermal growth factor receptor 2



**Table 3.2 Mean questionnaire scores at the three study time points**

	Baseline (n = 196)	Follow-up visit 1 (n = 186)			Sig.
	Mean	Mean	Estimate	95% CI	
<b>AUSCAN</b>					
Pain	2.46	3.2	0.83	0.32 to 1.33	*
Stiffness	0.76	0.91	0.16	0.02 to 0.29	*
Physical functioning	4.39	5.01	0.77	0.01 to 1.52	*
Total score	7.61	9.12	1.77	0.55 to 2.98	*
<b>WOMAC</b>					
Pain	2.92	3.58	0.74	0.26 to 1.22	*
Stiffness	1.59	2.03	0.44	0.20 to 0.68	*
Physical functioning	8.90	11.13	2.46	1.14 to 3.77	*
Total score	13.41	16.74	3.65	1.81 to 5.49	*

(Table 3.2 continued)

	Follow-up visit 2 (n = 138)			
	Mean	Estimate	95% CI	Sig.
<b>AUSCAN</b>				
Pain	3.63	1.44	0.87 to 2.00	*
Stiffness	1.10	0.37	0.23 to 0.52	*
Physical functioning	6.17	2.22	1.37 to 3.06	*
Total score	10.90	4.08	2.71 to 5.44	*
<b>WOMAC</b>				
Pain	3.95	1.20	0.66 to 1.73	*
Stiffness	2.10	0.54	0.28 to 0.81	*
Physical functioning	12.21	4.04	2.56 to 5.52	*
Total score	18.15	5.80	3.73 to 7.85	*

*AUSCAN* Australian/Canadian Osteoarthritis Hand Index; *WOMAC* Western Ontario and McMaster Osteoarthritis Index; *CI* confidence interval; *Sig.* Significant. Statistical test: linear regression model. All effect estimates are corrected for age, BMI, and genotype. Significance indicates that the 95% confidence interval for the effect estimate does not cross zero.

**Table 3.3 Association of arthralgia with clinical variables**

		Univariate analysis		
		Unadjusted <i>P</i> value	Odds Ratio	95% CI
Intercept				
Age (years)		0.584	0.990	0.956 to 1.026
BMI (kg/m <sup>2</sup> )		0.001	1.088	1.035 to 1.144
Aromatase inhibitor	Letrozole	<i>reference</i>		
	Anastrozole	0.023	0.504	0.279 to 0.910
Prior chemotherapy	Yes	<i>reference</i>		
	No	0.519	0.831	0.473 to 1.460
Prior taxane therapy	Yes	<i>reference</i>		
	No	0.557	0.843	0.476 to 1.491
Luminal status	Luminal A	<i>reference</i>		
	Luminal B	0.241	0.607	0.264 to 1.399
Cancer stage	I (A/B)	<i>reference</i>		
	II (A/B)	0.773	1.094	0.596 to 2.008
	III (A/B/C)	0.556	0.757	0.300 to 1.913

*CI* confidence interval; *BMI* body mass index

(Table 3.3 continued)

		Multivariable analysis		
		Adjusted <i>P</i> value	Odds Ratio	95% CI
Intercept		0.374	0.255	
Age (years)		0.757	0.993	0.952 to 1.036
BMI (kg/m <sup>2</sup> )		0.001	1.088	1.037 to 1.152
Aromatase inhibitor	Letrozole			
	Anastrozole	0.018	0.462	0.244 to 0.877
Prior chemotherapy	Yes	<i>reference</i>		
	No	0.720	0.765	0.178 to 3.294
Prior taxane therapy	Yes	<i>reference</i>		
	No	0.970	0.971	0.208 to 4.523
Luminal status	Luminal A	<i>reference</i>		
	Luminal B	0.285	0.611	0.248 to 1.508
Cancer stage	I (A/B)	<i>reference</i>		
	II (A/B)	0.453	0.761	0.372 to 1.555
	III (A/B/C)	0.276	0.534	0.173 to 1.651

*CI* confidence interval; *BMI* body mass index

Statistical tests: Logistical and linear regression models.

### 3.3.3 Clinical variables associated with AI arthralgia

BMI was significantly associated with AI-induced arthralgia ( $P = 0.001$ ; OR = 1.088 [CI 95%, 1.025 to 1.144], Table 3.3) and this significance was maintained in the multivariable analysis. The odds ratio for this clinical variable was quite small, indicating that although this is a precise predictor of arthralgia, it only influences its development to a small degree. BMI was not found to be a predictor for discontinuation of therapy ( $P = 0.113$ ; OR = 1.039 [CI 95%, 0.991 to 1.090]). The development of arthralgia symptoms was significantly less in patients prescribed anastrozole compared to letrozole in both the univariate ( $P = 0.023$ ; OR = 0.504 [95% CI, 0.279 to 0.910], Table 3.3) and multivariable analyses ( $P = 0.018$ ; OR = 0.462 [95% CI, 0.244 to 0.877], Table 3.3). Patients prescribed anastrozole were significantly less likely to discontinue their drug due to AI-induced arthralgia than patients prescribed letrozole ( $P = 0.032$ ; OR = 0.464 [CI 95%, 0.230 to 0.934]) after adjusting for age and BMI. The odds ratio indicates that patients on letrozole were more than twice as likely to discontinue their AI.

### 3.3.4 Pharmacogenetic variables associated with AI arthralgia

SNP genotyping information is summarized in Table 3.4. Four SNPs, one in *CYP19A1* (rs4775936) and three in *ESR1* (rs9322336, rs2234693, rs930799) were significantly associated with the development of arthralgia when controlling for the clinical variables of age and BMI (Table 3.5). Interestingly, the SNP in *CYP19A1* (rs4775936) was significantly associated with discontinuation of drug due to arthralgia when controlling for age and BMI (Table 3.6). Using a Cox regression analysis, *CYP19A1* (rs4775936) was also significantly associated with discontinuation of drug due to arthralgia (log

ranked  $P = 0.035$ , Figure 3.1). The SNPs in selected genes associated with inflammation (*OPG*, *TCLAI*, *RANKL*) were not associated with either arthralgia or early discontinuation of drug due to arthralgia. Because the results of previous studies on *RANKL* suggested that there might be a recessive effect of the G allele, we repeated the genetic association for this SNP using a recessive genetic model, adjusting for age and BMI, but did not find that it was associated with AI-induced arthralgia ( $P = 0.233$ ; OR = 1.464 [95% CI, 0.782 to 2.738]) or discontinuation of drug ( $P = 0.170$ ; OR = 1.581 [95% CI, 0.822 to 3.041]).

**Table 3.4 SNP information and genotyping quality control**

Gene	rs ID	SNP location or AA change	Call rate	MAF in this cohort	HWE <i>P</i> value
<i>CYP19A1</i>	rs4775936	Intronic	1.0	0.44	0.338
<i>ESR1</i>	rs2234693	Intronic	1.0	0.42	0.936
<i>ESR1</i>	rs9322336	Intronic	1.0	0.24	0.117
<i>ESR1</i>	rs9340799	Intronic	1.0	0.30	0.300
<i>OPG</i>	rs2073618	Asn3Lys	1.0	0.49	0.569
<i>TCL1A</i>	rs11849538	Downstream	1.0	0.12	0.140
<i>RANKL</i>	rs7984870	Intronic	1.0	0.43	0.560

*SNP* single nucleotide polymorphism; *MAF* minor allele frequency; *HWE* Hardy–Weinberg equilibrium

**Table 3.5 Genetic associations with AI arthralgia**

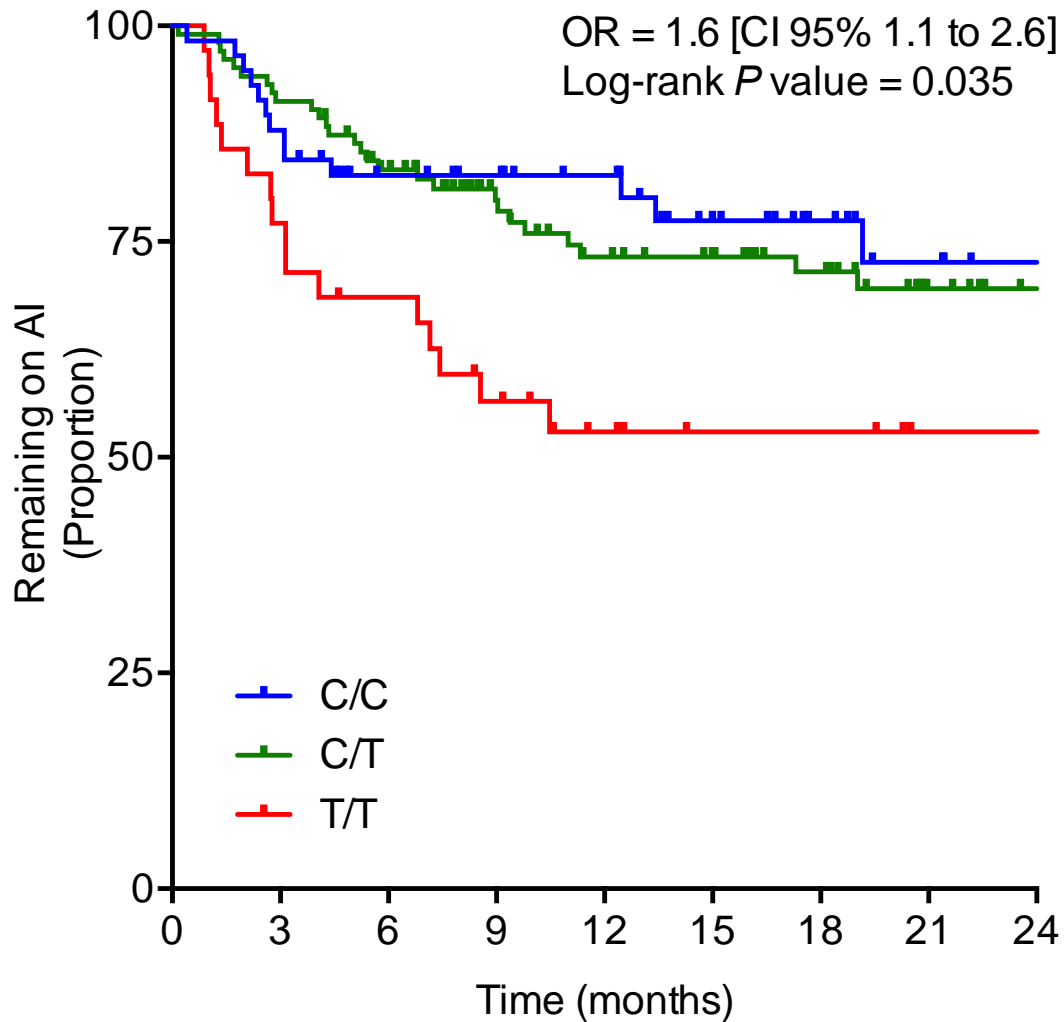
Gene	SNP	Odds Ratio	95% CI	P Value	Sig.
<i>CYP19A1</i>	rs4775936	1.719	1.115 to 2.692	<b>0.016</b>	*
<i>ESRI</i>	rs9322336	0.553	0.336 to 0.896	<b>0.018</b>	*
<i>ESRI</i>	rs2234693	1.706	1.109 to 2.673	<b>0.017</b>	*
<i>ESRI</i>	rs9340799	1.626	1.014 to 2.656	<b>0.047</b>	*
<i>OPG</i>	rs2073618	1.107	0.741 to 1.660	0.619	
<i>TCLIA</i>	rs11849538	1.146	0.637 to 2.089	0.649	
<i>RANKL</i>	rs7984870	0.874	0.574 to 1.324	0.525	

*SNP* single nucleotide polymorphism; *Sig.* significance. All associations are corrected for age and BMI. Statistical test: logistical regression model.

**Table 3.6 Genetic associations with early discontinuation of AI**

<b>Gene</b>	<b>rs ID</b>	<b>Odds Ratio</b>	<b>Confidence Interval</b>	<b>P Value</b>	<b>Sig.</b>
<i>CYP19A1</i>	rs4775936	1.618	1.019 to 2.607	<b>0.044</b>	*
<i>ESRI</i>	rs9322336	0.633	0.364 to 1.061	0.092	
<i>ESRI</i>	rs2234693	1.386	0.889 to 2.174	0.151	
<i>ESRI</i>	rs9340799	1.515	0.924 to 2.498	0.100	
<i>OPG</i>	rs2073618	0.976	0.630 to 1.509	0.912	
<i>TCLIA</i>	rs11849538	1.440	0.781 to 2.607	0.231	
<i>RANKL</i>	rs7984870	0.762	0.480 to 1.194	0.240	

*SNP* single nucleotide polymorphism; *Sig.* significance. All associations are corrected for age and BMI. Statistical test: logistical regression model.



**Figure 3.1 Time to treatment discontinuation for patient-reported AI arthralgia**

*CYP19A1* (rs4775936) genotype (C/C, C/T, and T/T). The proportion of patients remaining on the first aromatase inhibitor medication is given on the y-axis. AI, aromatase inhibitor; OR, odds ratio. Statistical test: Cox regression model.



### 3.4 Discussion

In this prospective analysis of arthralgia in early stage breast cancer patients treated with AIs, we observed that more than half of patients developed arthralgia symptoms during treatment with AIs. We found that nearly one-third of patients discontinued AI therapy due to severe and intolerable musculoskeletal adverse reactions. Our results are consistent with previous studies in which compliance to AI therapy was compromised by ADRs, primarily musculoskeletal in nature [6, 10].

The etiology of AI-induced arthralgia is relatively unclear, although different mechanisms and pathways have been identified [8]. Our study focused on mechanism-specific predictors of AI-induced arthralgia in the estrogen and inflammation pathways. *CYP19A1* is a gene that encodes aromatase, an enzyme responsible for the biosynthesis of estrogen and the protein target of AIs. A study by Garcia-Giralt *et al.* (2013) found that the *CYP19A1* SNP rs4775936 was associated with worsened arthralgia pain [11]. In our study, we found that rs4775936 was not only associated with arthralgia but also predicted early discontinuation of AI therapy due to arthralgia. SNPs in *ESR1* (rs9322336, rs2234693, rs930799) also have been found to be associated with the development of AI-related musculoskeletal symptoms [12, 13]. *ESR1* is a gene which encodes estrogen receptor alpha (ER- $\alpha$ ), which is an estrogen-activated transcription factor. ER- $\alpha$  is the primary receptor in the estrogen signalling pathway, and its expression is the defining feature of ER-positive breast cancer [14]. The intronic variant in *ESR1* (rs9322336) was found to be associated with AI-induced arthralgia in patients treated with exemestane [12]. Although our population was treated with letrozole and anastrozole only, we found

that rs9322336 was associated with the development of arthralgia, indicating that this SNP might predict arthralgia for all three AIs.

We were unable to validate previously reported findings that demonstrated that a SNP (rs11849538) near the *TCLIA* gene was associated with a decrease in arthralgia. This SNP was identified in a genome-wide association study of breast cancer patients in the MA.27 phase III clinical trial. Previous pharmacogenetic studies of *RANKL* (rs7984870) demonstrated that the G allele was protective of AI-induced arthralgia [15], and patients homozygous for the G allele of rs7984870 in *RANKL* had a lower risk of musculoskeletal ADR-associated treatment discontinuation [16]. The rs7984870 SNP was not significantly associated with arthralgia or discontinuation of therapy in our population when using either an additive genetic model or a recessive genetic model. *OPG* (rs2073618) is a missense Asn3Lys SNP that was originally found to be associated with decreased *OPG* expression and an increased risk of musculoskeletal ADRs in a population of breast cancer patients treated with AIs in a Chinese Han population [15]. A recent study was unable to replicate this association between rs2073618 and the development of musculoskeletal symptoms [16], which is consistent with our findings that there is no association between *OPG* (rs2073618) and the development of AI-induced arthralgia.

It was interesting to note that the clinical variable of BMI was significantly associated with the development of arthralgia, although the odds ratio was small, indicating that the impact of the variable is of lower importance. BMI has been previously linked to increased estrogen levels, as aromatase catalyzes biosynthesis of estrogen in the adipose tissues of post-menopausal women [5].

We observed a significant difference in tolerability between letrozole and anastrozole in our population. However, a large meta-analysis of clinical trial data detected no difference in musculoskeletal ADRs among patients treated with any of the three AIs [17]. However, a prospective study evaluating musculoskeletal symptoms in early-stage breast cancer patients treated with exemestane or letrozole found that time to treatment discontinuation was significantly shorter in patients prescribed exemestane [10]. It is possible that if the effect size between drugs were relatively small, it might not be detected in a meta-analysis and would require a study with more sensitive assessment tools of musculoskeletal symptoms. This finding requires further replication in order to determine whether it has clinical importance.

A previous study reported that patients who had been previously treated with taxane were more likely to report arthralgia symptoms [6]. However, two subsequent studies failed to replicate this finding [18, 19]. We also observed that prior treatment with taxane did not predict the development of AI-induced arthralgia.

In summary, more than half of our patients experienced treatment-emergent arthralgia symptoms, and almost one-third of our patients discontinued their AI treatment due to arthralgia. Both clinical and genetic factors help to explain the variation in tolerability of AIs. We replicated previously established associations of SNPs in genes within the estrogen-signalling pathway. Additional functional validation studies could aid in understanding the mechanism behind these genetic associations and lead to more accurate predictors of toxicity. Overall, our findings help to refine interventions to prevent AI-induced arthralgia and improve compliance with AI therapy.

### 3.5 References

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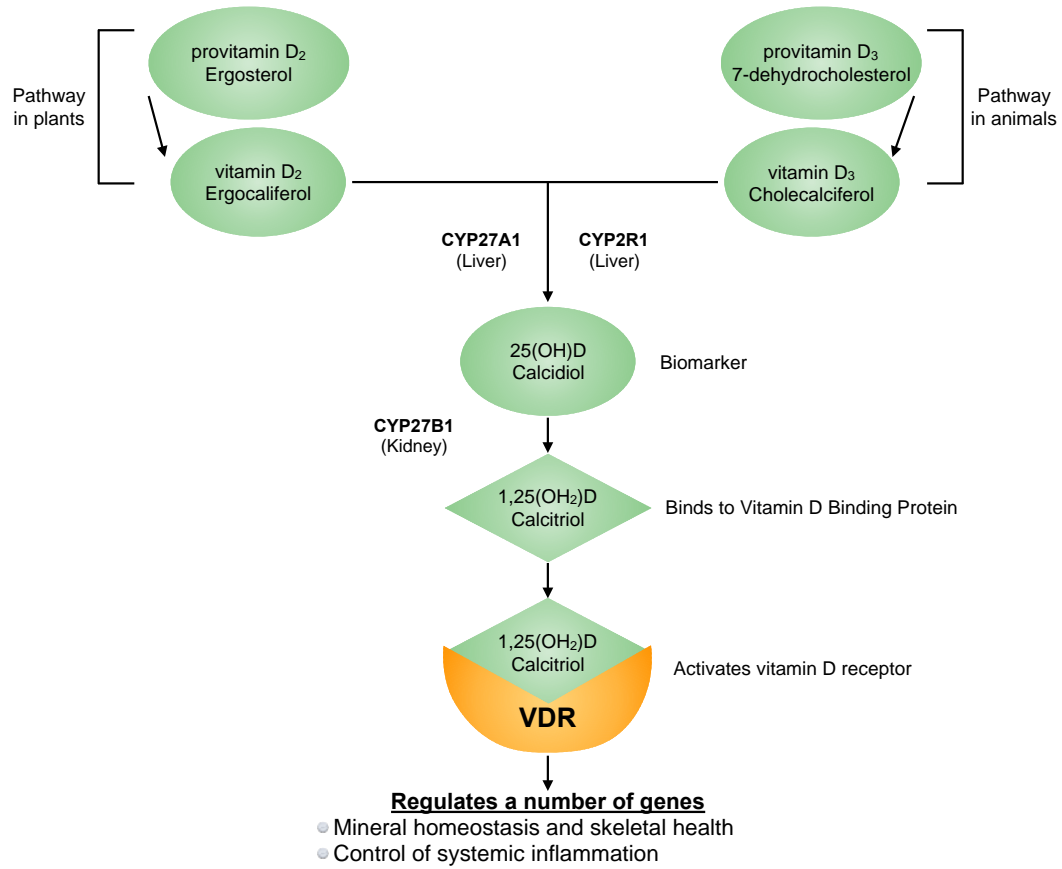
4 PROSPECTIVE ANALYSIS OF VITAMIN D LEVELS,  
VITAMIN D RECEPTOR POLYMORPHISMS, AND  
ARTHRALGIA IN POSTMENOPAUSAL WOMEN ON  
AROMATASE INHIBITORS

## 4.1 Introduction

Arthralgia pain, characterized by bilateral aches and pains affecting the shoulder, elbow, hand, hip and knee joints, is a frequent adverse drug reaction (ADR) among women prescribed AIs for treatment of estrogen receptor (ER) positive breast cancer [1-3]. The cause of AI arthralgia is unknown, however experts in the field theorize there may be multiple contributing factors, including vitamin D levels and SNPs in genes involved in the vitamin D metabolic pathway [4].

Vitamin D is a hormone that can be synthesized in the skin in the presence of ultraviolet-B light or consumed orally through supplementation of natural and fortified food [5]. Serum 25-hydroxyvitamin D [25(OH)D] provides the most accurate assessment of vitamin D status and is used as a biomarker in studies which measure vitamin D [6]. Vitamin D metabolism is illustrated in Figure 4.1.

Like other hormones, vitamin D plays a role in a wide range of processes in the body, including musculoskeletal health. Vitamin D deficiency is common in populations north of latitude 40 degrees north, in healthy postmenopausal women [7], and in women receiving adjuvant chemotherapy for breast cancer [8]. Low vitamin D intake and low 25(OH)D have been linked to higher prevalence of musculoskeletal symptoms, including arthralgia, in populations without breast cancer [9]. Vitamin D has been shown to play a role in the development of AI-induced arthralgia. Some studies have found that patients with low levels of vitamin D were more likely to develop arthralgia post-initiation on AI therapy. One study observed that patients on AIs with musculoskeletal symptoms were more likely to be vitamin D deficient at the time of AI initiation when compared to



**Figure 4.1 Schematic diagram of steroid and vitamin D signalling pathway**

Sterol 27-hydroxylase (encoded by *CYP27A1*) and vitamin D 25-hydroxylase (encoded by *CYP2R1*) both convert vitamin D<sub>2</sub> and D<sub>3</sub> to 25-hydroxyvitamin D (calcidiol). 1- $\alpha$ -hydroxylase (encoded by *CYP27B1*) catalyzes the hydroxylation of 25(OH)D (calcidiol) to the bioactive form 1,25(OH)<sub>2</sub>D (calcitriol). Calcitriol binds to the vitamin D receptor (encoded by *VDR*)



asymptomatic patients [10]. Servitja *et al.* found that vitamin D levels were closely related to the intensity of the arthralgia, with the most severely affected patients having the lowest vitamin D levels [11]. Body mass index (BMI) is a clinical factor which has been previously shown to be inversely associated with serum 25(OH)D levels [12, 13]. Low serum 25(OH)D levels are relatively common in adults over the age of 65 [14]. More research is needed to elucidate how these different factors interact to create painful joints in patients on AIs.

SNPs in genes in the vitamin D pathway have been previously investigated in genetic association studies in patients on AIs. Garcia-Giralt *et al.* demonstrated that variants in *VDR*, and *CYP27B1* genes predict the risk of AI arthralgia [15]. While vitamin D levels are known to impact the development of arthralgia, it is unclear how vitamin D levels, genetic variation, and arthralgia interact.

In our prospective study of women initiated on AI therapy, we investigated the effect of genetic variants in the vitamin D pathway, their effect on 25(OH)D levels, and whether either of these two factors contributed to the development of AI arthralgia in patients with ER-positive breast cancer.

## 4.2 Materials and Methods

### 4.2.1 Study population

196 postmenopausal breast cancer patients diagnosed with stage I-III ER-positive breast cancer who were considering AI therapy were enrolled at the London Regional Cancer Program in London Ontario from April 2015 – December 2017. All study participants

provided written informed consent. The Research Ethics Board at the University of Western Ontario approved the study.

#### 4.2.2 Sample collection and storage

Blood samples were obtained from each patient at three time points: prior to AI therapy initiation, approximately 2 months post-initiation, and 6 months post-initiation on 2.5mg of letrozole or 1mg of anastrozole. Date and time of blood collection and last AI dose were recorded and used to calculate the time (in hours) since the last dose. Blood samples were immediately stored at 4°C before centrifugation at 2000G for 10 minutes for plasma isolation. Plasma samples were stored at -80°C until analysis.

#### 4.2.3 Plasma vitamin D measurement

Plasma 25-hydroxy-Vitamin D (D<sub>2</sub> and D<sub>3</sub>) levels were measured by ELISA as per manufacture's protocol (BioVendor, Candler, NC). Dates of blood draw for each patient were categorized into seasons: Winter (October-March) when the sunlight levels are lower and summer (April to September) when sunlight levels are higher.

#### 4.2.4 Clinical data collection

Patient demographics including age, height, weight, ethnicity, and medication history were documented at the time of the first blood draw, and the letrozole start date was recorded. Cancer stage, as well as estrogen receptor (ER), progesterone receptor (PR), and HER2 status were collected from the patients' chart.

#### 4.2.5 Instruments

Patients were asked to complete two musculoskeletal questionnaires – AUSCAN version 3.1 and the WOMAC version 3.1, which were previously validated as sensitive measures for AI-induced arthralgia[16]. The AUSCAN is a 15-item questionnaire assessing pain, stiffness, and physical function in the upper body extremities, with possible scores ranging from 0-20 for pain, 0-4 for stiffness, and 0-36 for physical function [17]. The WOMAC is a 24-item questionnaire that assesses pain, stiffness, and physical function in the lower extremities, with possible scores ranging from 0-20 for pain, 0-8 for stiffness, and 0-68 for physical function [17]. For both instruments and their subscales, higher scores represent worse symptoms. The two questionnaires were administered to each patient on three separate occasions and completed at the time of each of the three blood draws: prior to AI therapy initiation, approximately 2 months post-initiation, and 6 months post-initiation.

#### 4.2.6 Genotyping

DNA was extracted from whole blood using either a standard DNA extraction protocol (QIAmp DNA Mini Kit, Qiagen, Valencia, California) or the MagNA Pure Compact instrument (Roche, Laval, Quebec, Canada) and DNA samples were stored at -20°C until analysis. *VDR* (rs11568820, assay ID: C\_\_\_2880808\_10) and *CYP27B1* (rs4646536, assay ID: C\_\_25623453\_10) Taqman allelic discrimination assays (Applied Biosystems, Carlsbad, CA) were used for genotyping. Hardy-Weinberg equilibrium was assessed for all genotypes using the chi-square goodness-of-fit test.

## 4.2.7 Statistical analysis

All statistical analyses were performed using R (version 3.31)[18] and GraphPad Prism (version 6.0, San Diego, CA) statistical software. All tests were two-sided and were considered statistically significant at  $P \leq 0.05$ .

## 4.3 Results

### 4.3.1 Patient demographics and medication history

196 postmenopausal women with breast cancer were enrolled into our study prior to initiation on AI therapy with patient demographics and medication history summarized in Table 4.1. Women were predominantly Caucasian (93.6%). The median age was 66 years and the mean age was 65 years (SD = 8.1; range = 31 - 88). All patients were initiated on the standard dose of 2.5mg of letrozole (n = 125) or 1mg of anastrozole (n = 71), with no adjustments in the dose. In our study, 186 patients (94.9%) completed at least 2 months of treatment returned for a follow-up 1 visit and 138 patients (70.4%) completed at least 6 months of AI therapy and returned to the clinic for a follow-up 2 visit.

**Table 4.1 Patient characteristics**

	Total (n = 196)
Age (Median (Min, Max))	65 (31, 88)
BMI (Median (Min, Max))	29.2 (18.6, 52.6)
Race/Ethnicity ( <i>N</i> (%))	
Caucasian	184 (93.9%)
Asian	3 (1.5%)
Other	9 (4.6%)
ER status ( <i>N</i> (%))	
Positive	196 (100%)
Negative	0
PR status ( <i>N</i> (%))	
Positive	165 (84.2%)
Negative	31 (15.8%)
HER2 status ( <i>N</i> (%))	
Positive	26 (13.3%)
Negative	170 (86.7%)
Stage of cancer ( <i>N</i> (%))	
I (A/B)	72 (36.7%)
II (A/B)	100 (51.0%)
III (A/B/C)	24 (12.2%)
IV	0

*BMI* body mass index; *ER* estrogen receptor; *PR* progesterone receptor; *HER2* human epidermal growth factor receptor 2

### 4.3.2 Vitamin D analysis

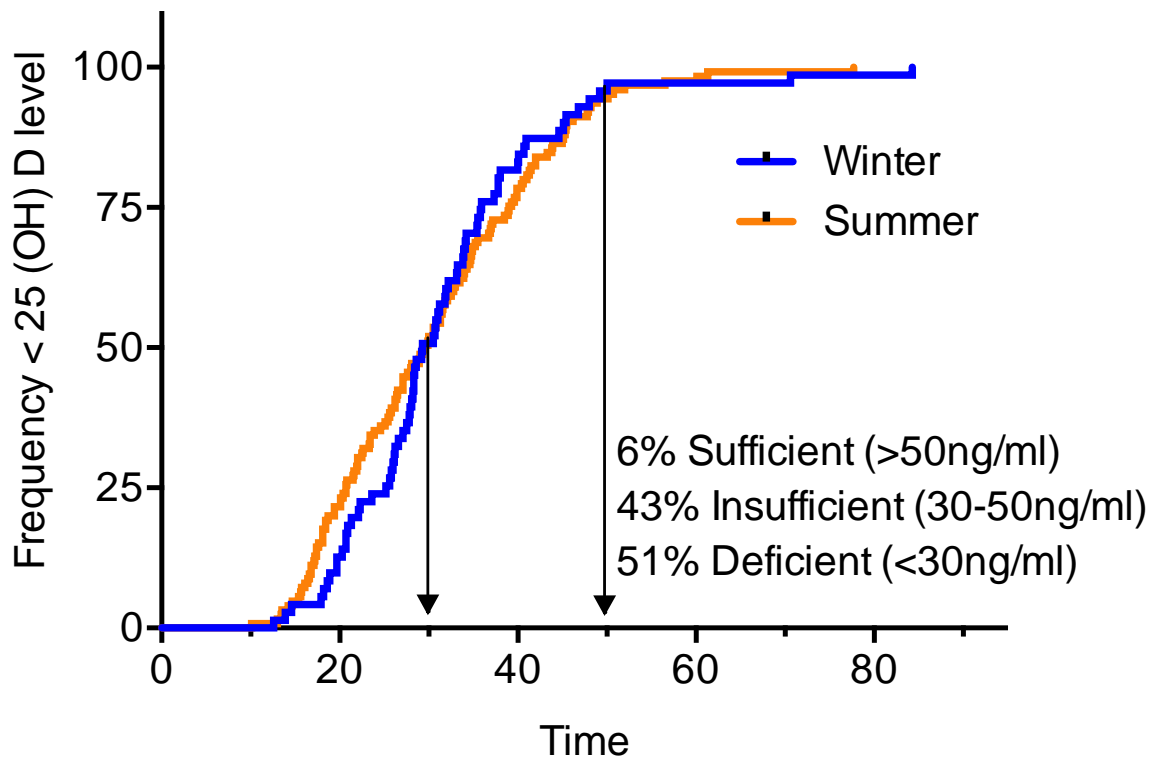
At the baseline visit, the median 25(OH)D level was 29.43ng/ml (10.11 – 84.26 ng/ml, 25.23 – 210.31nmol/l). One hundred women (51.0%) had vitamin D deficiency (<30 ng/ml, 75nmol/l); an additional 85 (43.3%) had vitamin D insufficiency (30 – 49 ng/ml; 75 – 125nmol/l). Only 5.6% of the women had levels that met sufficiency criteria (>50 ng/ml, >125nmol/l). Vitamin D levels are summarized in table 4.2. We did not observe a significant effect of season (summer versus winter) on baseline 25(OH)D level ( $P = 0.617$ , (Figure 4.2). BMI was significantly associated with vitamin D levels ( $P < 0.0001$ , estimate = -0.528, Figure 4.3). Interestingly, we found a significant association between the development of arthralgia and vitamin D sufficiency. Patients with a 25(OH)D level of at least 50ng/ml (>125nmol/l) were four times less likely to develop AI arthralgia, adjusted for age, BMI, and genotype ( $P = 0.048$ , estimate = 0.263 [95% CI, 0.070 to 0.988]).

### 4.3.3 Pharmacogenetic analysis

Genotyping information is summarized in Table 4.3. We found that there was a significant association between *CYP27B1* (rs4646536) and an increase in arthralgia score at both follow-up visit 1 ( $P = 0.010$ , estimate = -2.338 [95% CI, -4.104 to -0.572], Table 4.4) and follow-up visit 2 ( $P = 0.043$ , estimate = -2.588 [95% CI, -5.097 to -0.080, Table 4.4]). We did not find that *CYP27B1* (rs4646536) was significantly associated with early discontinuation of drug. We also did not find an association with *VDR* (rs11568820) and the development of AI arthralgia or early discontinuation of drug

**Table 4.2 Vitamin D levels**

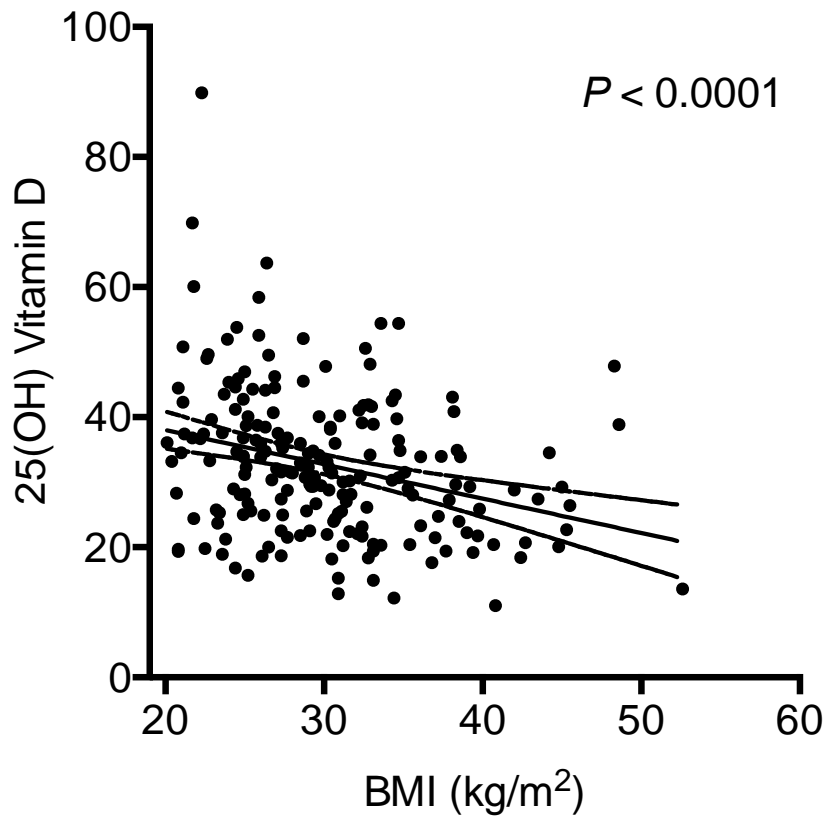
	Baseline (n = 196)	Visit 1 (n = 188)	Visit 2 (n = 145)
Median (ng/ml)	29.43	31.52	35.19
Minimum (ng/ml)	10.11	8.99	9.79
Maximum (ng/ml)	84.26	95.54	89.81
Mean (ng/ml)	30.95	32.47	35.51
Standard Deviation	11.89	12.12	12.44
Deficient (n (%))	100 (51.0%)	81 (43.1%)	49 (33.8%)
Insufficient (n (%))	85 (43.4%)	91 (48.4%)	79 (54.6%)
Sufficient (n (%))	11 (5.6%)	16 (8.5%)	17 (11.4%)



**Figure 4.2** Frequency distribution of patients' serum 25(OH)D levels

Levels of 25(OH)D were not significantly different during the winter (blue) than during the summer (orange)





**Figure 4.3 Serum 25(OH)D is negatively associated with BMI**

Effect estimate = -0.528,  $P < 0.0001$ . *Solid line* linear regression line; *dotted lines* 95% confidence intervals. Statistical test: Linear regression model.

**Table 4.3 SNP information and genotyping quality control (n = 196)**

Gene	rs ID	SNP location or AA change	Call rate	MAF in this cohort	HWE <i>P</i> value
<i>VDR</i>	rs11568820	Upstream	1.0	0.20	0.533
<i>CYP27B1</i>	rs4646536	Intronic	1.0	0.32	0.836

*SNP* single nucleotide polymorphism; *MAF* minor allele frequency; *HWE* Hardy–Weinberg equilibrium

**Table 4.4 Genetic associations with change in AUSCAN and WOMAC score**

Gene name	rs number	Follow-up visit	AUSCAN			
			Estimate	<i>P</i> value	95% CI	Sig.
<i>VDR</i>	rs4646536	1	0.823	0.438	-1.268 to 2.915	
		2	1.512	0.486	-4.048 to 1.936	
<i>CYP27B1</i>	rs11568820	<b>1</b>	<b>-2.338</b>	<b>0.010</b>	<b>-4.104 to -0.572</b>	<b>*</b>
		<b>2</b>	<b>-2.588</b>	<b>0.043</b>	<b>-5.097 to -0.080</b>	<b>*</b>

*AUSCAN* Australian/Canadian Osteoarthritis Hand Index; *WOMAC* Western Ontario and McMaster Osteoarthritis Index; *CI* confidence interval *Sig.* Significant. Statistical test: logistical regression

Table 4.4 continued

Gene name	rs number	Follow-up visit	WOMAC			
			Estimate	<i>P</i> value	95% CI	Sig.
<i>VDR</i>	rs4646536	1	0.223	0.878	-2.649 to 3.096	
		2	-0.334	0.883	-4.830 to 4.162	
<i>CYP27B1</i>	rs11568820	1	-1.759	0.159	-4.212 to 0.695	
		2	-2.083	0.281	-5.888 to 1.723	

*AUSCAN* Australian/Canadian Osteoarthritis Hand Index; *WOMAC* Western Ontario and McMaster Osteoarthritis Index; *CI* confidence interval *Sig.* Significant. Statistical test: logistical regression

## 4.4 Discussion

In our study, patients with serum 25(OH) vitamin D level of greater than 50ng/mL were 4 times less likely to develop arthralgia. Previous studies have investigated this as a potential intervention and have reported conflicting results. A double-blind placebo-controlled randomized study was performed with 60 women who were taking anastrozole to establish whether vitamin D supplementation could improve the symptoms of AI-induced arthralgia. The study found that weekly high dose (50,000 IU) vitamin D significantly improved musculoskeletal symptoms in patients on AIs [19]. A subsequent study of 160 patients on a weekly dose of 30,000 IU of vitamin D did not demonstrate a decrease in musculoskeletal symptoms [20]. In this study, patients in the vitamin D treatment arm reached a median dose of 53.0ng/mL, [range = 25.2 to 81.0ng/mL] at week 12 and 64ng/mL (range 23.0 to 87.0ng/mL). There was a significant portion of the treatment group that did not achieve 50ng/mL (>125nmol/l) levels on 30,000IU. It is possible that a higher dose to ensure the 25(OH)D level is above 50ng/ml is required to see a therapeutic effect. The patients in other studies which have found a positive effect of vitamin D supplementation on AI arthralgia achieved serum 25(OH)D levels of greater than 50ng/ml [21]. We found that *CYP27B1* (rs4646536) was associated with an increase in score on the AUSCAN scale, indicating worsening pain, stiffness, and physical function in the hands, arms, and shoulders. We did not replicate previous findings by Garcia-Gault that *VDR* (rs11568820) was associated with worsening musculoskeletal symptoms. Furthermore neither SNP was associated with discontinuation of drug. Vitamin D levels in our patients were not associated with either SNP, and 25(OH)D was not a significant covariate when we added it into multivariable models with either of

these SNPs. We did not observe a significant association between season and vitamin D level, however this may be due to sample size. Several studies have identified a seasonal impact on vitamin D level due to the changing levels of sunlight exposure, however these studies need to be quite large in order to detect this effect [22]. In conclusion, our findings suggest that sufficient vitamin D supplementation to reach a plasma level greater than 50ng/mL (>125nmol/l) could be a therapeutic strategy to help prevent AI arthralgia. Further research is needed to establish the vitamin D dose that is required to consistently produce this level in all patients, and to define other clinical variables, such as BMI, which may influence vitamin D level.

## 4.5 References

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## 5 DISCUSSION AND CONCLUSIONS



## 5.1 Summary and Discussion

### 5.1.1 Chapter Four

The aromatase inhibitor letrozole is a first-line drug in the adjuvant treatment of breast cancer in postmenopausal women. Adherence to AI therapy, including letrozole, remains problematic due to the development of debilitating AI-associated arthralgia. Letrozole is metabolized in the liver by *CYP2A6*. The aim of Chapter Four was to determine whether plasma letrozole levels or *CYP2A6* genetic variation is associated with the development of arthralgia. We hypothesized that genetic polymorphisms in *CYP2A6* were associated with altered letrozole plasma levels and arthralgia.

More than half of patients experienced a significant increase in their arthralgia symptoms. The clinical variables body mass index and age were negatively and positively associated with plasma letrozole concentrations, respectively. *CYP2A6* genotype was significantly associated with letrozole levels and increased plasma letrozole levels were observed in patients with *CYP2A6* reduced-function genotypes. We found that letrozole drug level and *CYP2A6* genotype were not significantly associated with a change in pain score from baseline. From our studies, we demonstrate that letrozole concentration is not responsible for the development of AI arthralgia. A prospective study of patients on exemestane and letrozole did not detect differences between steady-state drug concentrations and patient-reported quality of life outcomes or treatment discontinuation [1]. Though maintaining

systemic drug concentrations within a therapeutic range is essential for some drugs, this does not appear to be the case for AIs.

### 5.1.2 Chapter Five

Female patients with breast cancer develop arthralgia when treated with AIs. Though the mechanism of AI arthralgia is unknown, clinical factors and potential biomarkers have been identified that may predict their development. Replication and validation of these biomarkers are an essential next step to move these discoveries into implementation in patient care. The aim of this chapter was to investigate the clinical and genetic predictors of AI arthralgia in a prospective cohort of patients with estrogen receptor-positive breast cancer. We hypothesized that SNPs previously identified by genetic screens and genome-wide association studies would be significantly associated with arthralgia and early discontinuation of therapy in our population.

Of the 196 women, more than 50% experienced arthralgia symptoms. Genetic analysis revealed that four SNPs, in *CYP19A1* (rs4775936) and *ESR1* (rs9322336, rs2234693, rs930799), were associated with the development of arthralgia. BMI was also associated with the development of arthralgia symptoms compared to baseline. Patients prescribed letrozole were significantly more likely to develop arthralgia than patients on anastrozole, and also more likely to discontinue AI therapy due to arthralgia. One SNP, in *CYP19A1* (rs4775936), and BMI were significantly associated with discontinuation of drug due to intolerable arthralgia.

Our results suggested that BMI and AI drug (letrozole versus anastrozole) were clinical predictors of arthralgia, while genetic variants rs4775936, rs9322336, rs2234693, and rs930799 were the genetic predictors of AI arthralgia. Significantly, rs4775936 is also a predictor of discontinuation of drug.

### 5.1.3 Chapter Six

In Chapter Six, we hypothesized that the vitamin D pathway played a vital role in the development of arthralgia. The aim of this chapter was to attempt a replication of previously identified genetic predictors of AI arthralgia. We hypothesized that genetic variants in the vitamin D signalling and metabolism pathway and serum 25-hydroxyvitamin D [25(OH)D] levels contribute to the development of AI arthralgia in patients with ER-positive breast cancer. We found that a SNP in CYP27B1 previously identified in a candidate gene study was significantly associated with of pain, stiffness, and worsening physical functioning in the hands, arms, and shoulders. We also found evidence suggesting that a [25(OH)D] level of 50ng/ml or greater is protective against the development of AI arthralgia.

## 5.2 Therapeutic Interventions

To our knowledge, this is the first data set of its kind to prospectively measure AI arthralgia using the AUSCAN and WOMAC, while also collecting genotypic, clinical, and drug level data in patients on AIs. Our studies revealed that the AUSCAN and WOMAC were sensitive measures of AI arthralgia. The use of the AUSCAN and the

WOMAC may be applied in clinical practice to help clinicians assess patient musculoskeletal symptoms.

In addition, we demonstrated that a serum 25(OH)D level of 50ng/ml was the threshold at which the patients were 4 times less likely to develop AI arthralgia. Combined with findings from other vitamin D randomized clinical trials [2, 3], these data support vitamin D supplementation as an intervention to try to prevent AI arthralgia. Next steps could include a vitamin D randomized clinical trial with the goal of achieving at least 50ng/ml serum 25(OH)D level.

### 5.3 Limitations

We were enrolling patients from one hospital site in London, Ontario. This meant that our patient population predominantly Caucasian and we were somewhat limited in our sample size. Though we enrolled sufficient patients based on our power calculations, it would be interesting to include a greater number of patients in the future.

Enrolling patients in a busy clinic environment meant that it was not feasible to conduct an extensive battery of tests that may measure different aspects of arthralgia. Because of this, we selected previously validated questionnaires to measure pain, stiffness, and physical functioning. However, a limitation is that we could have used other measures, such as a 6-minute walk test and grip strength tests to measure arthralgia in our patients.

## 5.4 Future Directions

Collaborating with other Canadian, U.S., and international cancer centers would allow us to expand the study population and examine more patients from different ethnic backgrounds.

Other future studies could employ the use of a greater number of assessments in order to measure pain, stiffness, and physical functioning in patients in addition to the AUSCAN and WOMAC questionnaires. The 6-minute walk test or grip strength test could be added in order to further measure arthralgia in our patients. Previous studies have utilized magnetic resonance imaging (MRI) of hands and wrists to measure tenosynovial changes and intra-articular fluid in patients with AI induced arthralgia [4]. If more funding were available, it would be interesting to perform MRI on the hands, wrists, and other joints at baseline and follow-up appointments.

It is essential that we have a better understand the mechanisms underlying the associations with SNPs identified in our studies. Further in vitro investigations of estrogen metabolism and estrogen signalling could help to better understand the mechanism underlying AI arthralgia.

## 5.5 Conclusions

There is a growing recognition of the role of genetic factors in the development of adverse drug reactions and that genetic variability can predict drug response. The focus of this thesis was aromatase inhibitors, an important group of endocrine therapy drugs used to treat breast cancer in post-menopausal women. We focused on validating previously

identified molecular and genetic biomarkers. We also examined the role of different pathways that underlie the mechanism of AI arthralgia. Taken together, these studies increase our understanding of AI arthralgia and improve our ability to predict variability in the pharmacokinetics of and response to aromatase inhibitors.

## 5.6 References

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## Appendices



## Appendix A - Ethics Approval



Western  
Research

Research Ethics

### Western University Health Science Research Ethics Board HSREB Delegated Initial Approval Notice

**Principal Investigator:** Dr. Richard Kim

**Department & Institution:** Schulich School of Medicine and Dentistry\Medicine-Dept of, London Health Sciences Centre

**HSREB File Number:** 106413

**Study Title:** Evaluating musculoskeletal pain in patients treated with aromatase inhibitors

**Sponsor:** Canadian Institutes of Health Research

**HSREB Initial Approval Date:** April 17, 2015

**HSREB Expiry Date:** April 17, 2016

**Documents Approved and/or Received for Information:**

Document Name	Comments	Version Date
Other	WOMAC Questionnaire (received March 26, 2015)	
Other	AUSCAN Questionnaire (received March 26, 2015)	
Western University Protocol	Received April 14, 2015	
Letter of Information & Consent	Letter of Information & Consent - Clean Copy	2015/04/16

The Western University Health Science Research Ethics Board (HSREB) has reviewed and approved the above named study, as of the HSREB Initial Approval Date noted above.

HSREB approval for this study remains valid until the HSREB Expiry Date noted above, conditional to timely submission and acceptance of HSREB Continuing Ethics Review.

The Western University HSREB operates in compliance with the Tri-Council Policy Statement Ethical Conduct for Research Involving Humans (TCPS2), the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Guideline for Good Clinical Practice Practices (ICH E6 R1), the Ontario Personal Health Information Protection Act (PHIPA, 2004), Part 4 of the Natural Health Product Regulations, Health Canada Medical Device Regulations and Part C, Division 5, of the Food and Drug Regulations of Health Canada.

Members of the HSREB who are named as Investigators in research studies do not participate in discussions related to, nor vote on such studies when they are presented to the REB.

The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

Ethics Officer, on behalf of ~~Dr. Marcelo Kremenchtzky~~, HSREB Vice Chair

Ethics Officer to Contact for Further Information

Erika Basile	Grace Kellv	Mina Mekhail	Wikki Tran
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**Western  
Research**

Research Ethics

**Western University Health Science Research Ethics Board  
HSREB Annual Continuing Ethics Approval Notice**

**Date:** March 31, 2016

**Principal Investigator:** Dr. Richard Kim

**Department & Institution:** Schulich School of Medicine and Dentistry\Medicine-Dept of,London Health Sciences Centre

**Review Type:** Expedited

**HSREB File Number:** 106413

**Study Title:** Evaluating musculoskeletal pain in patients treated with aromatase inhibitors

**Sponsor:** Canadian Institutes of Health Research

**HSREB Renewal Due Date & HSREB Expiry Date:**

Renewal Due -2017/03/31

Expiry Date -2017/04/17

The Western University Health Science Research Ethics Board (HSREB) has reviewed the Continuing Ethics Review (CER) Form and is re-issuing approval for the above noted study.

The Western University HSREB operates in compliance with the Tri-Council Policy Statement Ethical Conduct for Research Involving Humans (TCPS2), the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Guideline for Good Clinical Practice (ICH E6 R1), the Ontario Freedom of Information and Protection of Privacy Act (FIPPA, 1990), the Ontario Personal Health Information Protection Act (PHIPA, 2004), Part 4 of the Natural Health Product Regulations, Health Canada Medical Device Regulations and Part C, Division 5, of the Food and Drug Regulations of Health Canada.

Members of the HSREB who are named as Investigators in research studies do not participate in discussions related to, nor vote on such studies when they are presented to the REB.

The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

\_\_\_\_\_  
Ethics Officer, on behalf of Dr. Joseph Gilbert, HSREB Chair

Ethics Officer to Contact for Further Information: Erika Basile \_\_\_ Katelyn Harris \_\_\_ Nicole Kaniki \_\_\_ Grace Kelly \_\_\_ Vikki Tran

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**Western  
Research**

Research Ethics

**Western University Health Science Research Ethics Board  
HSREB Annual Continuing Ethics Approval Notice**

**Date:** March 07, 2017

**Principal Investigator:** Dr. Richard Kim

**Department & Institution:** Schulich School of Medicine and Dentistry\Medicine-Dept of,London Health Sciences Centre

**Review Type:** Delegated

**HSREB File Number:** 106413

**Study Title:** Evaluating musculoskeletal pain in patients treated with aromatase inhibitors

**Sponsor:** Canadian Institutes of Health Research

**HSREB Renewal Due Date & HSREB Expiry Date:**

Renewal Due -2018/03/31

Expiry Date -2018/04/17

The Western University Health Science Research Ethics Board (HSREB) has reviewed the Continuing Ethics Review (CER) Form and is re-issuing approval for the above noted study.

The Western University HSREB operates in compliance with the Tri-Council Policy Statement Ethical Conduct for Research Involving Humans (TCPS2), the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Guideline for Good Clinical Practice (ICH E6 R1), the Ontario Freedom of Information and Protection of Privacy Act (FIPPA, 1990), the Ontario Personal Health Information Protection Act (PHIPA, 2004), Part 4 of the Natural Health Product Regulations, Health Canada Medical Device Regulations and Part C, Division 5, of the Food and Drug Regulations of Health Canada.

Members of the HSREB who are named as Investigators in research studies do not participate in discussions related to, nor vote on such studies when they are presented to the REB.

The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

Ethics Officer, on behalf of Dr. Joseph Gilbert, HSREB Chair

EO: Erika Basile \_\_\_ Nicole Kaniki \_\_\_ Grace Kelly  Katelyn Harris \_\_\_ Nicola Morphet \_\_\_ Karen Gopaul \_\_\_



**Date:** 19 March 2018

**To:** Richard Kim

**Project ID:** 106413

**Study Title:** Evaluating musculoskeletal pain in patients treated with aromatase inhibitors

**Application Type:** Continuing Ethics Review (CER) Form

**Review Type:** Delegated

**REB Meeting Date:** April 3, 2018

**Date Approval Issued:** 19/Mar/2018

**REB Approval Expiry Date:** 17/Apr/2019

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Dear Richard Kim,

The Western University Research Ethics Board has reviewed the application. This study, including all currently approved documents, has been re-approved until the expiry date noted above.

REB members involved in the research project do not participate in the review, discussion or decision.

Western University REB operates in compliance with, and is constituted in accordance with, the requirements of the TriCouncil Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2); the International Conference on Harmonisation Good Clinical Practice Consolidated Guideline (ICH GCP); Part C, Division 5 of the Food and Drug Regulations; Part 4 of the Natural Health Products Regulations; Part 3 of the Medical Devices Regulations and the provisions of the Ontario Personal Health Information Protection Act (PHIPA 2004) and its applicable regulations. The REB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

Please do not hesitate to contact us if you have any questions.

Sincerely,

Daniel Wyzynski, Research Ethics Coordinator, on behalf of Dr. Joseph Gilbert, HSREB Chair

*Note: This correspondence includes an electronic signature (validation and approval via an online system that is compliant with all regulations).*

## Appendix B - Copyright Approval



### Use of previously published works in a dissertation or thesis

2 messages

**Adrienne Borrie**

Fri, Feb 9, 2018 at 5:36 PM

To:

To whom it may concern,

I am a PhD student at the University of Western Ontario and I plan to submit my thesis in June/July of 2018. I am writing a manuscript-style thesis and I would like to include a previously published review article as one of the chapters:

Borrie AE, Kim RB. 2016. Molecular basis of aromatase inhibitor associated arthralgia: known and potential candidate genes and associated biomarkers. *Expert Opin Drug Metab Toxicol* 13(2):149-156

Could you please direct me as to how I would obtain permission to reproduce this article in my PhD thesis?

Thank you,  
Adrienne Borrie

**Symmons, Louise**

Wed, Feb 14, 2018 at 11:35 AM

Dear Adrienne,

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Best wishes,

Louise Symmons

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Institution name	Western University
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## Appendix C - Australian Canadian Osteoarthritis Hand Index

AUSCAN Survey

ID Number: \_\_\_\_\_

Instructions: In Sections A, B, and C, questions will be asked about your shoulder, arm or hand pain. Please mark each response with an X. If you are unsure about how to answer a question, please give the best answer you can.

A. Think about the pain you felt in your shoulders/arms/hands during the last 48 hours.

Question: How much pain do you have?	None	Mild	Moderate	Severe	Extreme
1. At rest	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
2. Gripping objects with your hands	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
3. Lifting objects	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
4. Turning objects	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
5. Squeezing objects	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

B. Think about the stiffness (not pain) you have in your shoulders/arms/hands during the last 48 hours. Stiffness is a sensation of decreased ease in moving your joint.

	None	Mild	Moderate	Severe	Extreme
6. How severe is your stiffness after first awakening in the morning?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

C. Think about the difficulty you had in doing the following daily physical activities due to your shoulders/arms/hands during the last 48 hours. By this we mean your ability to move around and look after yourself.

Question: What degree of difficulty do you have?	None	Mild	Moderate	Severe	Extreme
7. Turning tap/faucets on	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
8. Turning a round doorknob or handle	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
9. Doing up buttons	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
10. Fastening jewelry	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
11. Opening a new jar	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
12. Carrying a full pot with one hand	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
13. Peeling vegetables/fruits	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
14. Picking up large heavy objects	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
15. Wringing out wash cloths	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

## Appendix D - Western Ontario and McMaster Universities Arthritis Index

WOMAC Survey

ID Number: \_\_\_\_\_

Instructions: In Sections A, B, and C, questions will be asked about your hip or knee pain. Please mark each response with an X. If you are unsure about how to answer a question, please give the best answer you can.

A. Think about the pain you felt in your hip/knee during the last 48 hours.

Question: How much pain do you have?

	None	Mild	Moderate	Severe	Extreme
1. Walking on a flat surface	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
2. Going up and down stairs	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
3. At night while in bed, pain disturbs your sleep	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
4. Sitting or lying	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
5. Standing upright	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

B. Think about the stiffness (not pain) you have in your hip/knee during the last 48 hours. Stiffness is a sensation of decreased ease in moving your joint.

	None	Mild	Moderate	Severe	Extreme
6. How severe is your stiffness after first awakening in the morning?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
7. How severe is your stiffness after sitting, lying, or resting in the day?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

C. Think about the difficulty you had in doing the following daily physical activities due to your hip/knee during the last 48 hours. By this we mean your ability to move around and look after yourself.

Question: What degree of difficulty do you have?

	None	Mild	Moderate	Severe	Extreme
8. Descending stairs	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
9. Ascending stairs	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
10. Rising from sitting	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
11. Standing	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
12. Bending to the floor	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
13. Walking on flat surfaces	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
14. Getting in and out of a car, or on or off a bus	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
15. Going shopping	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
16. Putting on your socks or stockings	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
17. Rising from the bed	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
18. Taking off your socks or stockings	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
19. Lying in bed	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
20. Getting in or out of the bath	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
21. Sitting	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
22. Getting on or off the toilet	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
23. Performance heavy domestic duties	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
24. Performing light domestic duties	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

# Curriculum Vitae

Adrienne Elizabeth Borrie  
London Health Sciences Centre, University Hospital  
339 Windermere Road, C9-101  
London, ON, Canada

## Post-secondary Education and Degrees

Doctor of Philosophy, Pharmacology and Physiology  
2014 – 2018  
The University of Western Ontario  
London, Ontario, Canada

Master of Science, Medical Genetics  
2007 – 2010  
The University of British Columbia  
Vancouver, British Columbia, Canada

Bachelor of Science, Biology and Psychology  
2003 – 2007  
McMaster University  
Hamilton, Ontario, Canada

## Honours and Awards

**Canadian Institutes of Health Research (CIHR)**  
Canada Graduate Scholarships: Doctoral Research Award (\$105,000)  
2015 – 2018

**University of Western Ontario**  
Doctoral Research Excellence Award (\$20,000)  
2016 – 2018

**Government of Ontario**  
Ontario Graduate Scholarships (OGS) (\$18,000 – Declined)  
2015 – 2016

**Canadian Institutes of Health Research (CIHR)**  
CIHR Strategic Training Program in Cancer Research and Technology Transfer (CaRTT)  
Trainee award (\$26,700)  
2014 – 2015

**Breast Cancer Society of Canada**

Translational Breast Cancer Research Unit Trainee award (\$18,000)  
2014 – 2015

**Schulich School of Medicine & Dentistry**

Morris Kroll Memorial Scholarship in Cancer Research (\$500)  
2015

**University of Western Ontario**

Department of Oncology Research and Education Day Poster Award (\$200)  
2015

**Natural Sciences and Engineering Research Council (NSERC)**

Post Graduate Scholarship – Masters Award (\$17,300)  
2008 – 2009

**University of British Columbia**

Medical Genetics Graduate Entrance Scholarship (\$2750)  
2007

**Robarts Research Institute**

Cobban Studentship Award (\$1600)  
2006

**London Health Science Centre**

Harvey Sullivan Research Award (\$4000)  
2003

**Related Work Experience****Teaching Assistant, Department of Physiology and Pharmacology****University of Western Ontario, London, Ontario, Canada**

Pharmacology 4360 - Mechanisms of Cancer Chemotherapy

Taught chemotherapy mechanism related course material with a focus on breast cancer;  
supervised and graded quizzes and exams

2015 – 2018

**Teaching Assistant, Department of Physiology and Pharmacology****University of Western Ontario, London, Ontario, Canada**

Pharmacology 3580 - Pharmacology Laboratory

Led a weekly pharmacology laboratory course for third and fourth year students; marked  
laboratory assignments, supervised quizzes and exams

2014 – 2015

**Research Coordinator, Canadian Pharmacogenomics Network for Drug Safety, University of British Columbia, Vancouver, BC, Canada**

I was responsible for identifying and consenting patients, and clinically characterizing patients as cases and controls. I liaised with nationwide surveillance team, coordinating DNA supplies, DNA samples, and clinical information from 13 hospital sites across Canada. I analyzed combined clinical and genetic results and I assisted with writing ethics applications and grant proposals

2011 – 2014

**Research Coordinator, The Lung Centre, Department of Medicine, University of British Columbia, Vancouver, BC, Canada**

I was responsible for identifying and consenting patients, conducting study visits, maintaining case report forms (CRFs) and online E-CRFs. I facilitated monitoring visits and attended investigator meetings

2010 – 2011

**Publications**

**Borrie AE**, Rose RV, Choi YH, Perera FE, Read N, Sexton T, Lock M, Vandenberg TA, Hahn K, Dinniwell R, Younus J, Logan D, Potvin K, Yaremko B, Yu E, Blanchette PS, Lenehan J, Welch S, Tyndale RF, Teft WA, and Kim RB. Letrozole concentration is associated with CYP2A6 variation but not with arthralgia in patients with breast cancer. *Breast Cancer Research and Treatment*, epub Aug 9

**Borrie AE**, Maleki Vareki S. (2018). T lymphocyte based cancer immunotherapeutics. *International Review of Cell and Molecular Biology*, in press.

Drögemöller BI, Monzon JG, Bhavsar AP, **Borrie AE**, Brooks B, Wright GEB, Liu G, Renouf DJ, Kollmannsberger CK, Bedard PL, Aminkeng F, Amstutz U, Hildebrand CA, Gunaretnam EP, Critchley C, Chen Z, Brunham LR, Hayden MR, Ross CJD, Gelmon KA, Carleton BC. (2017) Association Between SLC16A5 Genetic Variation and Cisplatin-Induced Ototoxic Effects in Adult Patients With Testicular Cancer. *JAMA Oncology*, Nov 1;3(11):1558-1562.

**Borrie AE**, Kim RB. (2016) Molecular basis of aromatase inhibitor associated arthralgia: known and potential candidate genes and associated biomarkers. *Expert Opinion Drug Metabolism and Toxicology*, Feb;13(2):149-156.

Corso-Díaz X, **Borrie AE**, Bonaguro R, Schuetz JM, Rosenberg T, Jensen H, Brooks BP, Macdonald IM, Pasutto F, Walter MA, Grønskov K, Brooks-Wilson A, Simpson EM. (2012) Absence of NR2E1 mutations in patients with aniridia. *Molecular Vision*,18:2770-82.

**Borrie AE**, Ostrow DN, Levy RD, Swiston JR. (2011) Assessing response to therapy in idiopathic pulmonary arterial hypertension: a consensus survey of Canadian pulmonary hypertension physicians. *Canadian Respiratory Journal*. Jul-Aug;18(4):230-4.

### **Publications, in preparation for submission**

**Borrie AE**, Rose RV, Choi YH, Perera FE, Read N, Sexton T, Lock M, Vandenberg TA, Hahn K, Dinniwell R, Younus J, Logan D, Potvin K, Yaremko B, Yu E, Blanchette PS, Lenehan J, Welch S, Teft WA, and Kim RB. Genetic and clinical predictors of aromatase inhibitor drug toxicity in breast cancer patients.

**Borrie AE**, Rose RV, Choi YH, Perera FE, Read N, Sexton T, Lock M, Vandenberg TA, Hahn K, Dinniwell R, Younus J, Logan D, Potvin K, Yaremko B, Yu E, Blanchette PS, Lenehan J, Welch S, Teft WA, and Kim RB. Prospective analysis of vitamin D levels and vitamin D receptor polymorphisms in postmenopausal women on aromatase inhibitors.

### **Invited Oral Presentations**

“The impact of clinical and pharmacogenetic factors in the use of aromatase inhibitors in women with breast cancer” **Borrie AE**. Department of Physiology and Pharmacology Seminar Series, the University of Western Ontario, London, ON, December 2017.

“The path to implementation of personalized medicine in patients with ER+ breast cancer” **Borrie AE**. Division of Clinical Pharmacology Grand Rounds, University Hospital, London, ON, November 2016

“Pharmacogenetics of aromatase inhibitors: personalized medicine for patients with ER+ Breast Cancer” **Borrie AE**. Division of Clinical Pharmacology Grand Rounds, University Hospital, London, ON, March 2016

“Progress and Promise in Cancer Research: Hope for the future” **Borrie, AE**. McMaster Mini Medical School, McMaster University, Hamilton, ON, March 2015

“Building a framework for personalized AI therapy for breast cancer” **Borrie AE**. Division of Clinical Pharmacology Grand Rounds, University Hospital, London, ON, March 2015

“Adult CPNDS Studies” **Borrie AE**. Scientific Advisory Board Meeting for the Canadian Pharmacogenomics Network for Drug Safety, Vancouver, BC, April 2013

“DSEN active Surveillance and Evaluation of Adverse Drug Reactions in Canadian Healthcare team (DSEN-SEARCH)” **Borrie AE**, Jimenez R. Drug Safety and Effectiveness Network First Meeting, Ottawa, ON, October 2011

**Poster Publications**

“Pharmacogenomics of aromatase inhibitors: Personalized medicine for patients with ER-positive breast cancer” Borrie AE, Rose RV, Teft, WA, Kim, RB. Canadian Conference for Cancer Research, Vancouver, BC, November 2017

“Pharmacogenomics of aromatase inhibitors: Personalized medicine for patients with ER-positive breast cancer” Borrie AE, Rose RV, Teft, WA, Kim, RB. IMPAKT Breast Cancer Conference, Brussels, Belgium, May 2017

“Pharmacogenomics of aromatase inhibitors: Personalized medicine for patients with ER-positive breast cancer” Borrie AE, Teft, WA, Kim, RB Canadian Society for Pharmacology and Therapeutics, Vancouver, BC, September 2016