ABSTRACT

Title of Dissertation	EFFECTS OF TAMOXIFEN THERAPY ON BREAST CARCINOGENESIS: EPIDEMIOLOGICAL ASSOCIATIONS AND BIOMECHANISMS OF ACTION
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Background: Tamoxifen, a key chemopreventive and adjuvant endocrine therapy (ET) for breast cancer, is suggested to alter breast cancer risk factors including circulating hormones (estrogen and insulin-like growth factors (IGFs)) and breast density. However, the biological underpinnings of tamoxifen's effect on these factors remain unclear. This dissertation evaluated effects of tamoxifen on estrogen metabolites (EMs), explored associations between circulating IGFs (IGF-I and IGFBP-3) and volume average sound speed measures (VASS) of breast density, and synthesized evidence from real-world studies to meta-analyze adjuvant ET in relation to contralateral breast cancer (CBC) risk.

Methods: Within the Ultrasound Study of Tamoxifen, serial serum samples collected prior to and 12 months after tamoxifen treatment were used to assess longitudinal changes (paired t-tests) in 15 circulating EMs among postmenopausal women (n=23) (Aim 1), and changes in IGFs and VASS (n=53) (Aim 2). Multivariable linear regression examined associations between metabolites of tamoxifen and estrogen among pre- (n=33) and postmenopausal women (n=27) (Aim 1) and concomitant changes in IGFs and VASS (n=53) (Aim 2), 12 months after treatment

initiation. In Aim 3, a random effects meta-analysis of observational studies (n=17, 287,576 participants) estimated relative risks (RR) and 95% confidence intervals (CI) for associations between ET and CBC risk among primary breast cancer patients overall, by menopausal status and CBC estrogen receptor (ER)-subtype.

Results: Circulating 2-OH and 16-OH pathway EMs, IGF-I, and IGF-I:IGFBP-3 decreased 12 months after tamoxifen initiation ($p < 0.05$; Aims 1 and 2). No associations were observed between concomitant changes in IGFs and VASS among tamoxifen-treated patients (Aim 2). In meta-analyses (Aim 3), endocrine therapy was associated with reduced CBC risk (RR: 0.62, 95% CI: 0.53, 0.73), with a greater reduction observed among premenopausal (RR: 0.58, 95% CI: 0.43, 0.78) versus postmenopausal women (RR: 0.72, 95% CI: 0.60, 0.87). Endocrine therapy reduced the risk of ER-positive (RR: 0.55, 95% CI: 0.43, 0.70) but not ER-negative CBC.

Conclusion: The tamoxifen mediated decline of circulating 2-OH, 16-OH and IGF-I provides etiologic insight into the biomechanisms of tamoxifen on breast carcinogenesis. Meta-analyses of observational studies further support a chemopreventive role of endocrine therapy on CBC risk, particularly, ER-positive CBC.

EFFECTS OF TAMOXIFEN THERAPY ON BREAST CARCINOGENESIS: EPIDEMIOLOGICAL ASSOCIATIONS AND BIOMECHANISMS OF ACTION

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Dedication

To my family

This dissertation is dedicated to my family. Thank you for making endless sacrifices so that I can be here today. I could not have done this without the unwavering support of my parents, Sutapa and Prabir Ghosh. I would like to especially express my gratitude to my mother who stood like a rock and encouraged me throughout this process. Without your unconditional love and support, this would not have been a reality. This dissertation belongs to you.

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List of Abbreviations

ACR	American College of Radiology
ADH	Atypical ductal hyperplasia
AIs	Aromatase inhibitors
AJCC	The American Joint Committee on Cancer
ALH	Atypical lobular hyperplasia
BBD	Benign breast disease
BCFR	Breast Cancer Family Registry cohort
BIRADS	Breast imaging reporting and data system
BMI	Body mass index
CBC	Contralateral breast cancer
CLIA	Chemiluminescent immunoassay
CT	Chemotherapy
CURE	Computed ultrasound risk evaluation
CV	Coefficient of variation
CYP	Cytochrome P450
DAGs	Directed acyclic graphs
DCIS	Ductal in situ carcinoma
Endoxifen	4-hydroxy-N-desmethyl-tamoxifen
ELISA	Enzyme linked immunoassay
EMs	Estrogen metabolites
ER	Estrogen receptor
ER+	Estrogen receptor positive
ET	Endocrine therapy
E1	Estrone
E1S	Estrone sulfate
E2	Estradiol
E3	Estriol
FSH	Follicle stimulating hormone
ICC	Intraclass correlation coefficient
IGFs	Insulin-like growth factors
IGF-I	Insulin-like growth factor I
IGFBP-3	Insulin-like growth factor binding protein 3
IITRI	Illinois Institute of Technology Research Institute
IHC	Immunohistochemical
IRMA	Immunoradiometric assay
GM	Geometric mean
HER2	Human epidermal growth factor receptor 2
HFHS	Henry Ford Health Systems

HPLC	High performance liquid chromatography
HR	Hormone receptor
HR+	Hormone receptor positive
HT	Hormonal therapy
KCI	Barbara Ann Karmanos Cancer Institute
LCIS	Lobular carcinoma in situ
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LH	Luteinizing hormone
MHT	Menopausal hormone therapy
MRI	Magnetic resonance imaging
NCI	National Cancer Institute
NDtam	N-demethyltamoxifen
NDDtam	N-dedimethyltamoxifen
N-Destam	N-desmethyl-tamoxifen
NOS	Newcastle-Ottawa scale
OFS	Ovarian function suppression
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
PR+	Progesterone receptor positive
RCTs	Randomized clinical trials
RIA	Radioimmunoassay
RR	Relative risk
RT	Radiotherapy
SD	Standard deviation
SEER	Surveillance, Epidemiology, and End Results Program
SERMs	Selective estrogen receptor modulators
SERDs	Selective estrogen receptor down-regulators
SHBG	Sex hormone-binding globulin
TAM	Tamoxifen
TamNox	Tamoxifen-N-oxide
TNM	Tumor-node-metastasis system
UST	Ultrasound tomography
VASS	Volume average sound speed
2-OH	2-hydroxy
2-OHE1	2-hydroxyestrone
2-OHE2	2-hydroxyestradiol
2-ME1	2-methoxyestrone
2-ME2	2-methoxyestradiol
3-ME1	2-hydroxyestrone-3-methyl ether
4-OH	4-hydroxy
4-OHE1	4-hydroxyestrone
4-ME1	4-methoxyestrone
4-ME2	4-methoxyestradiol
4-OHT	4-hydroxy-tamoxifen

16-OH	16-hydroxy
16α-OH-E1	16α-hydroxy estrone
16-keto-E2	16-ketoestradiol
16-epiE3	16-epiestriol
17-epiE3	17-epiestriol

Chapter 1: Introduction

Breast cancer is the most commonly diagnosed cancer among women in the United States[1], with an annual incidence of over 200,000, accounting for 30% of all cancers among women[2]. Despite high incidence rates, the mortality rate of breast cancer is declining[3], and five-year survival rates of breast cancer patients in the United States have exceeded 90%[1]. This change has placed a growing population of breast cancer survivors at risk for second primary cancers. Contralateral breast cancer (CBC) is the most common second primary cancer among breast cancer survivors[4], accounting for approximately 30%-50% of all second cancers[5,6]. Recent advances in screening and treatment regimens have been pivotal in reducing breast cancer recurrence and improving prognosis[7-9]. One such therapeutic regimen that has revolutionized breast cancer treatment is adjuvant endocrine therapy. Tamoxifen and aromatase inhibitors (AIs), two of the most effective endocrine therapies, inhibit breast tumor proliferation via their antiestrogenic activity[10].

Endogenous estrogen is a key determinant of breast carcinogenesis, and elevated levels of circulating estrogens have been consistently linked to an increased risk of breast cancer[11-13]. The carcinogenic effects of estrogen are not only limited to the parent estrogens, estrone and estradiol, but also extend to estrogen metabolites (EMs) across three different pathways (2-OH, 4-OH, and 16-OH)[14-17]. The role of estrogen in breast cancer etiology is either mediated by the estrogen receptors (ERs) via the estrogen signaling pathway[18], or genotoxic effects of the EMs that lead to DNA damage and subsequent tumor formation[15,16,19]. Tamoxifen, a key chemopreventive and adjuvant endocrine therapy plays a crucial role in the prevention and management of ER-positive breast tumors[11]. The underlying mechanism by which tamoxifen selectively blocks estrogen signaling[18], suggests a possible association between tamoxifen

treatment and endogenous estrogens. However, little is known about tamoxifen's effect on estrogen metabolism or the relationship between metabolites of tamoxifen and estrogen, highlighting the need for research in this area.

In addition to endogenous estrogens, elevated breast density is a strong breast cancer risk factor, and women with dense breast tissues have a greater than 2-fold increased risk of breast cancer compared to women with less dense tissues[20-22]. However, breast density is a potentially modifiable determinant of breast cancer risk, and reductions in breast density are associated with improved breast cancer outcomes[23]. The tamoxifen mediated declines in breast density are a strong indicator of therapeutic efficacy in both preventative[24-26] and prognostic settings[27-29]. Hence, elucidating the mechanism that influences breast density is of paramount importance, as declines in breast density may lower the risk of breast cancer and improve outcomes among breast cancer survivors. Insulin-like growth factors (IGFs) are peptide hormones that play an important role in cell differentiation and proliferation[30,31] have been linked to both breast cancer[30,32,33] and elevated breast density[34-36]. Prior studies have reported that tamoxifen therapy alters circulating IGF levels among breast cancer patients[33,37-39], suggesting a positive effect of tamoxifen on breast cancer progression via changes in circulating IGFs. These finding indicate that, tamoxifen may play a significant role in breast carcinogenesis through modulation of both breast density as well as circulating IGFs. However, the mechanistic association between IGFs and breast density in relation to tamoxifen use remains unclear. Understanding how these peptide hormones (IGFs) influence breast density before and after tamoxifen initiation is crucial in elucidating breast cancer etiology.

The therapeutic efficacy of tamoxifen in breast carcinogenesis is not limited to the primary breast tumor. Breast cancer patients have more than twice the risk of developing CBC

compared to women in the general population[40]. Given the high incidence of CBC, improving our understanding of preventative treatments in a real-world setting is imperative. Evidence synthesized from randomized clinical trials support that adjuvant endocrine therapy, including tamoxifen, is associated with a reduction in contralateral breast cancer risk[41,42]. However, their controlled settings and selective enrollment of participants do not reflect the real-world treatment conditions, and therefore limit the generalizability of the findings. Population-based observational studies reflect the effects of the treatment therapy in a real-world setting, however, prior studies support differential relationships among subgroups of breast cancer patients, including menopausal status[43-47] and ER-status of CBC[47-52] leading to inconclusive findings. Given the observed heterogeneity among select subgroups of breast cancer patients, it is crucial to evaluate and synthesize evidence from prior literature that assesses the relationship between endocrine therapy use and CBC risk among breast cancer patients in the general population.

Objectives

The overarching objective of this dissertation was to explore the underlying mechanisms of tamoxifen, a key adjuvant therapy for breast cancer, and to critically assess and meta-analyze prior evidence from published observational studies of adjuvant endocrine therapy and CBC risk. More specifically, the dissertation analyses aimed to examine the biological underpinnings of tamoxifen's effect on breast cancer risk via its effect on circulating hormones and changes in breast density and potential heterogeneity in the protective effect of endocrine therapy on second cancer risk . The specific aims of this dissertation are:

Aim 1: To evaluate temporal changes in serum concentrations of parent estrogens and estrogen metabolites (EMs) among postmenopausal women diagnosed with or at risk of breast cancer, 12-months following tamoxifen initiation. Additionally, to explore the

relationship between the metabolites of tamoxifen and estrogen by menopausal status at 12 months following tamoxifen initiation.

Hypothesis: Tamoxifen use will influence circulating levels of EMs, and the metabolites of tamoxifen and estrogen will be differentially associated by menopausal status among women diagnosed with or at risk of breast cancer.

Aim 2: To determine if longitudinal changes in circulating levels of insulin-like growth factors, IGFs (insulin-like growth factor-I, IGF-I; insulin-like growth factor binding protein 3, IGFBP-3; and the ratio of IGF-I: IGFBP-3) are associated with concomitant changes in volume average sound speed (VASS) measures of breast density among women diagnosed with breast cancer, 12 months following tamoxifen initiation.

Hypothesis: Changes in circulating levels of IGF-I and the ratio of IGF-I: IGFBP-3 will be positively associated while changes in IGFBP-3 will be inversely associated with concomitant changes in breast density 12 months after tamoxifen therapy among women diagnosed with breast cancer.

Aim 3: To conduct a systematic review and a meta-analysis of published population-based observational studies that have assessed the association between endocrine therapy use in relation to CBC risk among women diagnosed with primary breast cancer. Further, to assess whether associations differ by: i) study design, ii) menopausal status, and estrogen receptor (ER)-status of contralateral breast cancer CBC.

Hypothesis: Endocrine therapy use will be associated with reduction in CBC risk in overall analyses and when stratified by study designs. The relationship between endocrine therapy and CBC risk will vary by menopausal status ant ER-status of CBC.

Aims 1 and 2 were conducted within the Ultrasound Study of Tamoxifen, an observational study aimed at assessing changes in volume averaged sound speed (VASS) measures of breast density via ultrasound tomography (UST), 12-months following tamoxifen treatment. The analytic population included women aged 30-70 years referred to tamoxifen therapy for clinical indications and an age, race, and menopausal status frequency matched control group[53,54]. Aim 3 utilized risk estimates from published population-based observational studies assessing the relationship between endocrine therapy and the risk of CBC.

Significance and innovation

The exploratory analyses conducted for Aims 1 and 2 were the first longitudinal studies to assess the influence of tamoxifen on EMs and the relationship between concomitant changes in IGFs and VASS using serial serum samples collected prior to and 12 months after tamoxifen treatment. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay[55,56] was used to measure a comprehensive panel of 15 EMs in relation to tamoxifen therapy (Aim 1). The high specificity and sensitivity of the assay allowed accurate measurement of low estrogen concentrations, common among postmenopausal women[55,56]. Importantly, the LC-MS/MS assay can concurrently measure circulating estrogens across three metabolic pathways (2-OH, 4-OH, and 16-OH), which provided crucial mechanistic insights into the previously unknown relationship of tamoxifen and/or its metabolites with estrogen metabolism. Assessment of the metabolites of tamoxifen in addition to tamoxifen treatment in relation to EMs, one of the crucial

factors of breast cancer risk, highlighted the significance of tamoxifen in breast cancer etiology. In Aim 2, VASS, a volumetric breast density measure, was measured using the novel imaging technique of whole breast ultrasound tomography (UST), a highly reliable approach for single timepoint measures as well as changes in breast density over time[54]. Unlike mammography, the most widely used breast screening technique, VASS is a more accurate measure of physical breast density[57-59] that allows repeated measurement before and after tamoxifen treatment without any exposure to harmful ionizing radiations[60]. Aim 3 is the first systematic review and meta-analysis to synthesize and re-analyze evidence from prior observational studies that assessed the effectiveness of endocrine therapies on contralateral breast cancer risk within real-world settings. Evidence of the heterogeneity of the association by menopausal status or ER-subtype of CBC furthers knowledge on the effectiveness of adjuvant endocrine therapy among different subgroups of breast cancer patients.

Chapter 2: Background

Breast cancer: Brief overview

Breast cancer, a malignant tumor that develops from the uncontrolled growth of breast cells, is one of the most common cancers diagnosed among women globally[61], with an annual incidence of approximately 2.3 million[61]. In the United States, breast cancer is the most commonly diagnosed cancer, and approximately 1 in 8 women develops a breast tumor in their lifetime[3]. Although breast cancer can also develop among men, it is far less common, with an incidence of 1.28 per 100,000[62]. Mammography is the most common screening method used for breast cancer detection, but other methods such as magnetic resonance imaging (MRI) are also used[63]. Such advanced techniques aid in the early detection of the disease, faster identification of women at risk, and improved prognosis and survival of the breast cancer patients. Despite such advances in screening, breast cancer is still the leading cause of cancer deaths among women both in the United States[3] and worldwide[61].

Breast tumors can be classified by histology, stage, and immunohistochemical features, including molecular subtypes. The different features of a breast tumor are described below:

Tumor histology

Tumor histology can be sub-divided into two categories: type and grade. The histologic type refers to the morphologic growth patterns of the breast tumor, while the histologic grade denotes the aggressiveness of the tumor growth[64].

- Histologic type: There are at least 17 known histological types of breast carcinomas[64,65], with ductal and lobular carcinoma among the two most common histological types. Breast tumors originating from the inner lining (epithelium) of the breast ducts are known as ductal breast

carcinoma, whereas those originating from the milk-producing glands, the lobules, are known as lobular breast carcinoma[66]. Breast cancer can also be categorized in terms of invasiveness. Tumors that spread to the surrounding breast tissues, including lymph nodes, and other parts of the body are known as invasive breast cancer while those that do not spread to the surrounding tissues are known as in-situ breast cancer[66]. Invasive ductal carcinoma accounts for approximately 55% of all breast cancers and 40-75% of all invasive breast tumors[66]. Ductal carcinoma in situ is the second most commonly diagnosed breast tumor, and accounts for 20-25% of all the newly diagnosed cases[67]. The third most common breast carcinoma is invasive lobular breast tumors, accounting for 5-15% of all invasive breast cancer cases[66]. Other histologic types of invasive carcinomas account for less than 5% of all breast cancers[66].

- Histologic grade: The degree of differentiation and proliferation of the tumor tissue characterizes the histological grade of the breast tumor[68]. The internationally-recommended Nottingham Grading system[69] evaluates the grade of breast tumors based on three morphological features: i) degree of tubule or gland formation, ii) nuclear pleomorphism, and iii) mitotic count[68]. Based on these cell characteristics, breast tumors can be categorized into three grades: i) Grade 1: slow-growing tumor cells (well-differentiated) which mostly appear like normal breast tissue, ii) Grade 2: moderately differentiated tumor cells that are between grade 1 and 3, and iii) Grade 3: the tumor cells that are either undifferentiated or poorly differentiated, and the tissue structure looks abnormal[68]. Grade 1 and 2 tumors are less likely to spread than grade 3 tumors, which are the fast-growing and rapidly spreading tumor cells that have the worst prognosis, given tumor size and cancer stage[68].

Immuno-histochemical features

Breast tumors can also be categorized based on the immunohistochemical (IHC) markers expressed on their surface:

- Hormone receptor (HR) status: Estrogen receptors (ER) and progesterone receptors (PR) are the breast tissue markers most widely used to classify breast tumors[70]. The breast tumors that expresses receptor proteins for the sex hormones, estrogen, progesterone, or both, are classified as hormone receptor positive (HR-positive) breast tumors. A hormone receptor positive (HR+) cancer can be either estrogen receptor positive (ER+), which has receptors for only estrogen, or progesterone receptor positive (PR+), which has receptors for only progesterone, or both (ER+/PR+). Breast tumor cells that do not express hormone receptors for either estrogen or progesterone (ER-negative/PR-negative) are known as hormone receptor negative (HR-) breast tumors. Prior studies have shown that ER- tumors are associated with worse prognosis than ER+ tumors[71]. When these sex hormones bind to their respective receptor proteins, they form a hormone-receptor complex that stimulates cancerous growth in those breast cells. HR+ breast cancer constitutes approximately 75% of all breast cancers[72]. The HR status of a breast tumor forms the basis of endocrine therapy in breast carcinogenesis[72].
- Human epidermal growth factor receptor 2 (HER2): A member of the epidermal growth factor (EGF) family, HER2 is a protein that controls cell growth and differentiation[73]. Overexpression of HER2 protein in breast cancer cells defines the HER2 positive (HER2+) subtype. HER2+ tumors are often associated with aggressive cancer, higher rates of recurrence, and shorter survival times among breast cancer patients[73]. Knowledge of the HER2 protein's presence is critical in determining if targeted anti-HER2 therapy drugs can

be used to treat a cancerous growth. HER2 protein is overexpressed in approximately 20-30% of all breast cancer cells[73].

Molecular subtypes

The molecular subtypes of breast cancer are defined mainly by immunohistochemical and proliferation markers, most important of which are the ER, PR, and HER2 status of the breast tissues[72]. Following the new St. Gallen's classification, breast cancer is divided into four main molecular subtypes: luminal A, luminal B, triple-negative/basal-like, and HER2-enriched/positive (Table 2.1)[74,75].

- Luminal A breast cancer: Defined as ER+|PR+|HER2- with low levels (< 14%) of the Ki-67 protein[74]. Based on 2012-2016 Surveillance, Epidemiology, and End Results Program (SEER) data, luminal A is the most common tumor subtype, accounting for approximately 66% of all breast cancers[3].
- Luminal B breast cancer: Defined as ER+|PR+|HER2-positive/negative tumors with high levels (≥14%) of the Ki-67 protein[74]. These tumors have higher growth rates and poorer prognoses compared to luminal A tumors[75].
- HER2-enriched breast cancer: Defined as tumors that are hormone receptor negative (ER- and PR-) but HER2 positive.
- Triple negative breast cancer: Defined as tumors that lack receptors for both estrogen and progesterone (ER-|PR-) and have lower expression of HER2 protein (HER2-) on cancerous cells[76]. Such cancers are usually more aggressive, and due to the lack of protein receptors, endocrine therapies or treatments targeted towards ER, PR, and HER2 protein are not effective. Triple-negative breast cancer are the second most common subtype, accounting for

10-20% of all breast cancer diagnoses and carrying the worst prognosis of all breast cancers[75,77].

Based on 2012-2016 Surveillance, Epidemiology, and End Results Program (SEER) data, the most common tumor subtype is the luminal A subtype, accounting for approximately 66% of all breast cancers[3], followed by the triple-negative subtype[78,79].

Table 2.1: Molecular subtypes of breast cancer

Molecular subtypes	**HR-status**		**HER2- status**
	ER Status	PR- Status	
Luminal A	ER+	PR+	HER2-
Luminal B	ER+	PR+	HER2 +/-
Triple-negative/ basal-like	ER-	PR-	HER2-
HER2-enriched	ER-	PR-	HER2+

HR: hormone receptor, ER: estrogen receptor, ER+: estrogen receptor positive, ER-: Estrogen receptor negative, PR+: progesterone receptor positive, PR-: progesterone receptor negative, HER2: human epidermal growth factor receptor 2, HER2+: human epidermal growth factor receptor 2 positive, HER2-: human epidermal growth factor receptor 2 negative

Tumor stage

The American Joint Committee on Cancer (AJCC) oversees the tumor-node-metastasis (TNM) system which is utilized to classify breast cancer based on the stage of the tumor[80]. The TNM staging of cancer is based on size of the primary tumors (Tis, T0, to T4), number and involvement of lymph nodes (N0 to N3), and distant metastases of the tumor (M0 or M1)[80].

This system broadly categorizes breast cancer into four stages, stage 0 through stage IV[80]. Stage 0 captures the lowermost stage where tumor cells are present but have not spread to nearby

tissues. Depending on the size and spread of the tumor to nearby tissues, stages I-III are determined. Stage IV is the highest stage where cancer has spread to distant body parts, irrespective of the tumor size, lymph node status, and other factors[80]. In 2017, the AJCC announced the latest (8th) edition of the breast tumor staging system, wherein new biomarkers pertaining to tumor grade, hormone receptor (HR) status, and human epidermal growth factor receptor 2 (HER2) status have been incorporated into the existing TNM system[80].

Breast cancer epidemiology

Breast cancer incidence and mortality

Globally, breast cancer is the most commonly diagnosed cancer, and the leading cause of cancer-related death among women[61]. Breast cancer is the most common cancer diagnosed among women in the United States, with an incidence of over 200,000 per year[2], and it accounts for 15.3% of all new cancer cases[3]. The incidence rate of breast cancer is highest among white women (131.3 per 100,000 cases), closely followed by African American women (124.8 per 100,000 cases)[3]. The median age of diagnosis is approximately 62 years, and most cases are diagnosed in the 55-64 age group[3]. The incidence of breast cancer is higher in developed countries compared to the less developed nations: 86-96 per 100,000 women in Northern American/European countries versus 26-33 per 100,000 women in Asian and Middle African countries[61].

Breast cancer is the second leading cause of cancer mortality in the United States. It has an annual mortality of over 40,000 deaths[2], which accounts for 7% of all cancer deaths[3]. In contrast to the incidence rate, the mortality rate is highest among African-American women (

27.6 per 100,000 cases), followed by White women (19.8 per 100,000 cases)[3]. The median age of death is 69 years with the majority of the breast cancer deaths occurring in the 65-74 year age group[3]. The incidence rate of breast cancer increases by age at diagnosis, older women have a higher incidence compared to younger women. Below are graphical representations of breast cancer trends using data from SEER[81]. Incidence increases until approximately age 70 years and then gradually declines (Figure 2.1). When stratified by race, the incidence rate is highest among non-Hispanic and Hispanic White women closely followed by African American women and is lowest among American Indian/Alaskan Native women (Figure 2.2). The mortality rate of breast cancer increases with increasing age at diagnosis and is highest among women aged 85 and above (Figure 2.3). When stratified by race, the mortality rate is highest among African American women followed by White women and is lowest among Asian/Pacific Islander women (Figure 2.4). The majority of incident breast cancers are diagnosed at the localized stage (64.4%) followed by the regional stage (27.7%) (Figure 2.5).

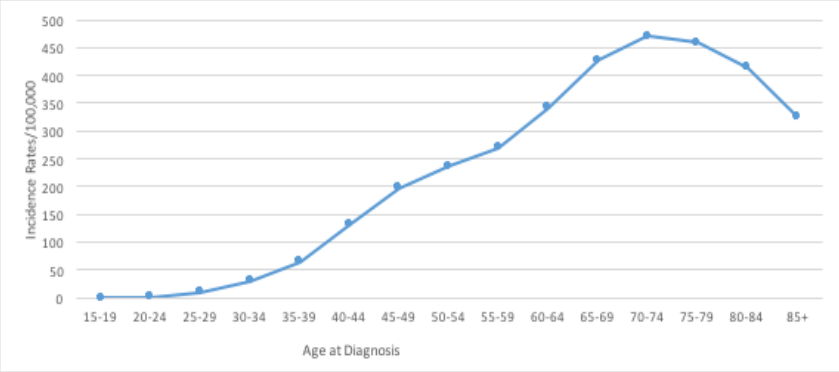

Figure 2.1: Breast cancer incidence rates by age at diagnosis: SEER, 2013-2017

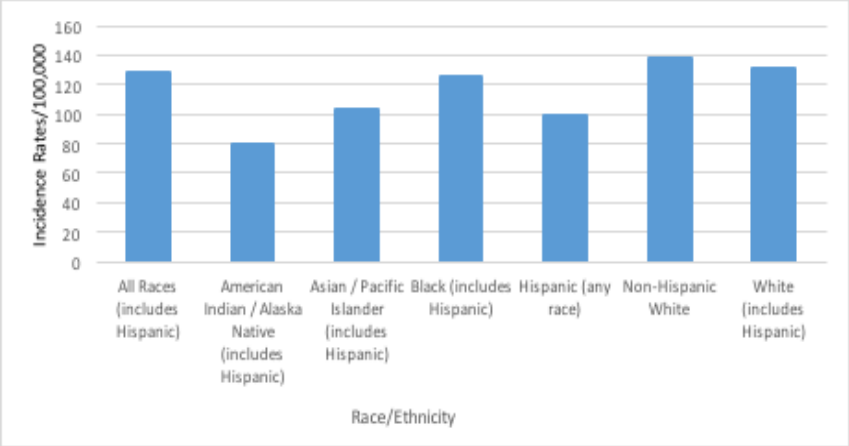

Figure 2.2: Breast cancer incidence rates by race: SEER, 2013-2017

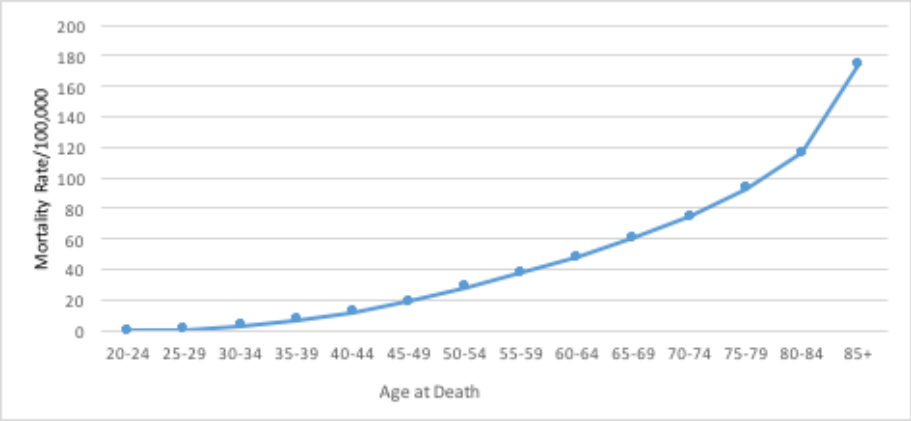

Figure 2.3: Breast cancer mortality rates by age at death: SEER, 2013-2017

Figure 2.4: Breast cancer mortality rates by race: SEER, 2013-2017

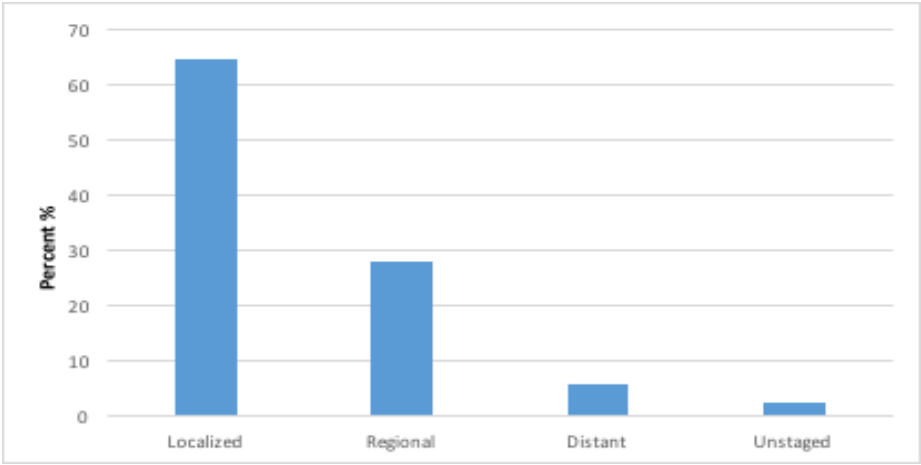

Figure 2.5: Distribution of incident breast cancer cases by stage at diagnosis: 2008-2017

The incidence of breast cancer has been gradually increasing in the United States, with an annual increase of 0.3% from 2012-2016[3]. In contrast, there has been a 40% decline in breast cancer mortality overall from the 1989 to 2017[3]. Currently, there are more than 3.8 million breast cancer survivors in the United States[3]. The steady increase in the incidence of breast cancer coupled with the increase of life expectancy among breast cancer patients has placed survivors of primary breast cancer at approximately 17% increased risk of second primary cancers[82], including contralateral breast cancer.

Contralateral breast cancer (CBC)

Contralateral breast cancer (CBC) refers to the occurrence of a second primary cancer in the contralateral (other) breast, and is, by definition, not a metastasis from the first primary cancer[40]. It is the most common form of second primary cancer, accounting for approximately half of all the malignancies among breast cancer survivors[6]. Women who are survivors of first primary breast cancer are at a 2-6-fold increased risk of contralateral breast cancer compared to the general population[51,83]. Evidence suggests that one-fourth of breast cancer survivors are at risk of CBC within 6 months of initial diagnosis[40], and approximately 10-15% of all patients develop contralateral breast cancer during the first 20 years of diagnosis with a first primary breast cancer[84]. Studies have also indicated that contralateral tumors have worse prognoses compared to primary breast tumors[84,85], among which ER- and ER-/PR- contralateral tumors are associated with worse prognoses compared to ER+ contralateral tumors[51].

Breast cancer risk factors

Primary breast cancer

Multiple factors have been associated with the risk of first primary breast cancer; the strongest and most consistent associations have been observed with age, breast density, family history, lifetime exposure to estrogen, reproductive factors (such as early menarche, late menopause, late age at pregnancy), and postmenopausal obesity[86]. Although BMI (body mass index) is an important risk factor, the association between BMI and breast cancer risk has been observed mostly among postmenopausal women but not in premenopausal counterparts[86,87]. The determinants of breast cancer risk can be broadly categorized as modifiable and non-modifiable factors[88] (Table 2.2). Potentially modifiable factors include: obesity, breast density, use of

exogenous hormones (i.e. menopausal hormone therapy), alcohol consumption, and physical activity/sedentary behavior, whereas non-modifiable factors include: age, genetics, reproductive factors, and family history of breast cancer[88] (Table 2.2).

The table below summarizes factors consistently associated with breast cancer, along with their direction of association[86,89].

Table 2.2: Summary table of risk factors of breast cancer

Risk factors	Comparison group	Direction of association
Non-modifiable risk factors		
Age at diagnosis	(>65 years vs ≤65 years)	Positive
BRCA1/2 mutations	No BRCA mutations	Positive
Early menarche	> 15 years	Positive
Late menopause	< 45 years	Positive
Early age at first childbirth	Nulliparous or first child ≥30 years	Inverse
Parity	≥ 1 live birth vs nulliparous	Inverse
Prolonged breastfeeding	No breastfeeding	Inverse
Family history of breast cancer	No family history of breast cancer	Positive
Presence of benign breast tumors	Absence of benign breast tumors	Positive
Potentially modifiable risk factors		
High breast density	Low breast density	Positive
Alcohol intake	No alcohol intake	Positive
Use of menopausal hormone therapy (MHT)	Never used	Positive
Postmenopausal BMI	>30 Kg/m^2 vs <25 Kg/m^2	Positive
Physical activity	Physically inactive	Inverse

Risk factors	Comparison group	Direction of association
Smoking	Non-smoker	Positive
Poor dietary habits	Healthy diet	Positive

Presented data abstracted from Singletary 2003 and Kaminska 2015 et al[86,89]

Contralateral breast cancer (CBC)

Several of the same determinants that influence the risk of primary breast cancer are also associated with the incidence of contralateral tumors, including age, family history of breast cancer, reproductive factors, and obesity (Table 2.3). It is important to note that one of the key determinants that has been significantly and consistently associated with reduced CBC risk is the use of adjuvant therapies for breast cancer, mainly endocrine therapy and chemotherapy[49,90,91]. Younger age at diagnosis (< 50 years) of primary breast cancer has been identified as one of the factors associated with increased risk of CBC[92]. One prior study has reported that per decade increase in age of primary breast cancer diagnosis is associated with a 7% decreased risk of CBC[91]. Evidence suggests that family history of breast cancer, especially among first-degree relatives is associated with an increased risk of CBC[93,94]. Having first-degree relatives with a history of breast cancer is associated with a 34% increased risk of CBC[40,83,95]. Being overweight (BMI $\geq$ 25 kg/m^2) or obese (BMI $\geq$ 30 kg/m^2) is associated with an increased risk of CBC when compared to normal weight (BMI < 25 kg/m^2)[91]. Several tumor characteristics are also associated with CBC. Lobular histology of primary cancer has been found to be associated with high CBC risk[91]. The HR status of the primary tumor is an important determinant of CBC risk; HR- (ER-/PR-) status of the primary breast tumors were observed to be associated with increased risk of CBC[91]. Among reproductive factors, late age at menarche ($\geq$ 13 years), having at least one full-term pregnancy, and high parity are associated with reduced CBC risk[91,96,97].

The table below summarizes the common risk factors and the magnitude of risk associated with contralateral breast cancer (Table 2.3). The information has been extracted from two systematic reviews and meta-analyses that have extensively reviewed the association between various factors and the incidence of contralateral breast cancer[91,97]. The meta-analysis by Akdeniz et al, 2020 (n=13 studies) only reviewed observational studies[97] while that by Akdeniz et al, 2019[91] reviewed both observational studies and randomized clinical trials (n=68 studies). All the risk factors had the expected direction of association with the risk of CBC, except tumor grade, where a non-significant inverse association was observed in the relationship between high tumor grade and CBC risk whereas, prior research shows that higher tumor grade is positively associated with CBC compared to lower grade[98].

Table 2.3: Summary of risk factors of contralateral breast cancer

Risk factor	**Comparison group**	**Direction of association**	**Magnitude of risk, RR (95% CI)***
Age (years)	Increase per decade	Inverse	0.93 (0.88, 0.98)
	Present vs none	Positive	1.72 (1.15, 2.57)
Family history	First degree vs none	Positive	1.54 (1.25, 1.90)
	Second degree vs none	Positive	1.17 (0.90, 1.52)
BMI	Overweight (BMI $\geq$ 25 kg/m^2) vs normal weight (BMI < 25 kg/m^2)	Positive	1.13 (0.86, 1.49)
	Obese (BMI $\geq$ 30 kg/m^2) vs normal weight (BMI < 25 kg/m^2)	Positive	1.54 (1.26, 1.87)
Breast density	Scattered vs almost entirely fatty	Positive	1.12 (0.79, 1.57)
Alcohol use	Ever vs never	Positive	1.15 (1.02, 1.31)
Smoking	Ever vs never	Positive	1.07 (0.97, 1.18)

Risk factor	Comparison group	Direction of association	Magnitude of risk, RR (95% CI)*
Reproductive factors			
Age at menarche (years)	≥13 vs < 13	Inverse	0.94 (0.83, 1.06)
Gravidity	Ever vs never	Inverse	0.92 (0.77, 1.09)
Parity	≥ 4 full term pregnancy vs nulliparous	Inverse	0.56 (0.42, 0.76)
Breastfeeding	Ever vs never	Inverse	0.87 (0.74, 1.01)
Menopausal status	Postmenopausal vs premenopausal	Positive	1.12 (0.97, 1.30)
Age at menopause (years)	<45 vs ≥ 45	Inverse	0.79 (0.67, 0.93)
Tumor characteristics			
Tumor size	T2/T3 vs T1	Positive	1.17 (1.03,1.34)
Nodal status	Positive vs negative	Positive	1.05 (0.95,1.16)
Tumor grade	III vs I/II	Inverse	0.93 (0.84, 1.02)
ER status	Negative vs positive	Positive	1.53 (1.04, 2.26)
PR status	Negative vs positive	Positive	1.23 (1.02, 1.48)
HER2 status	Negative vs positive	Positive	1.18 (0.96, 1.45)
Histology	Lobular vs ductal	Positive	1.43 (1.13, 1.82)
Treatment characteristics			
Endocrine therapy	Treated vs untreated	Inverse	0.61 (0.52, 0.71)
Chemotherapy	Treated vs untreated	Inverse	0.70 (0.62, 0.79)
Radiotherapy	Treated vs untreated	Inverse	1.05 (0.95, 1.17)

Presented data abstracted from Akdeniz et al. 2019 and Akdeniz et al. 2020[91,97]
*The relative risk (RR) presented were summary estimates calculated combining the OR, HR, and RR from the different articles used for the meta-analysis.

The findings of the meta-analyses suggest that some of the determinants associated with primary breast cancer (high breast density, younger age at menarche, high tumor grade, positive nodal status) were not significantly associated with CBC risk[91,97]. Additionally, while high postmenopausal BMI is a risk factor for primary breast cancer, no such distinction was reported in the association between BMI and CBC risk.

Breast density

Breast density is one of the strongest risk factors of primary breast cancer[21,22,99-102]. Women with elevated breast density (≥60%) are at 4-6-fold increased risk of breast cancer compared to women with less dense (≤5%) tissue[22,99]. Breast density, the amount of dense breast tissue, reflects the fibroglandular and adipose tissue composition of the breast, and is radiographically captured by mammography[22,99-103]. While the fibroglandular tissue is a mix of fibrous connective tissue, specifically the stroma and the epithelial cell lining the breast parenchyma (ducts), adipose tissue is mainly comprised of fat[103,104]. Variations in breast tissue composition often translate to differences in x-ray attenuation properties. Dense breast tissues attenuate more x-rays compared to the non-dense tissues and hence appear radiographically opaque[103]. More specifically, adipose tissue appears dark and radiologically lucent, while the fibroglandular tissue appears light and is radiologically dense in a mammogram[99-101]. The radiologically dense fibroglandular (stromal and epithelial) breast tissue is referred to as mammographic density, and is often expressed as the proportion of the dense area of the breast relative to the total breast tissue area, also known as mammographic percent density[102].

Methods of Measurement

Several methods, both qualitative and quantitative, have been developed for the measurement of mammographic density. Although there is no universally accepted standard classification system for mammographic density measurement, the most widely used processes are described below:

The first classification method of breast density was developed by John Wolfe in 1976, this method describes the relationship between the variations in parenchymal patterns of mammographic density and differences in breast cancer risk[105,106]. Wolfe's classification of breast density categorizes mammographic density into four qualitative categories: i) N1 (normal): predominantly fat, P1 (prominent 1): mostly composed of fat but the prominence of ductal tissues in <25% of the breast, iii) P2 (prominent 2): prominence of ductal tissues in ≥25% of the breast, iv) DY (dysplasia): extremely dense breast tissues with minor involvement of prominent ducts[105-107]. Based on this classification system, women in the N1 category have the lowest risk of breast cancer, while those in the DY category have the highest risk[105-107]. Prior literature supports the association between variations in mammographic density and differences in the associated breast cancer risk determined by the Wolfe method of classification[108]. Though Wolfe's classification system was the first developed method of breast density measurement, currently, the Breast Imaging-Reporting and Data System (BIRADS) developed by the American College of Radiology(ACR) is one of the most widely used and clinically accepted classification systems, both in the United States and internationally[109]. This measurement system provides a standardized method of reporting and interpreting radiographic breast images captured by a mammogram. Although developed as a qualitative system, a quantitative component was incorporated in the BIRADS 4th edition[110]. In the recently released 5th edition of the BIRADS

classification, the quantitative component was removed from the scoring system[111]. The BIRADS breast density classification has four categories: i) entirely fatty breasts, ii) scattered areas of fibroglandular densities, iii) heterogeneously dense breasts, and iv) extremely dense breasts[109]. Like Wolfe's method, the BIRADS classification system distinguishes the differences in breast cancer risk based on the variations in breast density, where the extremely dense category is associated with the highest risk while the non-dense category (predominantly fatty breasts) is associated with the lowest risk[20,112].

Below is a comparison of the BIRADS 4th and 5th edition

Table 2.4: BIRADS classification of breast density

	BIRADS 4th edition	**BIRADS 5th edition**
Category 1	The breasts are almost entirely fat (<25% glandular)	The breasts are almost entirely fatty
Category 2	There are scattered densities (approximately 25–50% glandular)	There are scattered areas of fibroglandular density
Category 3	The breast tissue is heterogeneously dense, which could obscure detection of small masses (approximately 51–75% glandular)	The breasts are heterogeneously dense, which may obscure detection of small masses
Category 4	The breast tissue is extremely dense. This may lower the sensitivity of mammography (>75% glandular)	The breasts are extremely dense, which lowers the sensitivity of mammography

Source: Qualitative Versus Quantitative Mammographic Breast Density Assessment: Applications for the US and Abroad. Author: Destounis et al., 2017 Source: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5489950/ License: CC BY 4.0

In addition to the qualitative methods of measurement, there are several quantitative approaches of breast density classification that produce more consistent results compared to the qualitative counterparts[20,108]. Quantitative methods to measure breast density usually involve computer assisted programs, some of the widely used programs are Cumulus[113], Volpara[114], and Quantra[115]. The Cumulus technique is a semi-automated approach that documents computerized

measurements of breast density on a digital mammogram. The Cumulus technique is often referred to as the gold standard for quantitative breast density measurements and is mainly used for research purposes[107]. Several fully-automated quantitative measurements of mammographic density have been developed, and include both two-dimensional (area-based) as well as three-dimensional (volumetric) approaches to measuring breast density[107]. The area-based measures include AutoDensity (developed by University of Melbourne), LIBRA (developed by University of Pennsylvania), and STRATUS (developed by the Karolinska Institute)[107]. The volumetric approaches of breast density measurement estimate the actual volume of the fibroglandular (dense) breast tissues[107], and the most widely used are Quantra[115] and VolparaDensity (Volpara)[114] methods of breast density measurement. Both Quantra, the first fully-automated volumetric breast density measurement tool[115], and Volpara[114] estimates the fibroglandular tissue volume, total tissue volume, as well as their ratio[107].

Breast Density Assessment

While mammography is the most common breast screening technique to measure mammographic percent density[116], the ratio of fibroglandular tissue to the total breast area or volume, it exposes women to harmful ionizing radiations[117] and there is no absolute standardized scale of mammographic density measurement[117]. Alternative imaging techniques to assess breast density include magnetic resonance imaging (MRI) and Ultrasound tomography (UST). Although MRI produces three dimensional images of the breast using the magnetic field and radiowave energy[118,119], it is an expensive procedure that requires intensive training, and generates more false positive results compared to a mammography. A newly emerging, safe, and

inexpensive imaging technique, Ultrasound tomography (UST), uses sound waves to characterize breast tissues and has shown significant promise in assessing breast density[53,120,121].

Ultrasound tomography (UST)

Ultrasound tomography (UST) is a novel imaging technique that uses sound waves to quantitatively measure the volumetric breast density of the uncompressed breast[53,120]. Essentially, UST is a type of ultrasound scanner that generates three-dimensional images of the uncompressed breast tissue using transmission properties of the sound waves known as sound speed[104,117]. Evidence indicates that there is a linear relationship between breast tissue density and sound speed measures[122,123], and the relationship can be expressed as ($v \propto \rho$), where v is the average speed of sound and ρ is the density of the tissue sound waves travel through[57,104]. The first UST device, an investigational prototype of the UST known as the Computed Ultrasound Risk Evaluation (CURE) was developed at the Karmanos Cancer Institute, Detroit Michigan, USA[120]. Later an upgraded version known as the SoftVue that built on the previous prototype was developed by Delphinus Medical Technologies, Plymouth, Michigan, USA[121].

The operation of the UST is summarized as follows: the patient lies in a prone position on a table with the breast submerged in an imaging tank filled with body temperature water that has a sound speed of approximately 1.52 km/s[54,57]; A UST sensor, a 20 cm ring shaped transducer mounted on an automatic motor gantry, surrounds the breast to be scanned[54,57]; the transducer progressively moves from the chest wall to the nipple capturing approximately 40-100 images depending on the size of the breast[54,57]. Typically, a whole breast UST scan takes about 1 min, generates sound speed data for each position of the sensor, and yields a stack of images of the sound speed breast density tomograms[54,57]. The sound speed measure of the UST is determined

by transmission and receipt of signals from portion of the sensor to the other, and is calculated in km/s on an absolute scale[57]. The sound speed images of the surrounding water bath, chest wall, and the nipple are not relevant to breast density measurements and must be manually segmented from the breast tissue images[54,57]. Once segmented, the clean stack of images is used to calculate the volumetric breast density measure, the volume averaged sound speed measure (VASS). The VASS is the mean value of the sound speed of the defined breast tissue area, and is calculated by summing all sound speed pixel values and dividing by the total number of pixels of the breast[54,57,104]. Prior literature has indicated that the VASS measures of breast density are strongly associated with mammographic present density[57].

Role of hormones

Estrogen

The sex steroid hormone, estrogen, is critical in the development and function of normal breast tissues[124]. Estrogens are mainly synthesized from low-density lipoprotein (LDL) cholesterol, which is metabolized and converted to pregnenolone, the precursor of all steroid hormones[125]. Pregnenolone is then metabolized into progesterone or androstenedione, which is further metabolized to other androgens or estrogens[125]. Estrogen is chemically comprised of a C18 steroid that consists of a benzene ring, a phenolic hydroxyl group at C3, and a hydroxyl group (17β-estradiol) or a ketone group (estrone) at C17[125]. There are two major forms of circulating estrogens, the parent estrogens, estrone (E1), and estradiol (E2, or 17β-estradiol). Parent estrogens are synthesized from cholesterol via a series of reactions in the estrogen biosynthesis process[126].

Estrogens play a key role in the female reproductive cycle, which includes, an increase in the proliferation of breast epithelium and stroma, development of sex organs, regulation of menstrual cycle, and reproduction[127-129]. They are produced in the ovaries, placenta, and adipose tissue by the enzyme aromatase (CYP19A1), a member of the cytochrome P450 family[126,127] and a key enzyme in the conversion of testosterone and androstenedione to estradiol and estrone[130]. Among premenopausal women, estrogen is produced mainly in the ovaries and estradiol is the major biologically active estrogen[126]. After menopause, the production of estrogen from the ovaries declines but continues in the peripheral tissues, such as in adipose tissues, skin, brain, etc.[126,130]. Among postmenopausal women, the main source of estrogen comes from the conversion of androstenedione to estrone in the adipose tissue[131]. This marks a shift from premenopausal women whose principal source of estrogen is the ovaries, while among postmenopausal women, extragonadal organs, such as adipose tissues serve as the primary source. Estrogens not only play a crucial role in the development and physiology of the female reproductive system[132] but also have protective effects on bone and cardiovascular health[132,133].

Role of estrogen in breast carcinogenesis

Breast cancer is a hormone-dependent disease, and elevated levels of endogenous estrogen have been consistently associated with breast cancer risk[129,134,135]. Both epidemiological and clinical studies have provided consistent evidence that higher levels of endogenous estrogens increase risk of breast cancer among postmenopausal women [14,15,129,134]. Although comparatively limited and less consistent findings have been reported among premenopausal women, in the pooled analysis of seven prospective studies, estrogen was associated with elevated breast cancer risk among premenopausal women[135]. The mechanism of estrogen signaling in breast carcinogenesis is mediated by the estrogen receptors (ER)[18]. ERs are proteins expressed on

certain breast cells that enable estrogen to bind to and initiate responses in those tissues. If a tumor cell expresses estrogen receptors, they are known as ER+ breast tumor; if a tumor cell does not express such receptors, they are known as ER- breast tumor. Approximately 70-80% of the breast cancer cells express estrogen receptors on their surface[136]. There are two types of estrogen receptors: ERα and ERβ[127]. ERα is more commonly expressed in the breast, uterus, and cervix, while ERβ is expressed mainly in the ovaries, prostate, spleen, lung, and hypothalamus[137].

There are two proposed mechanisms in which estrogen will act on target cells resulting in tumor formation. In the ER-dependent pathway, the mechanism of action is the estrogen receptor (mainly ERα) mediated stimulation of breast cells, that produces an increased mutation rate, cell proliferation, and finally, development of breast cancer[138]. When estrogen binds to the ERs, it activates the estrogen-ER complex that changes specific cell expressions, and stimulates uncontrolled breast cell growth[15]. In the ER-independent mechanism, the parent estrogens, estrone and estradiol are metabolized to their metabolites, increasing DNA mutations and production of the genotoxic metabolites[138]. The metabolites lead to DNA damage and stimulate subsequent tumor formation[138]. The genotoxic effects of estrogen on breast carcinogenesis are mediated by their respective metabolites[15], and are explained in detail below (Figure 2.6).

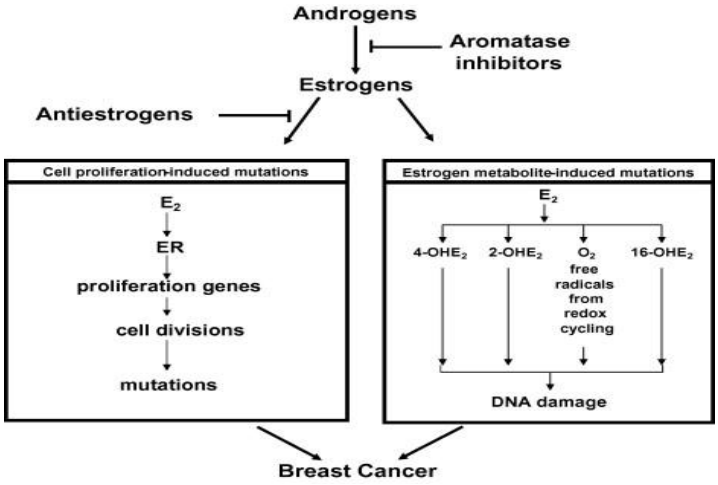

Figure 2.6: Pathways involved in ERα-mediated and ERα-independent effects of estradiol on breast cancer development

Source: Adapted from "Estrogen metabolites and breast cancer". Author: Richard J. Santen, Wei Yue, Ji-Ping Wang. Publication: Steroids. Publisher: Elsevier. Date: July 2015. (License no: 4873800450402; Date July 21, 2020)

Estrogen metabolism

Estrogen metabolism is the metabolic conversion of estrogen to its respective metabolites via oxidative and conjugated metabolism, sulfonation, and/or O-methylation[139]. Irreversible hydroxylation of the parent estrogens (estrone and estradiol) by the cytochrome P450 enzymes at the C-2, C-4, or C-16 positions of the steroid ring leads to production of estrogen metabolites across three different pathways[12,17,140] (Figure 2.7). The three pathways are: the 2-hydroxy (2-OH), 4-hydroxy (4-OH), and 16-hydroxy (16-OH) pathways (Table 2.7), and the cytochrome P450 enzymes, such as CYP 17, CYP3As, CYP 1A1, CYP 1A2, and CYP 1B1 are crucial to this metabolic process[141,142].

Estrogen metabolism is initiated by the reversible oxidation of estradiol to estrone at the C17 position[131]. Metabolism of parent estrogens occurs via either irreversible hydroxylation at the A-ring or D-ring. Metabolism on the A-ring mainly occurs at the C2 position, leading to the

production of the 2-pathway catechols, 2-hydroxy estrone (2-OHE1), and 2-hydroxy estradiol (2-OHE2)[12,140,143] (Table 2.5 and Figure 2.7). Methylation of these metabolites further yields the 2-methoxy estrone (2-ME1), 2-methoxy estradiol (2-ME2), and 2-methoxy estrone-3-methyl ether (3-ME1) (Table 2.5 and Figure 2.7). To a lesser extent, metabolism on the A-ring can also lead to the production of the 4-pathway catechol, 4 hydroxy estrone (4-OHE1), and methylation of which produces 4-methoxy estrone (4-ME1) and 4-methoxy estradiol (4-ME2)[12,140,143] (Table 2.5 and Figure 2.7). Lastly, hydroxylation of the D-ring at the 16α position leads to production of the 16-hydroxylation metabolites, estriol, 16α-hydroxy estrone (16α-OH-E1), 16-keto estradiol (16-keto-E2), 16-epiestriol (16-epiE3), and 17-epiestriol (17-epiE3)[12,140,143] (Table 2.5 and Figure 2.7). While most metabolites are found in conjugated forms as a sulfate or a glucuronide, some metabolites, such as estrone, estradiol, estriol, 2-methoxy estrone and 2-methoxy estradiol are also found in unconjugated forms[143].

Estrogen metabolites may have either agonistic or antagonistic estrogenic activities on surrounding tissues which translate to either pro-carcinogenic or anti-carcinogenic effects[125,142]. Evidence suggests that the 2-hydroxylated (2-OH) estrogen metabolites have reduced hormonal potency when compared to estradiol and may even have anti-carcinogenic effects[125,142]. The P-450 enzymes, CYP1A1, and CYP1B1 are the major enzymes that drive the 2-hydroxylation of estrogens and metabolize them into catechol estrogens[125]. Prior research indicates that select 4-OH and 16-OH estrogen metabolites are similar in hormonal potency when compared to estradiol[142]. Prior literature support that the hydroxylated catechol estrogen metabolites of the 4-OH pathway have tumorigenic properties[125]. The 16-hydroxylation pathway leads to the production of several important metabolites. Of which,16α-hydroxy estrone has been reported to be associated with tumorigenesis[125]. Among postmenopausal women, higher levels of the 2-OH

pathway metabolites and the ratio of 2-OH:16-OH metabolites have been consistently reported to be inversely associated with breast cancer risk, even after adjusting for total estrogen levels[12,17,143,144]. On the contrary, higher levels of 16-OH estrogen metabolites were associated with an increased risk of breast cancer [17,125,145]. However, evidence of the relationship between estrogen metabolites and breast cancer risk is limited and inconclusive among premenopausal women[145-149]. Prior studies that assessed the relationship between EMs and breast cancer risk have used both enzyme-linked immunoassay (ELISA)[145,146,148,150,151] and liquid chromatography mass spectrometry (LC-MS/MS) assay[147,149] to assess the estrogen metabolite concentrations. While one study has assessed the circulating EMs in serum[147], the remaining studies have assessed the urinary levels of these metabolites[145,146,148-151]. Kabat et al. observed a statistically significant inverse association between higher urinary levels of 2-hydroxyestrone:16α-hydroxyestrone (2-OHE1: 16α-OH-E1) ratio (as measured by ELISA) and breast cancer risk[146]. However, no associations were reported by Arslan et al. when circulating levels of 2-OHE1: 16α-OH-E1 ratio, measured by the highly sensitive and specific LC-MS/MS, were examined in relation to the risk of breast cancer[147]. Although a suggested inverse association between urinary concentrations of 2-OHE1: 16α-OH-E1 ratio and breast cancer risk has been reported by a few studies using both ELISA[145,148] and LC-MS/MS[149], no statistically significant associations were observed.

Table 2.5: Estrogen and estrogen metabolites

Parent Estrogens	**2-hydroxylation pathway**	**4-hydroxylation pathway**	**16-hydroxylation pathway**
Estrone	2-hydroxyestrone	4-hydroxyestrone	16α-hydroxyestrone
Estradiol	2-hydroxyestradiol	4-methoxyestrone	Estriol
	2-methoxyestrone	4-methoxyestradiol	17-epiestriol

	2-methoxyestradiol	16-ketoestradiol
	2-hydroxyestrone-3-methyl ether	16-epiestriol

Source: Adapted from "Endogenous Estrogens, Estrogen Metabolites, and Breast Cancer Risk in Postmenopausal Chinese Women". Author: Moore, Steven C.; Matthews, Charles E. Publication: Journal of the National Cancer Institute. Publisher: Oxford University Press. Date: May 2016 (License no: 4873771382136; Obtained On: July 21,2020).

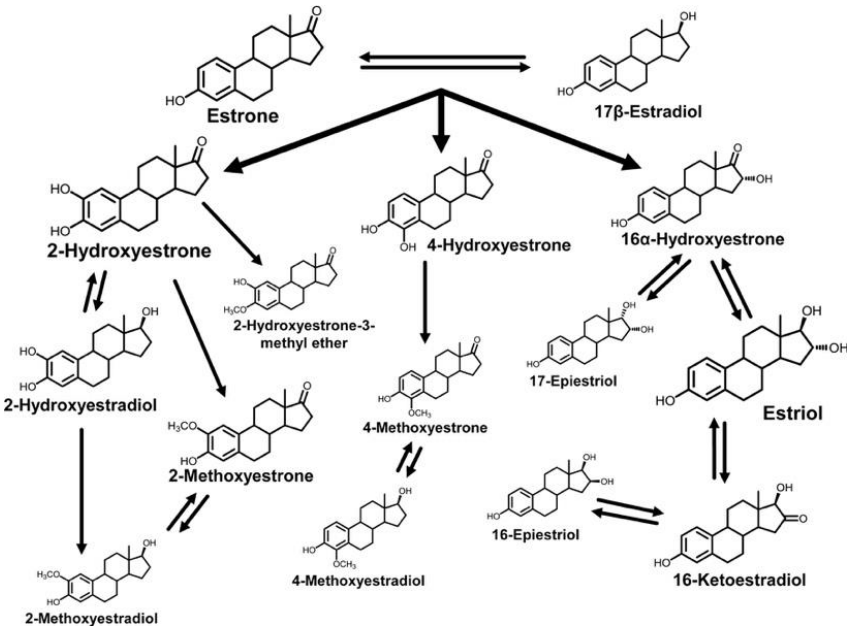

Figure 2.7: Estrogen metabolism pathways

The parent estrogens are irreversibly hydroxylated at the C-2, C-4, or C-16 position of the steroid ring.
Source: Adapted from "Epidemiologic studies of estrogen metabolism and breast cancer". Author: Regina G. Ziegler et al. Publication: Steroids. Publisher: Elsevier. Date: July 2015 (License no: 4878271481967; Obtained On: July 29, 2020).

Insulin like growth factors (IGFs)

Insulin like growth factors (IGFs) are peptide hormones that play a crucial role in human growth, development, and metabolism as well as in cell differentiation and proliferation[30,31]. The IGF system is comprised of growth factor ligands (IGFs I and II), their transmembrane growth factor receptors (type I and II receptors), IGF binding proteins (IGFBPs), and proteases[152-154]

(Figure 2.8). IGF-I, mainly produced in the liver, is the primary IGF in circulation among adults and has the properties of both a circulating hormone and a tissue growth factor[31]. IGFBPs have a strong affinity for IGF and modulate their bioavailability in circulation as well as their access to the potentially oncogenic IGF receptors[31,154]. The IGFBPs (1-7) differ based on size and functional motifs, including differences in ligand binding affinities, interactions with cell surface and extracellular proteins, and/or binding to the glycoprotein, acid labile unite (ALS)[155]. Of the IGFBPs, IGFBP-3 is the most abundant binding protein in circulation, and has both growth-promoting and inhibiting effects[31]. It is the main binding protein of IGF-I, and may function on its own or independently of the IGFs[31]. The proteases work to dissociate the IGF-IGFBP complex and prepare IGFs to bind to their high affinity IGF receptors. Upon binding to the receptors, IGFs activate the phosphatidylinositol-3-kinase and RAS-extracellular signal-regulated kinase pathways that play a significant role in cell differentiation and proliferation[31,156].

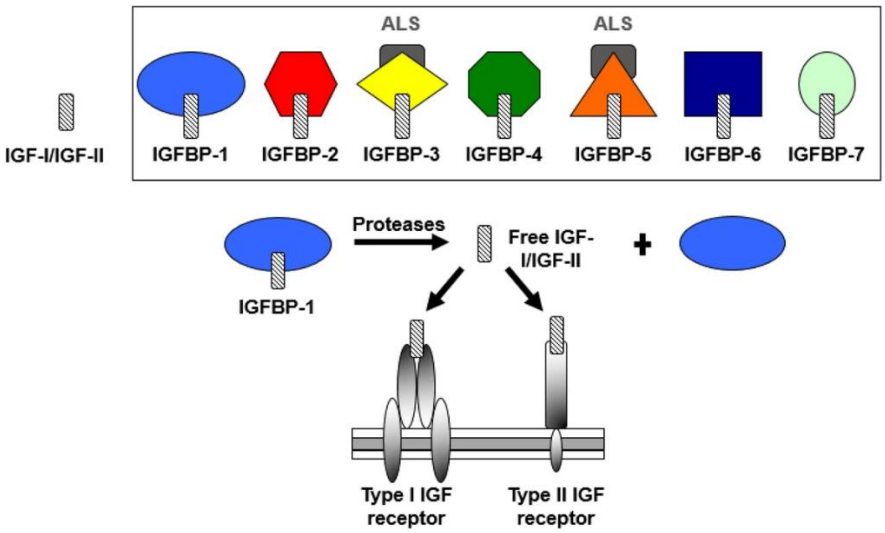

Figure 2.8: Insulin-like growth factor (IGF) system

Title: Adapted from "Relationship of circulating insulin-like growth factor-I and binding proteins 1–7 with mammographic density among women undergoing image-guided diagnostic breast biopsy". Author: Manila Hada et al. Publication: Breast Cancer Research. Publisher: BMC. Source: https://breast-cancer-research.biomedcentral.com/articles/10.1186/s13058-019-1162-8. License: CC BY 4.0
ALS: Acid labile subunit

IGFs and breast cancer risk

Evidence from previous literature support a strong mitogenic and anti-apoptotic effect of IGF on human tissues, including breast tissues[30,32]. Prior studies indicate that IGF-I and its main binding protein, IGFBP-3, play a major role in breast carcinogenesis by stimulating the proliferation of cancerous cells and inhibiting apoptosis[30,152,157]. Based on the evidence from previous literature, circulating IGF-I concentrations are positively associated with the risk of breast cancer[158-161] (Table 2.6 and 2.7). Some prior studies point towards heterogeneity by menopausal status in the association between IGF-I and breast cancer[30,162], with positive associations mainly noted among premenopausal women (Table 2.6 and 2.7). However, in the pooled analysis of 17 epidemiological studies[163], and epidemiologic studies conducted in the recent years[159,161], the association between IGF-I and risk of breast cancer did not vary by menopausal status. With regards to the association between IGFBP-3 and breast cancer risk, findings have been rather heterogenous[158-161,164-166]. Although evidence indicates a protective role of IGFBP-3 on cancer, including breast cancer[164-166], studies have primarily reported a positive association between circulating IGFBP-3 and an elevated risk of breast cancer (Table 2.6 and 2.7). This corroborates evidence from prior literature indicating that the binding protein, IGFBP-3 has both growth-promoting and inhibiting effects[31]. The anti-proliferative and pro-apoptotic functions of IGFBP-3 may or may not be mediated by IGFs[153]. In recent years, four studies that assessed the relationship between IGFs and breast cancer risk reported similar associations[158-161]. Tin et al. and Murphy et al.[158,159] recruited participants from the UK Biobank population database to conduct a retrospective cohort study assessing the association between IGFs and breast cancer, while Monson et al and Kaaks et al.[160,161] conducted nested case-control studies using data from the New York Breast Cancer Family Registry (BCFR) cohort and the

prospective European (EPIC) cohort, respectively. These studies reported a positive association between IGFs and breast cancer in overall and menopausal status-specific associations.

Table 2.6: Summary of studies that meta-analyzed the association between IGFs and breast cancer risk

Author, year	Study period	Country	Population	Study design	Sample size	Exposure	Main findings
Shi et al, 2004[30]	1960-2003	United States, Europe, China	Pre-and postmenopausal breast cancer cases and controls	Case-control	18	IGF-I, IGFBP-3 Measured using RIA, ELISA, IRMA, CLIA	Overall: OR (95% CI) IGF-I= 1.05 (0.94, 1.17) IGFBP-3 = 1.15 (1.00, 1.34) Premenopausal: OR (95% CI) IGF-I= 1.39 (1.16, 1.66) IGFBP-3 =1.42 (1.15, 1.74) Postmenopausal: OR (95% CI) IGF-I= 0.93 (0.80, 1.10) IGFBP-3= 1.23 (0.97, 1.55)
Renehan, 2004[162]	Jan 1996-Dec 2002	United States, Europe, China	Pre-and postmenopausal breast cancer cases and controls	Case-control	6	IGF-I, IGFBP-3 Measured using RIA, ELISA, IRMA,	Premenopausal: OR (95% CI) IGF-I= 1.65 (1.26, 2.08) IGFBP-3= 1.51 (1.01, 2.27) Postmenopausal: OR (95% CI) IGF-I= 0.95 (0.77, 1.17) IGFBP-3= 1.01 (0.74, 1.38)
Sugumar et al, 2004[167]	Jan 1990-March 2003	United States, Sweden, Italy, China	Premenopausal breast cancer cases and controls	Case-control	6	IGF-I, IGFBP-3 Measured using RIA, ELISA, IRMA,	Premenopausal: OR (95% CI) IGF-I= 1.74 (0.97, 3.13) IGFBP-3= 1.60 (0.84, 3.02)

This tables presents meta-analyses that examined the relationship between IGFs and breast cancer. ELISA: Enzyme linked immunosorbent assay; RIA: Radioimmunoassay; IRMAs: Immunoradiometric assay; CLIA: Chemiluminescent immunoassay

Table 2.7: Summary of observational studies that assessed the association between IGFs and breast cancer risk

Author, year	Study design	Population	Exposure	Statistical analyses	Covariates	Main findings
Tin et al, 2021[159]	Retrospective cohort	Women without breast cancer aged 40-69 years recruited between 2006-2010 from the UK Biobank • Premenopausal, n= 30,565 • Postmenopausal, n=133,294	Testosterone, SHBG, IGF-I Measured using CLIA	CPH	Ethnicity, educational level, smoking, alcohol use, physical activity, BMI, menstrual cycle, parity and age at first birth, time since last oral contraceptive pill, age at menopause, time since hormone replacement therapy use, presence of endocrine disorders, family history of breast cancer in first-degree relatives, total testosterone, SHBG and in pre-menopausal women total estradiol	<u>Premenopausal: HR (95% CI)</u> IGF-I= 1.18 (1.02, 1.35) <u>Postmenopausal: HR (95% CI)</u> IGF-I= 1.07 (1.01, 1.12)
Murphy et al, 2020[158]	Retrospective cohort	Women without breast cancer aged 40-69 years recruited between 2006-2010 from the UK Biobank • N=206,263	Testosterone, SHBG, IGF-I, IGFBP-3 Measured using CLIA	CPH	Physical activity, height, alcohol consumption frequency, smoking status and intensity, educational level, ever use of hormone replacement therapy, parity and age at first birth, and an interaction between menopausal status and BMI, CRP, testosterone, SHBG, and HbA1c	<u>Overall: HR (95% CI)</u> IGF-I= 1.11 (1.07, 1.16) IGFBP-3= 1.00 (0.97, 1.04)
Monson et al, 2020[161]	Nested case-control	Women diagnosed with breast cancer (cases) and an age, ethnicity, follow-up time matched controls selected from	IGF-I, IGFBP-3 Measured using	CPH	Obesity (BMI $\geq$ 30 kg/m2), smoking and alcohol consumption, menopausal status, and BOADICEA score	<u>Overall: OR (95% CI)</u> IGF-I= 1.37 (0.66, 2.85) IGFBP-3= 1.62 (0.81, 3.24)

Author, year	Study design	Population	Exposure	Statistical analyses	Covariates	Main findings
		the New York Breast Cancer Family Registry (BCFR) cohort. • Cases, n=80 • Controls, n=156	CLIA, ELISA			
Kaaks et al, 2014[160]	Nested case-control	Women diagnosed with breast cancer (cases) and an age and recruitment center matched control subjects identified from the prospective European EPIC cohort from 1992-2000 • Cases, n=938 • Controls, n=1,394	IGF-I Measured using ELISA	CPH	Number of full-term pregnancies, age at first FTP, age at menarche, age at menopause, BMI, smoking status, lifetime mean alcohol consumption, previous pill use, use of HRT, serum concentrations of C-peptide, estradiol, free estradiol, testosterone, free testosterone, SHBG and prolactin	Overall: OR (95% CI) IGF-I= 1.13 (1.01, 1.25)
The Endogenous Hormones and Breast Cancer Collaborative Group, 2010[163]	Pooled Analysis	Individual data was collected from 17 studies in 12 countries including USA, Netherlands, United Kingdom, Denmark, Norway, Sweden, Australia, Italy, and Europe • Cases, n=4790 • Controls, n=9428	IGF-I, IGFBP-3 Measured using ELISA, IRMA, RIA	CPH	Age at menarche, parity, age at first full-term pregnancy, BMI, previous use of oral contraceptives, and, for postmenopausal women only, type of menopause, time since menopause, previous use of hormone-replacement therapy	Overall: OR_{Q5vsQ1} (95% CI) IGF-I= 1.28 (1.14, 1.44) IGFBP-3= 1.13 (0.99, 1.28) Premenopausal: OR_{Q5vsQ1} (95% CI) IGF-I= 1.21 (1.00, 1.45) IGFBP-3= 1.00 (0.82, 1.22)Postmenopausal: OR_{Q5vsQ1} (95% CI) IGF-I= 1.33 (1.14, 1.55) IGFBP-3= 1.23 (1.04, 1.45)

CPH: Cox proportional hazards regression; ELISA: Enzyme linked immunosorbent assay; RIA: Radioimmunoassay; IRMA: Immunoradiometric assay; CLIA: Chemiluminescent immunoassay

IGFs and breast density

Evidence indicates that breast density is an intermediate marker of breast cancer risk[20,22,99], however, the underlying mechanism of association between breast density and breast cancer risk remains unknown. Past literature suggests that IGFs may be key in the etiologic pathway connecting breast density to breast cancer risk[34-36]. Prior studies indicate that IGFs, specifically, circulating levels of IGF-I and the ratio of IGF-I: IGFBP-3, are positively associated with elevated breast density, while IGFBP-3 levels are inversely associated (Table 2.8). Across studies, variations have been reported both by and within menopausal status. Positive[34-36] and null associations[168-170] have been reported among premenopausal women, while evidence of an association was limited among postmenopausal women[35,36,168-170]. Boyd et al. illustrated that IGF-I was significantly positively associated with breast density among premenopausal women but not postmenopausal women. While IGFBP-3 was inversely associated with breast density among premenopausal women, the association was not significant after adjusting for potential confounders[36]. A similar pattern of association was reported in studies by Byrne et al.[34] and Diorio et al.[35] where IGF-I and the ratio of IGF-I: IGFBP-3 were positively associated with breast density while IGFBP-3 was inversely associated, among premenopausal women. No significant associations were reported among postmenopausal women. In a cross-sectional study, Hada et al. examined the association of IGF-I and IGFBPs with breast density by menopausal status (premenopausal=189, postmenopausal= 101), among women referred to breast biopsy. A novel, positive association was reported between IGFBP-2 and mammographic density among both pre- and postmenopausal women[156]. Though positive associations were observed between IGF-I and IGF-I: IGFBP-3 and volumetric breast density in premenopausal women, the relationship was not statistically significant. Both Bremnes et al. and Aiello et al. assessed the

association between IGFs and breast density among postmenopausal women with conflicting findings[171,172]. In the study by Bremnes et al., elevated concentrations of IGF-I and the ratio of IGF-I: IGFBP-3 were associated with high breast density (p trend =0.02)[172], while inverse associations were reported between the IGF-I: IGFBP-3 ratio and breast density in the study by Aiello et al[171]. In contrast, some studies did not observe any associations between IGFs and breast density, among either premenopausal[168-170,173] or postmenopausal women[35,36,168-170].

Table 2.8: Summary of studies that assessed the association between IGFs and breast density

Author, year	Study design	Population	Exposure	Outcome	Covariates	Statistical analyses	Main findings
Hada et al, 2019[156]	Cross-sectional	Pre- and postmenopausal women aged 40-65 years with an abnormal mammogram were identified from the University of Vermont (UVM) College of Medicine and University of Vermont Medical Center from 2007-2010 in the National Cancer Institute (NCI) Breast Radiology Evaluation and Study of Tissues (BREAST) Stamp Project • N=296 •Premenopausal, n= 189 •Postmenopausal, n= 101	IGF-I, IGFBPs (1-7): IGF-I: IGFBPs (1-7) ratio, IGF-I: Total IGFBP ratio Measured using ELISA and CLIA	Volumetric density measures (MD-V), Area density measures (MD-A), non-dense volume, and non-dense area Measured via mammogram	Age and BMI	Multivariate linear regression	<u>Premenopausal</u> IGF-I and % MD-V: β=0.11, p=0.97 IGFBP-2 and % MD-V: β=1.49, p=0.02 IGFBP-3 and %MD-V: β= -0.20, p=0.15 IGF-I: IGFBP-3 ratio and %MD-V: β= 3.31, p=0.28 <u>Postmenopausal</u> IGF-I and % MD-V: β= -0.55, p=0.89 IGFBP-2 and % MD-V: β=2.04, p=0.003 IGFBP-3 and %MD-V: β= -0.13, p=0.37 IGF-I: IGFBP-3 ratio and %MD-V: β= 3.07, p=0.53
Rinaldi et al, 2014[173]	Cross-sectional	Premenopausal Mexican women were selected from the ESMaestras cohort established in 2006-2008 in Mexico • N=593	IGF-I, IGFBP-3, IGF-I: IGFBP-3 ratio Measured using IRMA	Percent Breast Density, absolute dense tissue area, absolute non-dense tissue area	Age, BMI, batch of analyses, state, family history of breast cancer, benign breast disease, age at menarche, oral contraceptive use, number of full-	Multivariate linear regression	No associations were observed between IGFs and percent breast density among premenopausal women. Inverse associations were observed between IGF-I and IGF-I:

Author, year	Study design	Population	Exposure	Outcome	Covariates	Statistical analyses	Main findings
				Measured via mammogram	term pregnancies, age at first full term pregnancy, alcohol intake, smoking status, physical activity, and social economic status		IGFBP-3 with lower absolute dense and non-dense tissue areas (p trend <0.05)
Rice et al, 2012[168]	Cross-sectional study	Women who were controls in nested case–control studies in the Nurses' Health Study (NHS) and NHSII •Premenopausal, n= 783 •Postmenopausal, n=436	IGF-I, IGFBP-3, IGF-I: IGFBP-3 ratio Measured using ELISA	Percent Breast Density Measured via mammogram	Age at mammography, month of blood draw, fasting status, time of day of blood collection, and cohort, history of bbd, family history of breast cancer, age at menarche, parity and age at first birth, alcohol consumption, smoking status, and BMI, and IGFBP-3 in the IGF-I model and IGF-I in the IGFBP-3 model	Generalized linear models	No association were observed between IGF-I, IGFBP-3, and IGF-I: IGFBP-3 ratio and breast density among premenopausal (p trend ≥ 0.33) or postmenopausal women (p trend ≥ 0.23).
Izzo et al, 2012[169]	Cross-sectional	Women without any prior history of breast cancer were selected from the	IGF-I, IGFBP-3, IGF-I:	Percent Breast Density		Logistic regression	No association were observed between IGF-1, IGFBP-3, and

Author, year	Study design	Population	Exposure	Outcome	Covariates	Statistical analyses	Main findings
		Breast Care section, Dept of Gynecology section of the University of Rome from 2005-2007. • N=341	IGFBP-3 ratio Measured using CLIA	Measured via mammogram			IGF-I: IGFBP-3 ratio and breast density among premenopausal or postmenopausal women
Bremnes et al, 2007[172]	Cross-sectional	Postmenopausal women aged 55-71 years undergoing breast cancer screening were recruited in 2001 and 2002 for the Mammography and Breast Cancer study • N=977	IGF-I, IGFBP-3, IGF-I: IGFBP-3 ratio Measured using ELISA	Percent Breast Density Measured via mammogram	Age at screening, number of children, age at menopause, BMI, and hormone therapy use	ANOVA	Elevated levels of IGF-I and IGF-I: IGFBP-3 ratio were positively associated with percent breast density (p trend= 0.02), among postmenopausal women.
Maskarinec et al, 2007[174]	Pooled analysis	Women who had no history of cancer were recruited from mammography clinics across four geographical locations in United States, Norway, and Japan. •N=1327	IGF-I, IGFBP-3, IGF-I: IGFBP-3 ratio Measured using ELISA	Percent Breast Density Measured via mammogram	Age, BMI, and reproductive factors	Generalized linear models	<u>Premenopausal</u> Mean $Diff_{Q4vsQ1}$ (p trend) IGF=16 (0.83) IGFBP-3= 0 (0.91) IGF-I: IGFBP-3= 0.01 (0.67) <u>Postmenopausal</u> Mean $Diff_{Q4vsQ1}$ (p trend) IGF= -3 (0.57) IGFBP-3 = 156 (0.67) IGF-I: IGFBP-3= -0.004 (0.67)

Author, year	Study design	Population	Exposure	Outcome	Covariates	Statistical analyses	Main findings
Silva et al, 2006[170]	Cross-sectional	Women from Guernsey, United Kingdom aged ≥ 34 years without any prior history of cancer • Premenopausal, n=215 • Postmenopausal, n=241	IGF-I, IGFBP-3, IGF-I: IGFBP-3 ratio, IGF-II: IGFBP-3 ratio Measured using ELISA and RIA	Percent Breast Density, Dense breast area Measured via mammogram	Age, time since blood collection, age at first birth, BMI, waist circumference, smoking habits, past oral contraceptive use, IGFBP-3 in the IGF-I/IGF-II model and IGF-I/IGF-II in the IGFBP-3 model	Linear regression	No associations were observed between IGFs and percent breast density, among pre- or postmenopausal women.
Aiello et al, 2005[171]	Cross-sectional	Postmenopausal women aged 50-75 years were recruited from the Physical Activity for Total Health study • N=173	IGF-I, IGFBP-3, IGF-I: IGFBP-3 ratio Measured using CLIA and RIA	Percent Breast Density Measured via mammogram	Age, ethnicity, years since menopause, and percent body fat	Multiple linear regression	<u>Postmenopausal</u> Inverse associations were reported between IGF-I: IGFBP-3 ratio and breast density. No association was noted between IGFBP-3 and breast density
Diorio et al, 2005	Cross-sectional	Pre- and postmenopausal women without breast cancer were identified from two private radiology clinics during screening mammography exams from February 2001-March 2002 in Quebec, Canada	IGF-I, IGFBP-3, IGF-I: IGFBP-3 ratio Measured using ELISA	Breast Density Measured via mammogram	Age, BMI, and IGF-I or IGFBP-3, and parity (postmenopausal women)	Spearman's partial correlation coefficients	<u>Premenopausal</u> IGF-I: r (p value) = 0.083 (0.021) IGFBP-3: r (p value) = -0.124 (0.0005) IGF-I: IGFBP-3 ratio: r (p value) = 0.069 (0.056)

Author, year	Study design	Population	Exposure	Outcome	Covariates	Statistical analyses	Main findings
		• Premenopausal, n=783 • Postmenopausal, n= 791					<u>Postmenopausal</u> IGF-I: r (p value) = 0.032 (0.365) IGFBP-3: r (p value) = -0.013 (0.724) IGF-I: IGFBP-3 ratio: r (p value) = 0.029 (0.410)
Maskarinec et al, 2003[175]	Cross-sectional	Premenopausal women with no history of cancer aged 34-46 years selected from two studies on mammographic density • N= 263	IGF-I, IGFBP-3, IGF-I: IGFBP-3 molar ratio Measured using ELISA	Percent Breast Density Measured via mammogram	Age, BMI, and reproductive factors	Spearman's partial correlation coefficients	<u>Premenopausal</u> r (p value) IGF-I= 0.11 (0.06) IGFBP-3= 0.15 (0.02) IGF-I: IGFBP-3 ratio= 0.13 (0.03)
Boyd et al, 2002[36]	Cross-sectional	Pre- and postmenopausal women without breast cancer were identified from the mammographic units of St. Michael's Women's College and Mount Sinai Hospitals in Toronto from 1994-1997 • Premenopausal, n=193 • Postmenopausal, n=189	IGF-I, IGFBP-3 Measured using RIA	Percent breast density, dense area Measured via mammogram	Age, waist circumference	Multiple linear regression	<u>Premenopausal</u> β (p value) IGF-I = 0.01 (0.03) IGFBP-3 = -0.02(0.95) <u>Postmenopausal</u> β (p value) IGF-I = 0.004 (0.48) IGFBP-3 = 0.14 (0.57)

Author, year	Study design	Population	Exposure	Outcome	Covariates	Statistical analyses	Main findings
Byrne et al, 2000[34]	Cross-sectional	Pre-and postmenopausal women without breast cancer were identified from the Nurses' Health study • Premenopausal, n= 65 • Postmenopausal, n= 192	IGF-I, IGFBP-3, IGF-I: IGFBP-3 ratio Measured using ELISA	Breast Density Measured via mammogram	Age, BMI, laboratory batch, and alcohol intake	Spearman's partial correlation coefficients	<u>Premenopausal</u> r (p value) IGF-I = 0.36 (0.007) IGFBP-3= -0.24 (0.07) IGF-I: IGFBP-3 ratio= 0.39 (0.004) <u>Postmenopausal</u> r (p value) IGF-I = 0.01 (0.92) IGFBP-3= -0.05 (0.52) IGF-I: IGFBP-3 ratio= -0.02 (0.83)

ELISA: Enzyme linked immunosorbent assay; RIA: Radioimmunoassay; IRMA: Immunoradiometric assay; CLIA: Chemiluminescent immunoassay; BBD: Benign breast disease

Breast cancer treatment modalities

The advancement of treatment modalities has drastically improved survival among breast cancer patients in recent years. Treatment regimens for breast cancer are often multidisciplinary, utilizing both primary and systemic therapies (adjuvant and neoadjuvant therapies).

Primary therapy: There are two types of breast surgeries, mastectomy, which is the surgery to remove the entire breast, and lumpectomy, a surgery to remove a tumor on the breast[176].

Systemic therapies: They are usually administered to breast cancer patients either before, or after surgery.

a) Neoadjuvant therapies are administered before the primary surgery to reduce the tumor size[177]. Possible neoadjuvant therapies include chemotherapy, radiation therapy, and endocrine therapy.

b) Adjuvant therapies are administered after primary surgery to kill cancerous cells and inhibit metastasis[177] to improve prognosis and survival among breast cancer patients. Prognostic factors such as lymph node involvement, tumor size, and predictive factors such as ER status are associated with risk of relapse and mortality[178]. These factors are critical in determining the appropriate adjuvant therapy treatment course. Adjuvant therapies may include radiotherapy (irradiation after mastectomy), cytotoxic chemotherapy, and endocrine therapy[71] which are summarized below.

Chemotherapy

Evidence suggests that adjuvant chemotherapy reduces the rate of recurrence and improves survival among breast cancer patients[71]. Anthracycline-based chemotherapy and taxane-based chemotherapy are the two most active classes of chemotherapeutic agents[179].

Among women aged less than 50 years, 10 years of chemotherapy use is associated with a 35% reduction in recurrence and a 27% reduction in mortality among breast cancer patients[71]. In addition, a 30-70% reduction in CBC risk has been reported among breast cancer patients receiving adjuvant chemotherapy, with or without tamoxifen treatment[93,180,181].

Radiotherapy

Radiotherapy is a process of irradiating tumor cells with high-energy radiation to destroy cancerous cells. Breast cancer is usually treated by two main types of radiation therapy: external beam radiation therapy (EBRT), the most prominent form of radiation therapy, and brachytherapy, also known as internal radiation therapy[182]. EBRT is administered by aiming high-energy radiation to the tumor location from outside the body, whereas brachytherapy is administered from inside the body into the tumor site[182]. If administered before primary surgery (neoadjuvant therapy), radiation is aimed to shrink the tumor. If administered after surgery (adjuvant therapy), its goal is to destroy the remaining tumor cells in the periphery[182]. Radiotherapy post mastectomy is usually recommended for patients with tumor size > 5 cm, and with ≥4 lymph node involvement[71]. When administered after surgery, radiotherapy in combination with chemotherapy prevents local cancer recurrence[177].

Endocrine therapy

Endocrine therapy, also known as hormone therapy, inhibits the action of sex steroid hormones, primarily estrogen, on the breast tumor cells[10]. This treatment course is targeted at lowering the levels of endogenous estrogen, or inhibiting it from binding to the cancer cells[10], slowing or stopping the growth of breast tumors. Endocrine therapy is one of the most effective

treatments for HR-positive tumors. However, due to absence of receptors for the respective hormones, HR-negative cancers do not respond to endocrine therapy.

There are four types of endocrine therapy for breast cancer: selective estrogen receptor modulators (SERMs), aromatase inhibitors (AIs), ovarian function suppression (OFS), and selective estrogen receptor down-regulators (SERDs)[183]. The most widely used endocrine therapies are SERMs, which block estrogen receptors on tumor cells, and AIs, which block the key enzymes required in the synthesis of estrogens[183].

Selective estrogen receptor modulators (SERMs)

Selective estrogen receptor modulators (SERMs) are a diverse group of nonsteroidal compounds that lack the chemical structure of estrogen but possess a structure that allows them to bind to the estrogen receptors typically present in breast and uterine cells[88,184]. Evidence suggests that while estrogen stimulates cancerous growth of the ductal epithelial breast cells, SERMs block estrogen's effect on breast tissues, inhibiting cancerous growth[185]. SERMs act as estrogen antagonists by binding to the breast cells via estrogen receptors (ER). This binding alters the conformation of the ER and interferes with estrogen's ability to bind to these receptors and stimulate the growth of cancerous cells[186], thereby inhibiting the development of breast cancer[185,187]. Although SERMs act as estrogen antagonists for breast tissues, they acts as estrogen agonists for bone and cardiovascular health[187]. The tissue specificity of the SERMs depends on a variety of factors, including the expression of ERs on the surface of the tissues[188].

Despite their many benefits, SERMs have been associated with numerous side effects such as: thromboembolic events, endometrial cancer, increased risk of stroke, hot flashes, etc.[188]. Although the major clinical application of SERMs is the prevention and treatment of breast cancer, its estrogen agonistic properties lend it to use in the prevention of osteoporosis, and

maintenance of beneficial lipid profiles among postmenopausal women[188]. The discovery of SERMs paved a new way in the application of estrogen-based endocrine therapy. Major SERMs are detailed below:

Tamoxifen

Tamoxifen (TAM), a nonsteroidal triphenylethylene compound[188], is the first SERM successfully used in breast cancer prevention and treatment[189] (Figure 2.9). It was approved in 1977 by the US Food and Drug Administration (FDA)[190], and has since been one of the primary choices of endocrine therapy for breast cancer patients[191]. Evidence shows that it not only prevents the incidence of breast cancer but also improves disease prognosis among breast cancer patients[192-194].

Tamoxifen selectively blocks estrogen signaling and inhibits proliferation of ductal cells of the breast[183], both *in vitro* and *in vivo*[193]. The compound acts by intercepting estrogen-ER signaling, thus inhibiting the proliferation of breast tumor cells. The beneficial effect of tamoxifen has been primarily observed among ER-positive breast cancer patients[188]. However, there is inconsistent evidence regarding the effect of tamoxifen on the ER- tumors, where select studies reported a beneficial effect[195] while others observed null associations[196,197]. In addition to its beneficial effects on[198], evidence indicates tamoxifen's protective effect on the reduction of contralateral breast cancer[41]. Animal studies have provided evidence that there are two isomers of tamoxifen, the cis (E)-isomer and the trans (Z)-isomer. The trans (Z) -isomer of tamoxifen is a potent antiestrogen used in breast cancer treatment, while the cis (E)-isomer has no anti-estrogenic actions and pure estrogenic properties[199].

Tamoxifen serves as both an estrogen antagonist and an estrogen agonistic in the bone and uterus. In addition to its role in the prevention and treatment of breast cancer, tamoxifen has

beneficial effects on bone density post menopause, lipid metabolism, and total cholesterol levels due to its estrogenic properties[71,187].

Pharmacokinetics

Tamoxifen is usually prescribed as an oral dose in the form of tamoxifen citrate. A single dose of tamoxifen reaches its highest concentration in serum at about 40 ng/ml approximately 5 hours after the dose[200]. It has a long half-life of 5-7 days that causes the serum levels to differ after initial dosage between continuous users. The standard dosage of tamoxifen as an endocrine therapy approved by FDA is 20 mg/day for 5-10 years[177]. It is clinically recommended for both pre- and postmenopausal ER positive breast cancer patients[183].

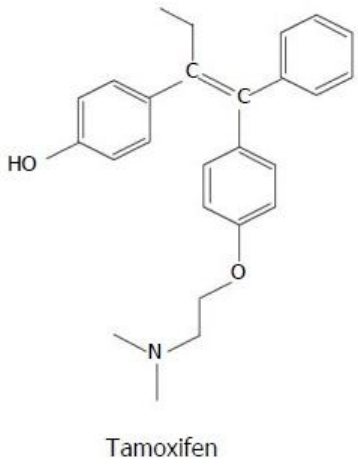

Figure 2.9: Chemical structure of tamoxifen

Source: Adapted with permission from "Current medical treatment of estrogen receptor-positive breast cancer". Author: Franco Lumachi et al. Date: August 26, 2015 [183]. Permission Obtained On: July 24, 2020(https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4549764/)

Tamoxifen metabolism

Tamoxifen is metabolized in the liver by enzymes in the cytochrome P450 (CYP) family into its respective metabolites[201] (Figure 2.10). There are two principal routes of tamoxifen

metabolism, the 4-hydroxylation pathway, and the progressive degradation of the dimethyaminoethane side chain[202]. Metabolism of tamoxifen leads to synthesis of its active metabolites, including N-desmethyl-tamoxifen, 4-hydroxy-N-desmethyl-tamoxifen (endoxifen) and 4-hydroxy-tamoxifen (4-OHT)[203]. Tamoxifen is catalyzed to N-desmethyl-tamoxifen and 4-hydroxy tamoxifen by the cytochrome P450 (CYP) enzymes, 2C19, 2D6, 3A5[204]. N-desmethyl-tamoxifen is further metabolized to endoxifen by the CYP2D6 enzyme, while 4-hydroxy tamoxifen is metabolized to endoxifen via the enzyme CYP3A4/5[203]. The biologic effects of tamoxifen and its metabolites are mostly similar, only differentiated through the increased potency of hydroxylated forms[199]. Research indicates that the hydroxylated derivatives of tamoxifen, endoxifen and 4-OHT have far greater binding affinity towards the estrogen receptors (ER) and are more potent in suppressing tumor growth, compared to tamoxifen in its original form [199,205,206]. The plasma concentration of endoxifen is 5-10-fold higher than that of 4-OHT. This concentration coupled with a high affinity for estrogen receptors makes endoxifen the most clinically relevant tamoxifen metabolite among active metabolites of tamoxifen[207-210].

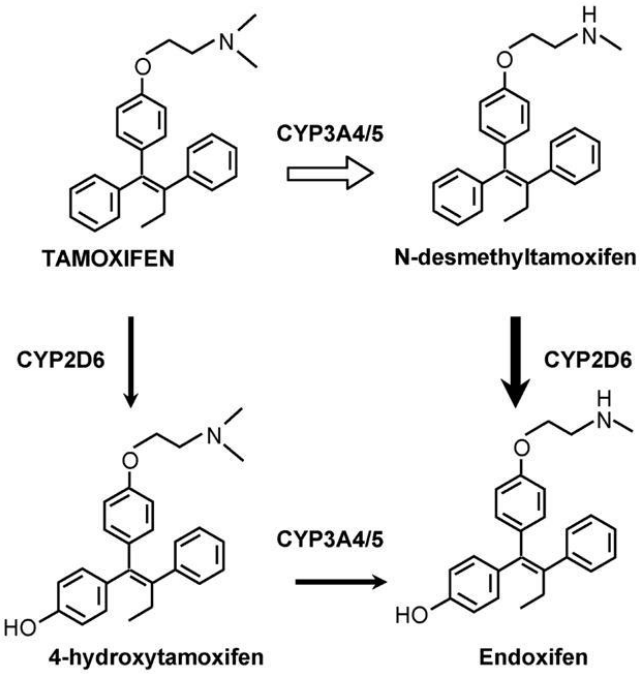

Figure 2.10: Tamoxifen metabolism

Source: Adapted from "Author: V. Craig Jordan. Publication: Steroids. Publisher: Elsevier. Date: November 2007 (License no: 5312651260376; Date obtained: May 19, 2022).
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2740485/

Tamoxifen and endogenous estrogens

Tamoxifen's mechanism of action is mediated by its anti-estrogenic activity, competitive inhibition of estrogen binding to its receptors (ER), displacement of estrogen, and inhibition of estrogen's function in breast tissue[211]. The association of tamoxifen with breast cancer via estrogen signaling and the relationship between endogenous estrogen with breast cancer risk, points towards a mechanistic association between tamoxifen use and endogenous estrogen levels. However, there are limited studies investigating this association and their results are somewhat inconsistent. To date, eight studies have examined the effect of tamoxifen therapy on levels of estrogen (Table 2.9). Of the eight studies, three were conducted among postmenopausal patients

only, three among premenopausal patients only, and the remaining among both pre and postmenopausal women. Sample sizes across these studies ranged from n=5 to n=93 (Table 2.9). Among the studies that examined this association, Lum et al.[212] and Lenning et al.[213] were the most detailed and reported crucial findings. In the study by Lum et al., the effect of tamoxifen was studied among both pre- and postmenopausal women. Estradiol levels significantly increased post tamoxifen use among all women, but when stratified by menopausal status, the effect remained significant only among postmenopausal women[212]. The increase in estrone levels among all women, however, was not statistically significant[212]. In contrast, Lenning et al. reported a decrease in plasma estradiol and estrone levels after tamoxifen use was observed among postmenopausal women [213]. Levin et al. reported a small decrease in circulating parent estrogen levels post tamoxifen use, with no change in urinary estrogen glucuronides[214]. Evidence from prior studies among premenopausal women points towards a significant increase in serum estrogen levels post tamoxifen use[215,216]. The study by Jordan et al. reported a 3-4 fold increase in serum levels of estradiol and total estrogen among tamoxifen users in premenopausal women[216]. The only study that assessed the relationship between circulating metabolites and estrogens reported positive associations between tamoxifen metabolites and endogenous parent estrogen levels, among postmenopausal women[204]. These findings indicate the plausibility of tamoxifen modulating circulating estrogen levels. However, limited studies have examined this association, and no known studies have evaluated the effect of tamoxifen or its metabolites on estrogen metabolism, making this a crucial research area to be investigated further.

Table 2.9: Tamoxifen and its association with endogenous estrogen levels

	Author, year	Study population	Study design/ sampling procedure	Exposure measurement	Outcome measurement	Methods	Findings
Observational studies							
1	J Gjerde et al 2010[204]	-Post-menopausal women treated with TAM 20 mg daily -Postmenopausal status determined by FSH levels >20 IU/L and E2 levels <70 pmol/L -N=90 -Population taken from a study of both pre- and post-menopausal women Inclusion criteria: -Patients treated for at least 80 days	-Cross sectional study -Frozen whole blood/serum sample	-Genotypes of CYP P450 enzymes and SULT1A, and FSH and SHBG -TAM use *TAM use was not treated as the main exposure variable -Median duration of TAM treatment: 571 days	**Estrogens** -Estradiol (E2) -Estrone (E1) -Estrone sulfate (E1S) -Radioimmunoassay **Tamoxifen** -Tamoxifen citrate -4OHtam - the internal standard deuterated5-tamoxifen (D_5tam) -4OHNDtam -NDtam -NDDtam -TamNox -Measured by LC-MS/MS	Spearman rank correlations	-Significant positive correlations between serum estrogen with TAM and its non-hydroxylated metabolites -TAM and NDDtam were correlated with E1 and E2, and tamNox with E1 and E1S -Genotype predicted CYP2C19 was associated with both tamoxifen metabolites and estrone
2	P.E. Lenning et al 1995[213]	-Postmenopausal patients receiving tamoxifen therapy (30 mg) -N=32 -Median age = 64 years (range 49-79 years)	-Cross sectional study -Serum and urine samples. -Blood samples	-Tamoxifen and its major metabolites -Measured by HPLC	-Plasma E1, E1S, and E2. -Urine estrogen metabolites using GC-MS method -Determined by radioimmunoassay	Pearson correlations	-Plasma E2 significantly decreased (geomean 18.9 pM to 16.6 pM) -Plasma E1S significantly elevated (geomean 424.5 pM to 501.7 pM)

Author, year	Study population	Study design/ sampling procedure	Exposure measurement	Outcome measurement	Methods	Findings
	-No other ET or drugs to influence hormone disposition	-Collected before tamoxifen and following 3-12 months on treatment -24-hour urine collected from 10 patients before TAM initiation and once during treatment				-Nonsignificant decreases of E1 (geomean 74.4 pM to 70.9 pM) -Plasma E1S/E1 ratio (5.7 to 7.17 pM) and E1S/E2 ratio (22.3 to 30.0 pM) significantly increased -Nonsignificant decrease in plasma E1 -Significant increase in plasma E1/E2 ratio (3.88 to 4.18 pM). -Nonsignificant reductions in urinary catechol EM excretion, and slight increase in other EM excretion. -No change in total urinary estrogen excretion (33.9 to 35.8 nM). - Significant reduction in the ratio of urinary 2OHE1, to E1 (0.97 nM to 0.60 nM). -Nonsignificant reduction in the ratio of total 2-OH to: 16a-OH urinary estrogens

	Author, year	Study population	Study design/ sampling procedure	Exposure measurement	Outcome measurement	Methods	Findings
3	V Craig Jordan et al. 1991[216]	-Premenopausal BC patients with stage I-II BC treated with adjuvant TAM use as single agent. -Aged 41-47 years Control: Healthy premenopausal 24-45 years of age N=12	-Case Control study	-TAM 10 mg/twice daily. -4-72 months' post mastectomy. -Serum levels of TAM, 4-OHTAM, N-Destam. -HPLC	-LH, FSH, prolactin, and SHBG -Estradiol, total estrogen (estradiol plus estrone), and progesterone -Radioimmunoassay		-Estradiol, estrone, and progesterone increased 1-3-fold among TAM users. - 4-5-fold increase in total estrogen levels among TAM users. - 3-fold increase in estradiol levels
Clinical Trials							
1	S Lum et al. 1997[212]	-BC patients treated with long term TAM therapy from 1984-1993 -Mean age 60 years (range 30-83) -N=47 (41 postmenopausal, 3 premenopausal)	-Clinical trial - Serum samples collected for estrogens	-Tamoxifen 10 mg daily (twice a day) - Follow-up period 2 years (at 3 months interval)	-Estrone -Estradiol -DHEA -Measured by HPLC and radioimmunoassay - Baseline concentrations compared to peak hormone concentrations	-Paired T-test. -Stratified by menopausal status	-Serum E1 levels increased by 264% from mean of 42 ± 94 pg/ml to 153 ± 422 pg/ml post TAM therapy. Diff not significant -Serum E2 levels increased 239% from mean of 28 ± 33 pg/ml to 95 ± 247 pg/ml post TAM therapy. Diff was significant -Among premenopausal patients, the increase in E1 (mean of 172 ± 271 pg/ml to 345 ± 452 pg/ml) and E2 (mean of 94 ± 60 pg/ml to 493 ± 574 pg/ml) was not significant

	Author, year	Study population	Study design/ sampling procedure	Exposure measurement	Outcome measurement	Methods	Findings
							-Among postmenopausal patients, the increase in E1 (mean of 26 ± 13 pg/ml to 129 ± 418 pg/ml) was not significant while the increase in E2 (mean of 18 ± 6 pg/ml to 37 ± 43 pg/ml) was significant
2	R Pommier et al 1987[217]	-BC patients receiving TAM citrate therapy -N=15	-Clinical Trial	-Tamoxifen therapy	-Serum levels of estrone (E1), estradiol (E2), DHEA -BC progression		-Discontinuation of TAM reduces E1 and E2 by 49% and 42% -TAM stimulates production of DHEA, the later aromatized to E1 and E2
3	J Levin et al 1982[214]	-Postmenopausal women with metastatic breast cancer -N=12	-Clinical Trial	-Tamoxifen therapy	- Plasma estrone -Plasma Estradiol -Urinary estrone glucuronides -Urinary estradiol glucuronides Urinary estriol glucuronides	*Info not found	-No change in urinary excretion of estrogen glucuronides - Small but consistent decrease in plasma estrone (47 to 36 pg/ml) and estradiol (15 to 13 pg/ml)
4	Sherman et al 1979[215]	-Premenopausal women aged 28-43. -N= 5	-Clinical Trial - 10 ml blood sample collected on alternate days for 3	-TAM treatment 10 mg twice daily for 2 consecutive cycles. (1st cycle was non-	-Serum E2, P, LH, FSH, TSH, and PRL -Urinary estrone, E2, estriol - Radioimmunoassay	Paired T – test and ANOVA	-During TAM treatment serum E2 and P greatly increased compared to non-treatment cycle -Mean serum E2 levels during TAM treatment

	Author, year	Study population	Study design/ sampling procedure	Exposure measurement	Outcome measurement	Methods	Findings
			consecutive menstrual cycle -Urine sample collected from2 of the 5 participants	treatment of TAM)			(585.9 ng/ml ± 135.1) significantly increased 2-3-fold compared to before treatment (196.5 ng/ml ± 15.4) -Urinary E2 levels markedly increased during TAM treatment as well
5	G V Groom et al 1976[218]	-Six women volunteers	-Clinical Trial	-TAM (orally 20 mg/day)	-Plasma levels of LH, FSH, prolactin, estradiol and progesterone -Determined during two consecutive menstrual cycles		-2-8-fold increase in E2 levels during mid-cycle and mid-luteal hormone peaks

LC-MS/MS= liquid-chromatograph-tandem mass spectrometry, HPLC: high performance liquid chromatography, LH= Luteinizing hormone, FSH= follicle stimulating hormone, SHBG= sex hormone-binding globulin, E2 =estradiol, E1 = estrone, E3= estriol, E1S: Estrone Sulfate, TAM: tamoxifen, 4OHtam: 4-hydroxytamoxifen, 4OHNDtam: 4-hydroxy-N-demethyltamoxifen, NDtam: N-demethyltamoxifen, NDDtam: N-dedimethyltamoxifen, tamNox: tamoxifen-N-oxide, N-Destam: N-desmethyl-tamoxifen

Tamoxifen and risk of CBC

Use of tamoxifen has been consistently associated with reduced risk of contralateral breast cancer among primary breast cancer survivors[47,48,219]. Tamoxifen use has been examined in relation to contralateral breast cancer risk in both observational studies and randomized clinical trials (RCTs) (Appendix A and B), however, the majority of studies conducted to date have been RCTs. Among observational studies, both retrospective cohort[43,45,47,48,50] and case control studies[44,46,49,51] have consistently observed a significant reduction in contralateral breast cancer risk among tamoxifen users ranging from 30%-60% (Appendix A and B). This reduction in CBC risk was observed primarily among women with ER+ primary breast cancer[47,48,50,51,220]. A meta-analysis of 20 clinical trials that examined five years of tamoxifen use compared to no tamoxifen use reported a significant decreased risk of CBC among those with ER+ primary tumors, but not among breast cancer patients with ER- tumors[41]. This finding aligns with the observed mechanism of action of tamoxifen that involves the compound binding to estrogen receptors on ER+ breast tumor cells, blocking the effect of estrogen on the breast cells, and inhibiting cancerous proliferation of the breast tumor. Furthermore, observational studies that examined the association of endocrine therapy with CBC risk by the ER-status of CBC reported that tamoxifen is associated with reduced risk of ER-positive CBC, but not ER-negative CBC[47,48,220].

Aromatase inhibitors (AIs)

Aromatase inhibitors (AIs) are a class of drugs that block the activity of the enzyme aromatase, and thereby reduces estrogen production[221]. Aromatase converts androgens, such as testosterone and androstenedione to the estrogens, estrone and estradiol[183] (Figure 2.10). AIs

prevent the biosynthesis of estrogens from androgens[186], leading to a reduction in estrogen production, and subsequently a reduction of the proliferation of ER-positive cancerous breast cells.

Post-menopause, the production of estrogen in the ovary declines and the majority of estrogen production continues in a number of extragonadal sites such as the adipose tissues of the breast, bone, and some sites in the brain[130,131,222]. Aromatase inhibitors block the conversion of adrenal steroids in the adipose tissue and hence, are effective in the reduction of estrogen levels among postmenopausal women only. AIs function by depleting estrogen among postmenopausal women, but do not inhibit ovarian estrogen production among premenopausal women, which makes them effective treatment for postmenopausal but not for premenopausal women[189]. There are two different classes of aromatase inhibitors: type I steroidal permanent aromatase inhibitor, and type II reversible nonsteroidal aromatase inhibitor (Table 2.10). Type I AIs are testolactone (1st generation), formestane (2nd generation), and exemestane (3rd generation). Type II AIs are aminoglutethimide (1st generation), fadrozole (2nd generation), anastrozole and letrozole (3rd generation)[183] (Figure 2.10 and 2.11). Aromatase Inhibitors (AIs) have been approved by the FDA for use among HR-positive postmenopausal breast cancer patients[183].

Table 2.10: Type I and II aromatase inhibitors (AIs)

Type	Chemical structure	Action	First generation	Second generation	Third generation
I	Steroidal	Permanent	Testolactone	Formestane	Exemestane
II	Nonsteroidal	Reversible	Aminoglutethimide	Fadrozole	Anastrozole Letrozole

Source: Adapted with permission from "Current medical treatment of estrogen receptor-positive breast cancer". Author: Franco Lumachi et al. Date: August 26, 2015 [183]. Permission Obtained On: July 24, 2020 (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4549764

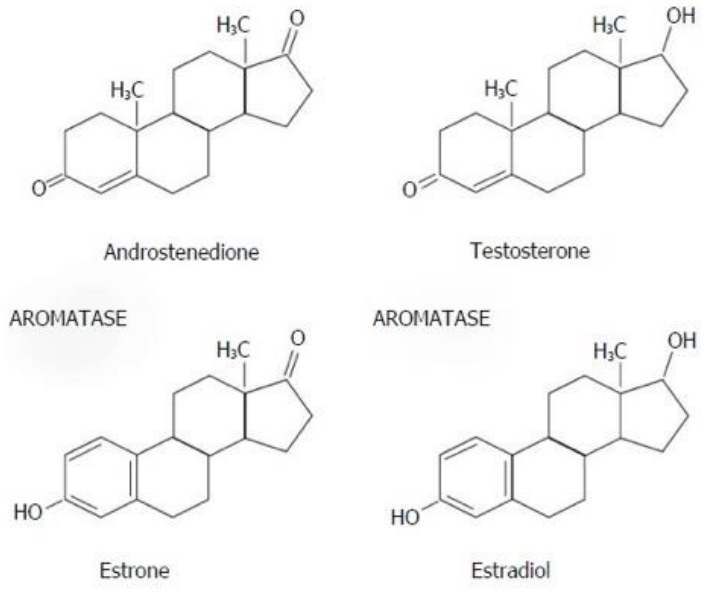

Figure 2.10: Action of the enzyme aromatase on steroid hormones

Source: Adapted with permission from "Current medical treatment of estrogen receptor-positive breast cancer". Author: Franco Lumachi et al. Date: August 26, 2015 [183]. Permission Obtained On: July 24, 2020 (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4549764/)

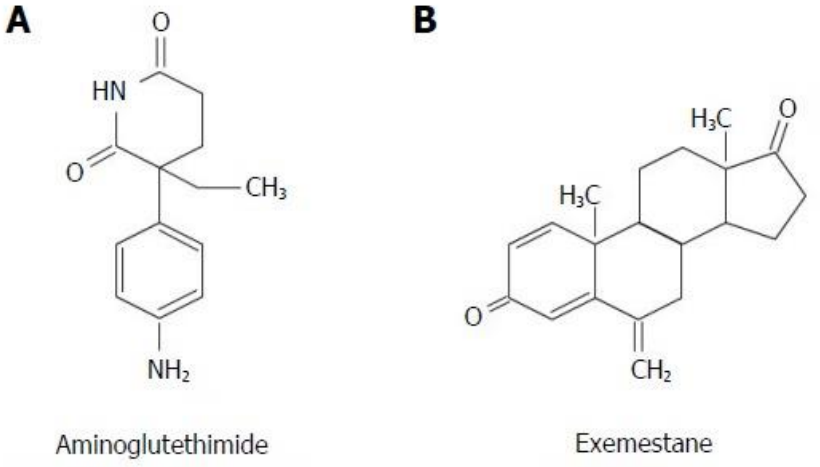

Figure 2.11: Chemical structure of aminoglutethimide and exemestane

Source: Adapted with permission from "Current medical treatment of estrogen receptor-positive breast cancer". Author: Franco Lumachi et al. Date: August 26, 2015 [183]. Permission Obtained On: July 24, 2020 (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4549764/)

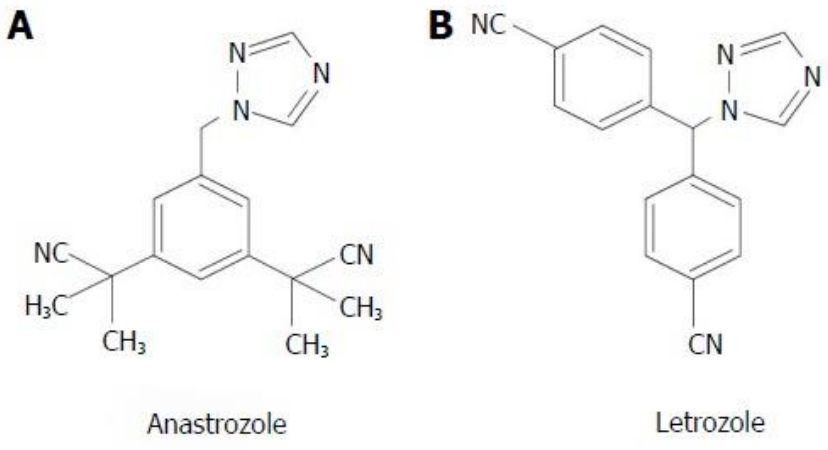

Figure 2.12: Chemical structure of anastrozole and letrozole

Source: Adapted with permission from "Current medical treatment of estrogen receptor-positive breast cancer". Author: Franco Lumachi et al. Date: August 26, 2015 [183]. Permission Obtained On: July 24, 2020 (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4549764/)

Aromatase inhibitors and risk of CBC

Aromatase Inhibitors, the most common endocrine therapy among postmenopausal women, have been assessed in relation to contralateral breast cancer risk, primarily by clinical trials[223]. Prior literature indicates that AI use is associated with reduced CBC risk when compared to non-users of endocrine therapy[47] as well as when compared to tamoxifen users[48,224-227]. Both clinical trials[224-226,228-230] and observational studies[47,48] have investigated the association of AIs and CBC risk. AI use has been consistently reported to be associated with an approximately 40-60% reduction in CBC risk, both in observational studies and clinical trials (Appendix A and B).

Chapter 3: Methods

Aims 1 and 2: Ultrasound Study of Tamoxifen

Study population

The women included in the study populations for aims 1 and 2 were drawn from the Ultrasound Study of Tamoxifen[53,104], an observational study aimed at assessing changes in breast density, measured using an ultrasound tomogram (UST), among tamoxifen treated and untreated women. In brief, women who were at high risk or diagnosed with breast cancer and referred to tamoxifen therapy (n=82), and a matched comparison group (n=165) who screened negative for breast cancer, were identified from the Henry Ford Health Systems (HFHS) and Barbara Ann Karmanos Cancer Institute (KCI) in Detroit, MI[53,54]. Participants were ineligible for enrollment in the study if they: i) weighed ≥350 lbs., ii) were pregnant or lactating, iii) had active skin infections lesions in the breast or open chest wounds, or iv) had a breast size ≥22 cm in diameter (due to ultrasound transducer size limitations). Women with breast implants or those taking endogenous hormones (menopausal hormone therapy or oral contraceptives) were also ineligible for enrollment, the latter due to its modulating effects on breast density[231].

Selection of cases (tamoxifen-treated women): Women who had elevated breast cancer risk, defined by a 5 year predicted risk of ≥1.66% according to Gail model[232] or were diagnosed with incident unilateral ductal carcinoma in situ (DCIS), lobular carcinoma in situ (LCIS), atypical lobular or ductal hyperplasia (ALH/ADH), invasive ER-positive breast cancer and referred to tamoxifen therapy, were selected as cases. Breast cancer patients diagnosed with bilateral

invasive breast cancer or contralateral breast cancer were ineligible for enrollment in the study as no breast without cancer would be available for UST scan.

Selection of comparison group (untreated women): Women who had no personal history of breast cancer and were identified through routine mammography screening at KCI or HFHS with a recommendation to continue screening (i.e., Breast Imaging Reporting and Data System (BI-RADS) diagnostic score of "1" or "2") were selected as the comparison group. Women who were not taking tamoxifen or raloxifene to lower breast cancer risk and did not receive medication or radiation for any cancer were eligible for enrollment in this group. Women in the comparison group were frequency matched to the breast cancer cases on age, race, and menopausal status.

Study design

Aim 1

In a longitudinal analysis, temporal changes in serum concentrations of parent estrogens and EMs from baseline (prior to tamoxifen initiation) to 12 months following tamoxifen initiation were evaluated among postmenopausal tamoxifen-treated women. Additionally, cross-sectional associations between the metabolites of tamoxifen and estrogen were assessed by menopausal status, 12 months after tamoxifen initiation.

Aim 2

A longitudinal analysis was conducted to explore the association between changes in circulating IGFs and concomitant changes in breast density among tamoxifen-treated breast cancer cases, 12 months following treatment initiation. The associations between baseline measures (prior to treatment initiation) of circulating IGFs and breast density were evaluated in a

cross-sectional analysis, among tamoxifen-treated breast cancer cases and the untreated comparison group. A supplemental case-control study was conducted to assess the association between circulating IGFs and the odds of breast cancer, prior to tamoxifen initiation (Appendix C).

Measurement of breast density

In the Ultrasound Study of Tamoxifen, the novel UST scanner was used to measure VASS (m/s), a quantitative measure of volumetric breast density using previously described methods[53,54,233]. Among breast cancer cases, breast density was measured in the contralateral (healthy) breast to avoid any influence of the treatment on sound speed measures[234]. Among the comparison group, a breast was randomly selected for the UST scan, as concurrent measurements of breast density of the left and right breast from the same individual are highly correlated[235]. VASS measures were estimated by calculating the average pixel counts of the common volume measures in each tomogram in meters per second (m/s)[54]. To analyze the changes in VASS 12 months following tamoxifen initiation, the sound speed images were restricted to a common volume contained within the scans, prior to and 12 months after tamoxifen use[54].

Strengths

In contrast to mammography, the most widely used breast screening technique, UST directly assesses physical breast density using sound speed measures without exposure to harmful ionizing radiations, allowing for repeated screenings to monitor changes in breast density over time without increasing breast cancer risk[60]. This is especially important, since breast density, a potential intermediate marker of breast cancer risk, is modulated by

antiestrogenic breast cancer treatments such as tamoxifen, and repeat measurements may provide insights into the biomechanisms of tamoxifen mediated density declines in breast cancer risk and progression[24,27-29,236]. Additionally, the UST technique has high reliability for both measurement of VASS at a single time point measurements (ICC =93.4%) and for changes in VASS over time (ICC =70.4%) [54]. Prior literature suggests that sound speed breast density measures are strongly associated with mammographic density measures[57-59,104]. This strong association indicates that VASS measures may be considered as a quantitative surrogate marker of mammographic breast density, and is more accurate and prone to fewer errors compared to mammography[57-59].

Sample collection and laboratory assays

Blood samples were collected at the baseline visit (prior to tamoxifen initiation) and 12 months following tamoxifen treatment by a trained phlebotomist at KCI. The blood specimens for this study were collected in two 10 ml tubes to measure circulating hormones and growth factors among all women, and tamoxifen metabolites among the tamoxifen-treated women. All the containers were labeled with a participant ID and a biospecimen inventory ID, unique to each participant, and up to two serum samples were collected at each visit (baseline and 12-month follow-up visit). After blood collection, the tubes were kept at room temperature for 30-60 minutes to allow the formation of a blood clot. De-identified blood samples labeled with unique codes were processed as serum using standard operating procedures, stored at -80℃ at the KCI pharmacy core laboratory and then shipped to the National Cancer Institute (NCI) biorepository on dry ice and stored until further analyses were conducted.

Liquid chromatography-tandem mass spectrometry

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to measure a comprehensive panel of 15 EMs across three different estrogen metabolism pathways in serial serum samples, prior to and 12 months after tamoxifen treatment, and four circulating tamoxifen metabolites 12 months after treatment initiation, among pre-and postmenopausal women (aim 1). Evidence from prior literature indicates that estrogen metabolism plays a key role in breast carcinogenesis[15]. However, until recently, the role of individual EMs and their respective mechanistic pathways in breast cancer development was largely unexplored due to the absence of a robust and reliable analytic technique to simultaneously measure all 15 EMs in circulation. Prior studies have used either radioimmunoassay (RIA) or enzyme-linked immunoassay (ELISA) to measure limited endogenous EMs[56], mainly 2-hydroxyestrone (2-OHE1) and 16α-hydroxyestrone (16α-OH-E1). Although these assays were efficient in processing serum samples at a comparatively low cost, they had limitations. Both RIA and ELISA have limited sensitivity, specificity, and reproducibility to accurately measure low estrogen concentrations, of particular importance among postmenopausal women[55,56]. Since each metabolite was assayed independently, a considerable quantity of the serum sample was required and resulted in a substantial cost per sample[56]. Given the availability of the RIA and ELISA assays to measure 2-OHE1 and 16α-OH-E1 coupled with evidence from laboratory studies that indicated that the ratio of 2-OHE1: 16α-OH-E1 is a biomarker of reduced risk of breast cancer[237-240], prior epidemiological studies mainly focused on examining these EMs in relation to breast cancer risk[12,17,140,143,145,241]. The development of the LC-MS/MS assay facilitated the simultaneous measurement of a comprehensive panel of fifteen EMs[55,242] in population-based studies.

The LC-MS/MS is a highly sensitive and specific assay that quantitates EMs across three different (2-OH, 4-OH, and 16-OH) pathways in both serum and urine, and allows accurate measurement of low estrogen concentrations, common among postmenopausal women[55,56]. Although the concentrations of EMs quantified by RIA and ELISA moderately correlate with LC-MS/MS measures among premenopausal women, the association is weaker among postmenopausal women[56,243]. The ability of LC-MS/MS to detect lower levels of circulating EMs among postmenopausal women is crucial in the study of estrogen-dependent diseases such as breast cancer, cardiovascular diseases, and osteoporosis[56]. Importantly, the LC-MS/MS assay has high laboratory reproducibility and an excellent ability to detect interindividual variation in circulating levels of EMs[244], and is often considered the gold standard for the quantification of endogenous estrogens and its metabolites[55]. The accuracy and precision of the LC-MS/MS assay is determined by using replicate aliquots of standardized quality control samples assessed in multiple batches. Assay accuracy is measured as the percent recovery of the known added amount of the EMs[55]. Intra-and inter-batch precision of the assay is estimated by between and within batch coefficients of variation (CV), respectively[55]. The interindividual variability compared to within laboratory variations in analyte measurements is determined by the interclass correlation coefficient (ICC)[244,245]. An ICC close to 1 (100%) indicates that assay variability is small relative to between-person variability and the assay can efficiently discriminate the variability between individuals. Using the LC-MS/MS assay, the CVs (inter-and intra-batch) and ICC were measured to be <2% and >99%, respectively for all serum EMs among pre-and postmenopausal women[244]. Although LC-MS/MS is a robust analytical method and has several strengths, it is an expensive technique with high maintenance and operational costs that relies on highly trained personnel[246].

Enzyme linked immunoassay (ELISA)

In aim 2, IGFs were measured using the enzyme linked immunoassay (ELISA) which has high sensitivity and specificity to measure IGF concentrations in serum[247]. ELISA is a simple and highly efficient cost-effective assay that does not require pre-treatment of the samples. However, there is a possibility of false positive/negative result that may affect the measurement of the analytes[248].

Assessment of potential biases

Information bias

In aims 1 and 2, the exposures (tamoxifen metabolites and IGFs) and outcomes (EMs and VASS) were obtained based on objective measures. The metabolites of estrogen and tamoxifen were measured using the LC-MS/MS assay (aim 1), while the circulating IGFs and VASS measures were estimated using ELISA and the ultrasound tomography (UST) scanner (aim 2), respectively. Despite the strengths highlighted above for each of these techniques, limitations remain and may introduce information bias.

Certain methodological complexities are inherent in molecular epidemiologic studies evaluating biomarkers that may lead to measurement errors. Error may stem from sample collection or storage, laboratory variability, and/or biological variability.

Collection and storage of samples

In most molecular epidemiologic studies, the same biological sample is used to conduct different analyses over an extended period, therefore, any variation in sample collection,

processing, and storage profoundly affects the results of the biomarker-based analyses. Long-term storage of samples and repeated freeze-thaw cycles are two of the most important factors that may adversely affect the stability of biomarkers and influence the results of the assay[249]. Methodological studies pertaining to estrogen have yielded inconsistent findings with regards to storage[250-252]. Although one study indicated that the storage duration does not alter the levels of estrogen in circulation[250], a few studies have suggested that levels of sex steroid hormones, including estrogen, may change during long term sample storage[251-254]. Prior literature has also indicated that IGFs (IGF-I and IGFBP-3) levels decrease with storage time[252,255]. The storage duration of serum samples in the Ultrasound Study of Tamoxifen, defined as the time since blood collection and measurement of the metabolites may have affected the circulating hormone (EMs, IGFs) levels and led to measurement error. Serum samples were collected at baseline and 12-month follow-up visits over a period of 3-4 years from 2011-2014 and 2012-2015, respectively. The average storage duration of the baseline samples was 6.5 years, while that of the 12-month follow-up samples was 5.5 years. Although the period of blood collection (and subsequently, storage duration) varied slightly given the three year window of blood collection, any storage effects would likely be nondifferential. Although evidence suggests that tamoxifen metabolites, the exposure, are quite stable in serum during both short and long-term storage[256], the difference in timing of blood collection and metabolites measurement may influence the concentrations of EMs, the outcome (aim 1). Tamoxifen metabolites (the exposure) and EMs (the outcome) were measured in different time period (years), and this would translate to different storage duration for each metabolite. Due to differences in the duration of storage between the metabolites of estrogen and tamoxifen, it was not possible to account for storage duration as a single variable in the aim 1 analyses. Using simple linear regression, year of blood collection at 12-month follow-

up was used as a proxy for storage duration and assessed as a confounder in the cross-sectional associations between the metabolites of tamoxifen and estrogen. Year of blood collection was not associated with tamoxifen metabolites (exposure) or EMs (outcome) among pre-or postmenopausal women at a significance level of 0.05 or 0.1. Hence, blood collection year (proxy for storage duration) was not identified as a confounder in the association between the metabolites of tamoxifen and estrogen and was not included in the final models. With regards to Aim 2 analyses, storage duration was not related to the outcome, VASS measures of breast density, and thus, not considered a confounder in the association between concomitant changes in IGFs and breast density.

An additional issue associated with long-term storage is the repeated free-thaw cycles. In the Ultrasound Study of Tamoxifen, the whole blood samples collected from the participants were separated into serum samples and stored in small aliquots in liquid nitrogen at -80°C. When conducting the assays for each of the biomarkers (EMs, tamoxifen metabolites, and IGFs), previously stored samples were first thawed, a smaller volume of the sample was obtained, and the original sample was refrozen for future analyses. Repeated free-thaw cycles may lead to degradation of the analytes, and the levels of metabolites in the subsequent cycles may differ from levels when the sample was thawed for the first time[249]. Although the impact of this variation can be substantial, evidence from prior literature indicates that both circulating estrogen[257,258] and IGFs[259] remain quite stable despite multiple freeze-thaw cycles. Prior evidence also suggests that tamoxifen metabolites are stable in plasma for a minimum of three freeze-thaw cycles[256,260].

Finally, any temperature differences in the freezer where the samples were stored may affect the biomarker level[249]. The serum samples collected as part of the Ultrasound Study of

Tamoxifen were first stored at KCI and then shipped to the NCI repository for long-term storage, and any changes in the temperature between the two locations may have affected the quality of the samples. However, since the samples were stored in liquid nitrogen at -80°C, one of the most consistent storage conditions, the probability of temperature issues affecting the quality of the samples is low.

Laboratory variability

In molecular epidemiology, a group of biological samples is analyzed simultaneously in batches, under a given set of conditions remaining consistent across each batch[249]. Batch effects are sources of variation introduced by non-biological factors in a batch, and these changes can limit the efficacy of biomarker-based analyses[249]. Both intra-and inter-batch variations may mask the true measurement of the samples, irrespective of biological variations, and result in erroneous conclusions. In the LC-MS/MS assay, the samples were measured in batches, and to ensure that the results of the assay were not influenced by batch effects, standardized quality control (QC) serum samples were used in each batch to quantify inter-and intra-batch variations in the measurement of the metabolites. The coefficients of variation (CV) that measures the variability of the biomarker measurement, assesses reliability. In the aim 1 analyses, the inter-and intra-batch coefficients of variation (CV) of the EMs and tamoxifen metabolites measured using the LC-MS/MS were <3% and ≤4%, respectively, for all metabolites (EMs and tamoxifen metabolites), except for 4-OH tamoxifen which was 20%. The low inter-and intra-batch CVs of the metabolites indicate excellent reliability and precision of the LC-MS/MS assay for measurement of a comprehensive panel of EMs and tamoxifen metabolites. However, the high inter-batch variability of the 4-OH tamoxifen may lead to measurement error, and due to the non-

differential nature of the misclassification may bias findings towards null. In the aim 2 analyses, the IGFs measured using ELISA also had a low inter-and intra-batch variability (<4%), suggesting high reliability of the IGFs measured using the ELISA technique.

Biological variability

The menstrual cycle status of premenopausal women is a pivotal factor that influences the measurement of sex steroid hormones such as estrogen, among the many sources of biological variability that affect the analyses of circulating hormones. Missing menstrual cycle data in the Ultrasound of Tamoxifen is a key limitation of the aim 1 analyses and discussed below:

Limitation of missing menstrual cycle phase data

Menstruation is a cyclic event that is influenced by hormonal changes, where steroid hormones, including estrogen and progesterone levels, undergo substantial fluctuations[261] . The menstrual cycle is characterized by two phases, i) the follicular or proliferative phase, and ii) the luteal or secretory phase. The follicular phase ranges from the first day of the menstrual cycle until the start of the ovulatory period, after which the luteal phase starts and, typically lasts 14 days[261]. Throughout the menstrual cycle, the steroid hormone levels of women undergo continuous changes, with major changes in estrogen and progesterone levels. Early in the follicular phase, the concentrations of both estrogen and progesterone are low. Throughout the follicular phase, the concentration of estrogen increases, reaches a peak in the late follicular phase and drops right before the ovulatory phase. After ovulation, concentrations of both estrogen and progesterone continue to increase and reaches a peak at the mid-luteal phase. Finally, in the late luteal phase levels of both hormones decrease again. Based on the estrogen

and progesterone levels, the phases of the menstrual cycle can be subdivided into, i) early follicular phase (low estrogen and progesterone), ii) late follicular phase (high estrogen and low progesterone), and iii) mid-luteal phase (high estrogen and progesterone)[262].

One key limitation of the aim 1 analyses was the exclusion of premenopausal women from the longitudinal analyses assessing changes in EMs, from baseline to 12 months after tamoxifen initiation. The primary reason for this exclusion was the absence of menstrual cycle phase information at the time of blood collection, pre-and post-tamoxifen therapy. Without controlling for menstrual cycle, the estimated changes in the panel of 15 EMs, after 12 months of treatment, could be due to the differences in the menstrual cycle phase rather than the true effect of tamoxifen.

Biological variability of tamoxifen may also stem from the inter-relationship between the half-life of tamoxifen and differences in the timing of tamoxifen intake among women in the Ultrasound Study of Tamoxifen. The half-life of tamoxifen is 5-7 days, and it reaches its highest concentration in the serum approximately 5 hours after the dose[200,263]. The half-life of tamoxifen metabolites varies and for the standard daily dose of 20 mg[177], circulating endoxifen and N-desmethyltamoxifen have a half-life of 2-3 days and 14 days, respectively[200,263-265]. Hence, the differences in timing between tamoxifen intake and measurement of circulating hormones (EMs and IGFs) among study participants may lead to variability in the findings. It is important to note, however, that the effect of tamoxifen treatment and timing of subsequent blood collection is only relevant for the serum samples collected at the 12-month follow-up, as the baseline measurements were conducted prior to tamoxifen initiation and were unaffected by the timing of tamoxifen intake. At the 12-month follow-up visit, serum samples were collected from 9 am - 5 pm and 8.40 am - 4.19 pm among pre-and postmenopausal women, respectively. Although the

exact timing of tamoxifen intake among women in the study population was not known, patients are generally advised to take tamoxifen at the same time each day[266]. Hence, it is assumed that any variability and subsequent measurement error would be minimal. However, the timing of intake in relation to tamoxifen half-life and blood collection, cannot be dismissed a possible source of variability in the levels of EM or IGFs.

An additional factor to be considered while interpreting the findings of aims 1 and 2 is potential differences in the daily dose of tamoxifen prescribed among women in the analytic population. The recommended dose of tamoxifen is 20 mg/day[177], however, it is unknown whether all women in the analytic population were prescribed the same dose by the KCI and HFHS, and hence is noted as a potential limitation of this study.

Selection bias

The participants of the Ultrasound Study of Tamoxifen were recruited from KCI and HCH after routine medical screening, and neither the exposures (tamoxifen metabolites, or IGFs) and, nor the outcomes (EMs, or breast density) influenced the selection of the study participants. Specifically, in the longitudinal analyses, the measured values of the outcomes, EMs (aim 1), and breast density (aim 2) did not influence participant selection and/or retention, limiting selection bias in the findings. Similarly, in the cross-sectional analyses of aims 1 and 2, the exposures and/or outcomes did not affect the selection of the study participants. Selection bias can arise from differential loss to follow-up (attrition), which is common in longitudinal studies, and may have affected the findings of aims 1 and 2. However, it should be noted that within the short follow-up period of 1 year, the proportion of participants lost to follow-up was <10% of the study population, and hence the probability of selection bias from selective loss to follow-up will

be limited. Among the tamoxifen-treated breast cancer cases missing 12-month serum samples (n=7), four women were missing 12-month follow-up data and may have been lost to follow-up. Since the study population consisted of women diagnosed with (aims 1 and 2), and/or at high risk (aim 1) of breast cancer, either recurrence and/or death among breast cancer patients, or detection of breast cancer among the high-risk group may explain loss to follow-up. Depending on the reason for attrition, women who were lost to follow-up may suffer from poorer health compared to those who remained in the study, which may bias the risk estimates. The analytic population for both aims 1 and 2 were derived after excluding participants who were missing data on key variables which could have contributed to selection bias, however, the proportion of missing values in aims 1 and 2 was <10%. Therefore, selection bias due to missing information, if any, was limited. Among the tamoxifen-treated breast cancer cases, women missing 12-month serum samples (n=7) or a baseline UST scan (n=1), were excluded. Twenty-four women (n=24) who were missing IGF measurements were excluded from the untreated comparison group. These women were excluded from the analysis (aims 1 and 2) under the assumption that their serum samples and/or IGF measurements were missing at random and were not associated with any unmeasured factors related to the participant. However, the association between the tamoxifen treatment and EMs (aim 1) and changes in IGFs and changes in breast density after tamoxifen treatment (aim 2) may be different among the excluded women compared to women in the analytic population. In the Ultrasound Study of Tamoxifen, the women in the comparison group were frequency matched to the breast cancer cases, making the groups comparable with respect to the matching factors, which may introduce selection bias. In the supplemental case-control analyses assessing the association between IGFs and the odds of breast cancer, the

matching factors age, race, and menopausal status were included in all models to further limit selection bias (Appendix C).

In addition to the biases described above, temporal ambiguity bias may influence the interpretation of the findings of the cross-sectional analyses assessing the relationship between IGFs and breast density, prior to tamoxifen initiation (aim 2). In presence of this bias, it is possible that the relationship observed may reflect the effect of breast density on IGFs, and not vice-versa. The findings of the analyses would need to be interpreted cautiously in this context.

Confounding bias

Aim 1

Within-person comparisons of EMs, before and after 12 months of tamoxifen initiation, among the same postmenopausal women, reduced the potential for confounding by key participant characteristics, including age, race, BMI, and the reproductive factors (early age at menarche, parity, and age at first birth). Although this approach controlled for the within-person variability, the between-person variability still needs to be considered. Hence, the possibility of residual confounding may exist in the longitudinal relationship between tamoxifen treatment and estrogen metabolism. In the cross-section analyses to assess the association between tamoxifen metabolites and EMs, age and BMI were identified as confounders and further described in the methods described in Chapter 4. As age had a quadratic relationship with the EMs, it was added as a quadratic term to the final model to better control for confounding by age. Additionally, given that EMs are derived from the parent estrogens and that tamoxifen treatment influences the

concentrations of estrone and estradiol[212-216], to account for baseline concentrations of parent estrogens, the sum of estrone and estradiol was added as an adjustment factor to the final models.

An additional important consideration for the Aim 1 analyses is the metabolism of both estrogen and tamoxifen by the CYP P450 enzymes[142,267-271]. Therefore, it was important to consider the role of these enzymes and/or a proxy for enzymatic activity in the cross-sectional associations between the metabolites of tamoxifen and estrogen at the 12-month follow-up visit. However, the absence of data on the CYP 450 enzymes and/or their genotypes in the Ultrasound Study of Tamoxifen limited the ability to account for these enzymes or their proxies. The missing menstrual cycle phase data among premenopausal women was not a limitation in this analysis conducted at a singular time point as it is not associated with tamoxifen metabolites, the exposure, and hence, by definition, was not a confounder in the association.

Aim 2

In this analysis, estimation of the changes in IGFs and changes in VASS among the breast cancer cases, using paired samples before and after tamoxifen treatment, minimized within-person variability of potential confounders. The absence of menstrual cycle phase data likely did not bias the findings of this analysis, as the menstrual cycle phase is not associated with IGFs or breast density. For the association between concomitant changes in IGFs and VASS, confounding was assessed by estimating associations of participant characteristics with mean IGFs and VASS at baseline (cases and comparison group) and changes in IGFs and changes in VASS (cases) 12 months after tamoxifen treatment using t-tests and ANOVA/Kruskal-Wallis tests, as appropriate. Due to the small sample size, variations in levels of changes in VASS and/or changes in IGFs across categories of participant characteristics were

also reviewed. The covariates associated with the exposure (changes in IGFs) and the outcome (changes in VASS) at a significance level of 0.1 were identified as potential confounders. Although the variable race was not associated with baseline VASS and/or IGFs at a significance level of 0.1, evidence from prior literature indicates that race is associated with both the exposure, IGFs[272-274], and the outcome, breast density[275,276]. Therefore, race was included as an adjustment factor in the final models assessing the association between baseline IGFs and VASS. Based on the aforementioned methods, age, race, menopausal status, and BMI were identified as confounders in the association between IGFs and VASS at baseline (prior to tamoxifen initiation), while age, change in BMI, menopausal status, and baseline VASS were identified as confounders in the association between changes in IGFs and concomitant changes in VASS, 12 months following tamoxifen initiation. Additional details of the assessment of confounding are provided in the methods section of Chapters 4 and 5.

Directed acyclic graphs (DAGS)

The directed acyclic graphs presented below depict the inter-relationship between tamoxifen treatment and breast cancer risk factors, breast density and the circulating hormones (EMs and IGFs) (Figure 3.1). The associations between tamoxifen and estrogen metabolism (aim 1), and the effect of tamoxifen on the relationship between changes in IGFs and changes in breast density (aim 2), among tamoxifen-treated women, are presented in Figures 3.2 and 3.3, respectively.

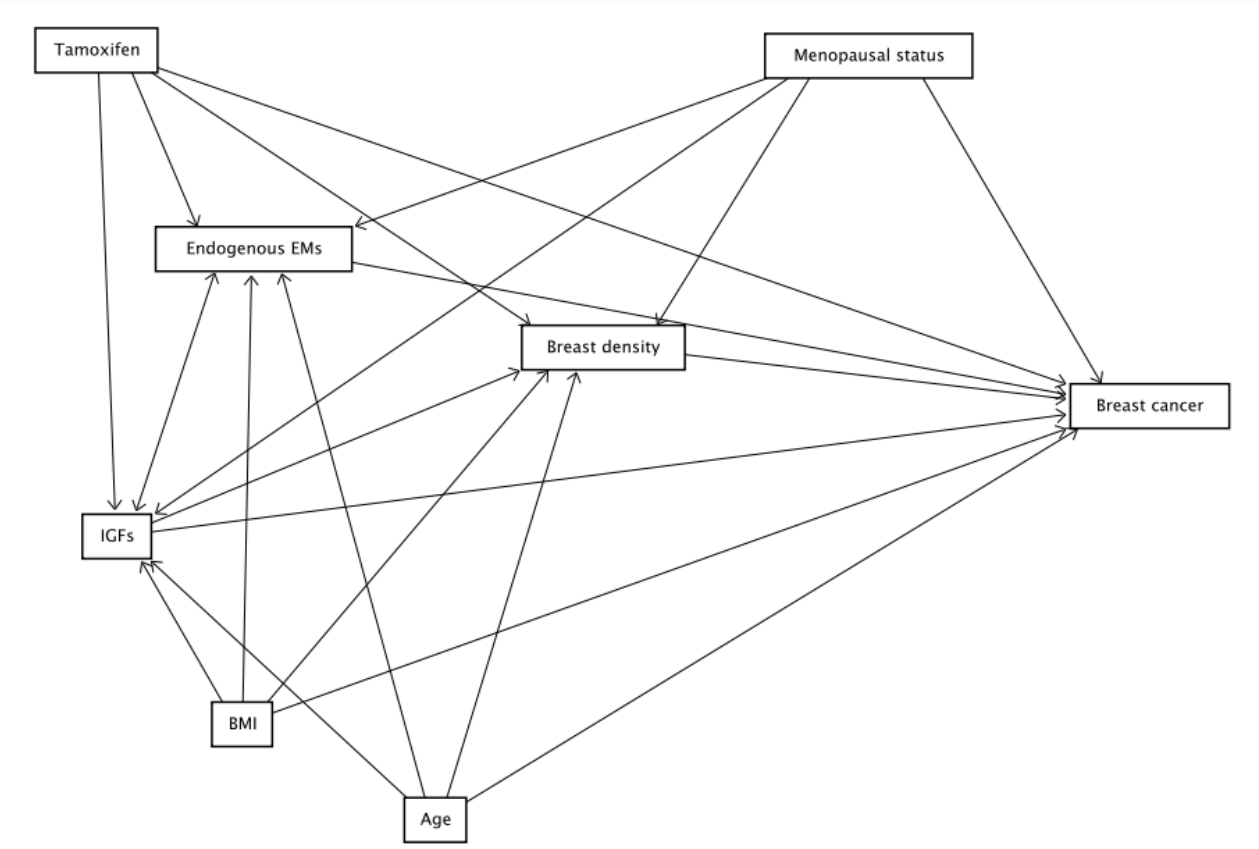

Figure 3.1: Overall associations depicting the inter-relationship between tamoxifen use, circulating hormones (estrogen and IGFs), breast density, and breast cancer

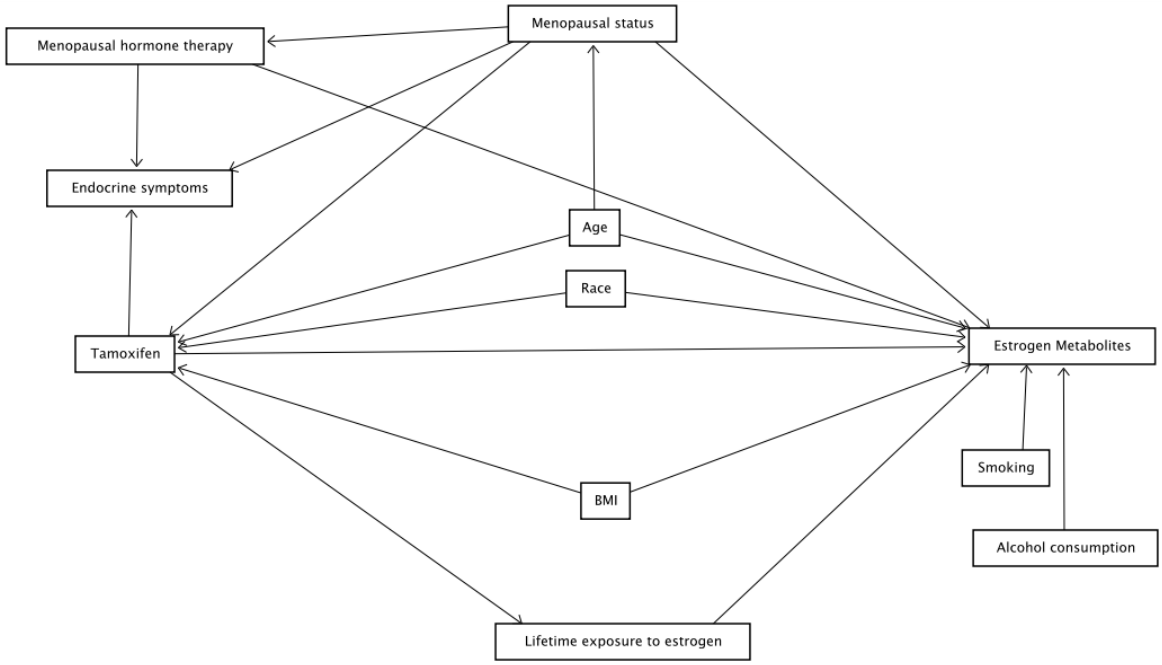

Figure 3.2: Directed acyclic graph of the association between tamoxifen and estrogen metabolism

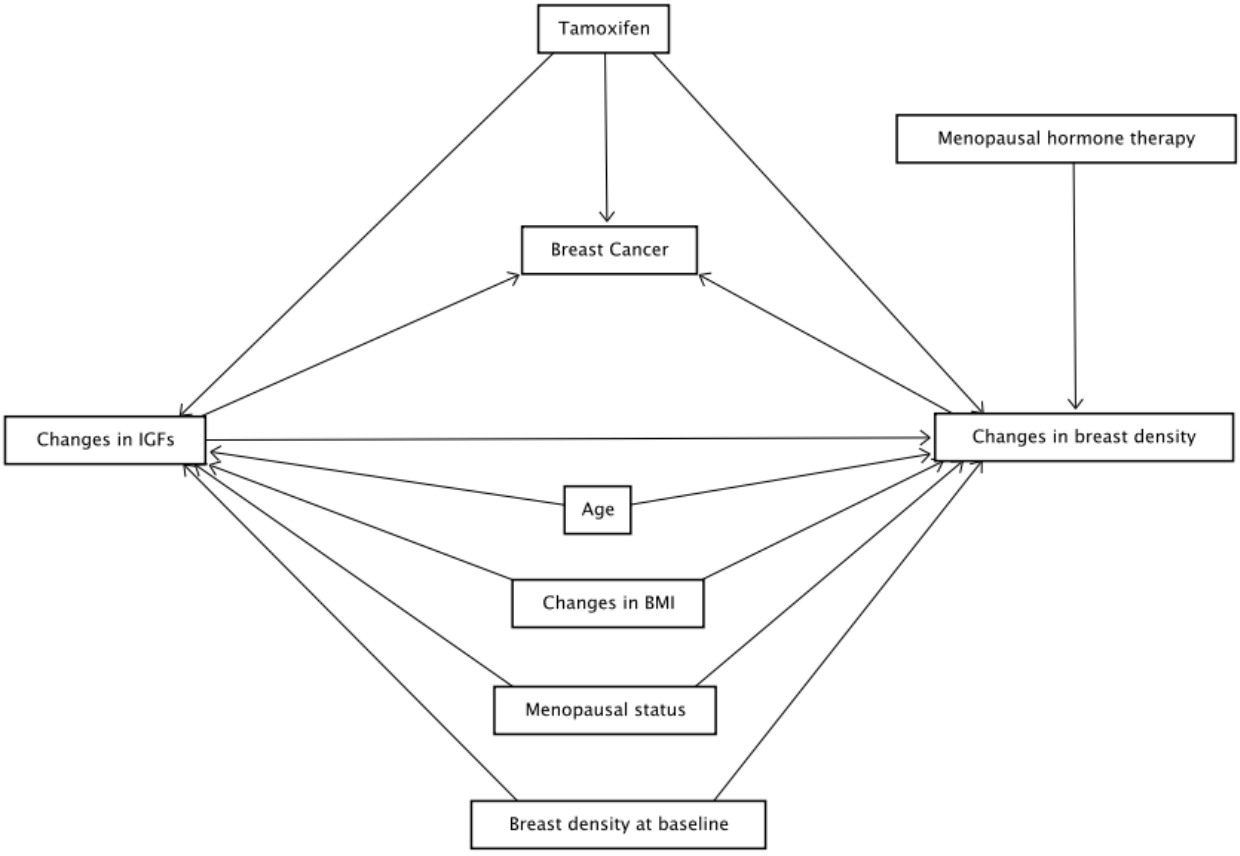

Figure 3.3: Directed acyclic graph of the association between changes in IGFs and changes in breast density, after 12 months of tamoxifen treatment

Statistical analysis

Aim 1

The levels of circulating estrogen metabolites in relation to the concentrations of total EMs among postmenopausal women are presented in Appendix D. Natural-log transformation was applied as the parent estrogens and their metabolites followed a right-skewed distribution. Paired t-tests were used to assess temporal changes in EMs among postmenopausal women 12-months following tamoxifen treatment, and multivariable linear regression was used to assess the relationship between tamoxifen and EMs, stratified by menopausal status at 12 months following treatment initiation. Additional details of the analyses are provided in the methods section of Chapter 4.

Aim 2

Multivariable linear regression analyses were conducted to assess the following: i) the relationship between IGFs and VASS, among tamoxifen-treated breast cancer cases and untreated comparison group, prior to tamoxifen initiation, and ii) to determine if the longitudinal changes in IGFs were associated with concomitant changes in VASS, 12 months after treatment initiation, among tamoxifen-treated breast cancer cases. Additional details of the analyses are provided in the methods section of Chapter 5.

Supplemental analyses (Aim 2)

For the supplemental case-control analysis, t-tests (continuous variables) and Chi-square tests (categorical variables) were used to evaluate the differences in participant characteristics by case-comparison group status (Appendix E). The odds ratios (ORs) and 95% confidence

intervals (CIs) of the association between circulating IGFs and the odds of breast cancer were estimated using multivariable logistic regression models, prior to tamoxifen initiation (Appendix C). The IGFs were assessed both on a continuous scale and in tertiles, based on the distribution among controls.

Power analysis

Aim 1:

Longitudinal change in circulating EMs after 12 months of tamoxifen initiation

A longitudinal study was conducted among n=23 postmenopausal tamoxifen-treated women who were at high risk of, or diagnosed with breast cancer. The null hypothesis was that tamoxifen treatment would not influence the circulating levels of EMs, while the alternative hypothesis was that tamoxifen treatment would decrease circulating EM levels after 12 months of follow-up. Using a one-sided paired t-test and fixed sample size, the minimum detectable change in mean circulating EMs levels from baseline to 12 months following tamoxifen treatment was estimated with 80% power at a significance level of 0.05. The power analysis was conducted using the PS software developed by Vanderbilt University[277].

SD	0.5	1.0	1.5	2.0
Minimum detectable change in EMs (pmol/l)	0.31	0.61	0.92	1.22

For the fixed sample size of n=23 postmenopausal women, a minimum decrease ranging from 0.31-1.22 pmol/l of serum EM concentrations (range of standard deviation of paired difference:

0.5-2) from baseline to 12 months following tamoxifen initiation, can be detected with 80% power at a significance level of 0.05.

Cross-sectional association between the metabolites of tamoxifen and estrogen, by menopausal status, at 12 months following tamoxifen initiation

A cross-sectional analysis was conducted among premenopausal (n=33), and postmenopausal (n=27) tamoxifen-treated women, who were at high risk of, or diagnosed with breast cancer, at 12 months following tamoxifen initiation. The null hypothesis was that there would be no association between the metabolites of tamoxifen and estrogen, while the alternative hypothesis was that the metabolites of tamoxifen and estrogen would be differentially associated by menopausal status. The power analysis was conducted using Stata 17.

Premenopausal women

A post hoc power analysis was conducted based on multivariable linear regression assessing associations between tamoxifen metabolites and EMs at 12-month follow-up, adjusted for potential confounders (age, BMI, and baseline parent estrogens). Estimations were based on a sample size of n=33 premenopausal women and R^2 of the full (R^2_{Full}) and reduced model ($R^2_{Reduced}$). For the fixed sample size of n=33 premenopausal women with R^2_{Full} of the association between metabolites of tamoxifen and estrogen ranging from 0.2 to 0.55 and the $R^2_{Reduced}$ ranging from 0.1 to 0.3, the power to detect a change in EMs for every 1 ng/ml increase in tamoxifen metabolite concentrations ranged between 67-98% at a significance level of 0.05.

Postmenopausal women

A post hoc power analysis was conducted based on multivariable linear regression assessing associations between tamoxifen metabolites and EMs at 12-month follow-up, adjusted for potential confounders (age, BMI, and baseline parent estrogens). Estimations were based on a fixed sample size of n=27 postmenopausal women, and R^2 of the full (R^2_{Full}) and reduced model ($R^2_{Reduced}$). For the fixed sample size of n=27 postmenopausal women with R^2_{Full} of the association between metabolites of tamoxifen and estrogen ranging from 0.2 to 0.4 and the $R^2_{Reduced}$ ranging from 0.1 to 0.3, the power to detect a change in EMs for every 1 ng/ml increase in tamoxifen metabolite concentrations ranged between 55-91% at a significance level of 0.05.

Aim 2

Association between changes in IGFs and changes in breast density from baseline to 12 months following tamoxifen treatment

Based on a fixed sample size, power was estimated based on multivariable linear regression analyses to assess the association between changes in circulating levels of IGFs and concomitant changes in breast density from baseline to 12 months after tamoxifen initiation, at a significance level of 0.05. The null hypothesis was that there would be no association between changes in circulating levels of IGFs and changes in breast density, while the alternative hypothesis was that the changes in IGF levels would be significantly associated with concomitant changes in VASS, after 12 months of tamoxifen therapy. The sample size (n=53) of tamoxifen-treated breast cancer cases was fixed based on the data available from the Ultrasound Tamoxifen Study. The power analysis was conducted using Stata 17.

IGF-I

A post hoc power analysis was conducted based on multivariable linear regression assessing associations between concomitant changes in IGF-I and VASS, adjusted for age, menopausal status, BMI change at 12 months, and baseline VASS measures. Estimation was based on a fixed sample size of n=53 tamoxifen-treated breast cancer cases, and R^2 of the full (R^2_{Full}) and reduced model ($R^2_{Reduced}$). For the fixed sample size of n=53 tamoxifen-treated breast cancer cases with R^2_{Full} of the associations of between concomitant changes in IGF-I and VASS of 0.35 and the $R^2_{Reduced}$ of 0.28, the power to detect per ng/ml change in IGF-I for every 1 m/s change in VASS measures was 65%, at a significance level of 0.05.

IGFBP-3

A post hoc power analysis was conducted based on multivariable linear regression assessing associations between concomitant changes in IGFBP-3 and VASS, adjusted for age, menopausal status, BMI change at 12 months, and baseline VASS measures. Estimation was based on a fixed sample size of n=53 tamoxifen-treated breast cancer cases, R^2 of the full (R^2_{Full}) and reduced model ($R^2_{Reduced}$). For the fixed sample size of n=53 tamoxifen-treated breast cancer cases with R^2_{Full} of the associations of between concomitant changes in IGFBP-3 and VASS of 0.31 and the $R^2_{Reduced}$ of 0.25, the power to detect per ng/ml change in IGFBP-3 for every 1 m/s change in VASS measures was 50%, at a significance level of 0.05.

Assessment of model assumptions

The assumptions of the linear regression models for aims 1 and 2 were assessed using the following methods. The assumption of linearity was assessed using scatter plots that indicated a

linear relationship between the metabolites of tamoxifen and estrogen (aim 1) as well as concomitant changes in IGFs and VASS (aim 2). Multicollinearity was assessed using the variance inflation factor (VIF) for each variable in the model[278]. Variables across all models in aims 1 and 2 had VIF <5 and <3, respectively, indicating lack of multicollinearity in the models. Potential outliers were estimated using Cook's Distance and were visually detected using scatter plots with regression lines. There were only a few (1-3) potential outliers in any given model, and upon excluding these observations, there was only a minor change in the slope of the relationship. Since these points represent a very small proportion of the sample size, they were included in the analyses. The assumption of normality was tested using a Q-Q plot, and the residuals did not deviate substantially from normality. The assumption of homogeneity of variance, homoscedasticity was assessed by visual inspection of the residuals plotted against predicted values in a scatter plot, and no specific trends or patterns were observed indicating that the residuals were approximately equally distributed across the regression line.

Aim 3

New Castle Ottawa scale: The Newcastle-Ottawa Scale (NOS) which is an assessment tool for the risk of bias for observational studies is recommended by the Cochrane Handbook for Systematic Reviews[279]. This dissertation used the NOS scale to assess the quality of the 17 eligible observational studies included in the systematic review and meta-analysis (Chapter 6). The scale and the rationale for quality scoring each study is presented in Appendix F.

Chapter 4: The effects of tamoxifen and its metabolites on circulating concentrations of estrogen metabolites among pre- and postmenopausal women

Abstract

Background: Prior studies suggest tamoxifen modulates serum estrogen levels; however, little is known about the influence of tamoxifen on endogenous estrogen metabolism or associations between circulating metabolites of tamoxifen and estrogen.

Methods: Among women referred to undergo clinically-indicated tamoxifen therapy, serum concentrations of 15 estrogens and estrogen metabolites (EMs) were measured at baseline (pre-tamoxifen) and 12 months post-tamoxifen initiation using liquid chromatography-tandem mass spectrometry. At the 12-month follow-up visit, serum concentrations of tamoxifen and its metabolites were measured. EMs were analyzed individually, by metabolic pathways (2-OH, 4-OH, and 16-OH) and as a sum (total EMs). In longitudinal analyses among postmenopausal women (n=23), changes in EMs between baseline and follow-up were assessed with paired t-tests. Cross-sectional associations between four tamoxifen metabolites (tamoxifen, endoxifen, 4-hydroxy-tamoxifen, N-desmethyl tamoxifen (NDT)) and EMs measured at the 12-month follow-up visit were assessed using linear regression models adjusted for age and BMI among pre- (n=33) and postmenopausal women (n=27).

Results: Among postmenopausal women, mean circulating levels of total EMs significantly decreased ($p < 0.05$) between baseline and 12 months post-tamoxifen initiation, a finding that largely seemed to be driven by decreases in levels of 2-OH and 16-OH pathway EMs. Among premenopausal women at the 12-month follow-up, serum levels of tamoxifen

(ng/ml) were positively associated with parent estrogens, estrone (β=1.24, p=0.004) and estradiol (β=1.39, p=0.01), the 2-OH metabolites, 2-OHE1(β=0.72, p=0.04) and 2-ME1 (β=1.15, p=0.01), and metabolites of the 16-OH pathway (p<0.05). Similarly, circulating endoxifen (ng/ml) was positively associated with estrone (β=10.1, p=0.01), estradiol (β=12.4, p=0.01), and select EM metabolites of the 2-OH (2-OHE1, 2-OHE2, 2-ME1) and 16-OH (16α-OH-E1, E3, 16-epiE3, 16-OH EM) pathways (p<0.05). Among postmenopausal women only, NDT was positively associated with the 2-OH pathway metabolite, 3ME1 (p=0.02),

Conclusion: Within the first year of tamoxifen therapy significant reductions in circulating 2- and 16-OH pathway EMs were observed among postmenopausal women. Positive cross-sectional associations of the metabolites of tamoxifen with 2- and 16-OH pathway EMs at 12-month follow-up, provides insight into the biomechanism of tamoxifen and/or its metabolites in breast cancer etiology. Elevated circulating EMs are crucial determinants of breast carcinogenesis, and modulation of EM levels in circulation may represent a marker of tamoxifen-mediated treatment response that varies by menopausal status.

Keywords: Estrogen, estrogen metabolites, tamoxifen, tamoxifen metabolites, menopausal status.

Introduction

Endogenous estrogens are strong risk factors for breast cancer[134,135] and act on target breast cells by binding to the ERs and forming an estrogen-ER complex that induces the proliferation of cancerous cells[18]. Tamoxifen, one of the most widely used endocrine therapies for estrogen receptor (ER)-positive breast tumors[192,193], selectively blocks estrogen signaling and subsequently inhibits the proliferation of breast cells[185,190]. The mechanism of action of tamoxifen, which involves competitive inhibition of estrogen binding to ERs and blocking the formation of the estrogen-ER complex, supports a possible association between tamoxifen use and circulating estrogens.

Recent evidence indicates that the carcinogenic effects of estrogens are not only limited to parent estrogens, estrone and estradiol, but also involve hydroxylated derivatives of the same[14-16]. In addition to ER-mediated tumor cell proliferation, estrogens stimulate breast cancer risk via the genotoxic effects of the EMs that lead to DNA damage and tumor formation[15,16,19]. Estrone and estradiol are irreversibly hydroxylated by cytochrome P450 (CYP) enzymes at the C-2, C-4, or C-16 positions of the steroid ring resulting in corresponding metabolites in the 2-hydroxy (2-OH), 4-hydroxy (4-OH), and 16-hydroxy (16-OH) pathways[142,267]. Prior literature has suggested that the estrogen metabolism pathways are differentially associated with breast cancer risk[237-239]. While elevated concentrations of metabolites of the 2-OH pathway are linked to a reduced risk[237,238], increased 16-OH pathway metabolites are associated with an increased risk of breast cancer[239,280]. Like estrogens, tamoxifen is primarily metabolized by the CYP enzymes into its active metabolites, 4-hydroxy-N-desmethyl-tamoxifen (endoxifen), 4-hydroxy-tamoxifen (4-OHT), and N-desmethyltamoxifen (NDT)[200,268-270]. Research indicates that compared to tamoxifen, the hydroxylated derivatives have a far greater binding affinity towards

the ER [199,205,206], suggesting that the metabolites of tamoxifen play an important role in breast cancer prevention and management.

Despite the plausibility of a mechanistic association between tamoxifen therapy and circulating estrogens, findings are limited and inconsistent. Few previous studies suggest that tamoxifen alters endogenous estrogen levels[212-216], however, evidence varies by menopausal status. While tamoxifen therapy was associated with an increase in the circulating parent estrogens (estrone and estradiol) among premenopausal women[212,215,216], conflicting findings have been reported among postmenopausal women[212-214]. Among postmenopausal tamoxifen-treated women, both a 6-23% decrease[213] and >200% increase in serum parent estrogen levels[212] were observed after 1 and 2 years of treatment, respectively. Although estrogen metabolism may play an important role in breast carcinogenesis [12,125,140,241], little is known about the effect of tamoxifen therapy on a comprehensive panel of fifteen circulating estrogen metabolites (EMs). Furthermore, the importance of the tamoxifen metabolites in estrogen signaling[199,205,206] and the commonality of the cytochrome P450 enzymes that mediate the metabolism of both estrogens and tamoxifen to their respective metabolites[142,267-270] support a possible association between the metabolites of tamoxifen and estrogen. Limited data exists on the influence of tamoxifen treatment on estrogen metabolism, warranting further investigation.

The broader objective of this exploratory analysis was to improve the etiologic understanding of the role of tamoxifen and its metabolites on estrogen metabolism, an important determinant of breast carcinogenesis. This study aimed to (1) evaluate the temporal changes in serum concentrations of parent estrogen and EMs among postmenopausal women, using serum samples taken at baseline (pre-tamoxifen) and 12 months after tamoxifen initiation, and (2)

explore the relationship between the metabolites of tamoxifen and estrogen by menopausal status, at 12 months following tamoxifen initiation.

Methods

Study design and population

The study population for this analysis was drawn from the previously described Ultrasound Study of Tamoxifen[53,104]. In brief, participants included women aged 30-70 years who were at high risk or diagnosed with breast cancer and referred to tamoxifen therapy for clinical indications (tamoxifen-treated), and untreated women who screened negative for breast cancer. Participants were recruited from the Barbara Ann Karmanos Cancer Institute (KCI) and Henry Ford Health Systems (HFHS) in Detroit, Michigan.

For this study, tamoxifen treated women (n=82) diagnosed with (n=65), or at high risk (n=17) of, breast cancer formed the basis of the analytical population summarized in Figure 4.1. Women were excluded if they never started tamoxifen or had missing serum samples at the 12-month visit (n=13). The menstrual cycle phase at the time of blood draw was unknown for premenopausal women (n=46), and subsequently, premenopausal women were excluded from the longitudinal analyses assessing change in circulating levels of EMs 12 months after tamoxifen initiation (Aim 1). For the cross-sectional analyses assessing associations between tamoxifen and estrogen metabolites at 12 months following tamoxifen initiation (Aim 2), women who either discontinued tamoxifen or had no detectable tamoxifen metabolites at 12 months (n=9) were excluded (Figure 4.1). After applying the above exclusions, final analytic populations for the longitudinal and cross-sectional analyses, respectively, consisted of (1) postmenopausal

women at baseline (n=23), and (2) both premenopausal (n=33) and postmenopausal women (n=27) at 12 months following tamoxifen initiation (Figure 1). Informed consent was obtained from all participants and study procedures were approved by the Institutional Review Boards of KCI, HFHS, and the National Cancer Institute (NCI).

Laboratory assays

Serial serum samples collected prior to and 12 months after tamoxifen initiation (Figure S4.1) were stored at -80°C and sent to the Frederick National Laboratory for Cancer Research (Frederick, MD) to measure EMs. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to measure a comprehensive panel of 15 EMs (pmol/l) which included the parent estrogens, estrone (E1) and estradiol (E2), and metabolites in the i) 2-hydroxylation (2-OH) pathway: 2-hydroxyestrone (2-OHE1), 2-hydroxyestradiol (2-OHE2), 2-hydroxyestrone-3-methyl ether (3-ME1), 2-methoxyestrone (2-ME1), and 2-methoxyestradiol (2-ME2); ii) 4-hydroxylation (4-OH) pathway: 4-hydroxyestrone (4-OHE1), 4-methoxyestrone (4-ME1), and 4-methoxyestradiol (4-ME2), and iii) 16-hydroxylation (16-OH) pathway: 16α-hydroxyestrone (16α-OH-E1), estriol (E3), 16-ketoestradiol (16-keto-E2), 16-epiestriol, and 17-epiestriol. Details of the assay have been published previously[55]. Blinded duplicate quality control samples were included within and across batches; all samples were randomized across batches. The coefficients of variation (CV, within and between batches) were <3% for all the estrogen metabolites. The limit of detection of each estrogen metabolite ranged from 0.33-0.37 pmol/L[55,281]; no samples had undetectable levels of EMs.

Concentrations of four tamoxifen metabolites (ng/ml), including tamoxifen, 4-hydroxy-N-desmethyl-tamoxifen (endoxifen), 4-hydroxy-tamoxifen (4-OHT), and N-desmethyltamoxifen (NDT), collected at 12 months following tamoxifen initiation were measured in serum using LC-

MS/MS by the Illinois Institute of Technology Research Institute (IITRI). The limit of quantitation of the tamoxifen metabolites was 5-500 ng/ml for circulating tamoxifen and NDT and 0.5-50 ng/ml for 4-OHT and endoxifen. For 4-OHT, 7 samples had levels below the lower limit of quantitation of 0.5 ng/ml. For these, the concentration was replaced by half of the lower limit of quantitation of 4-OHT. The coefficients of variation were <5% for each of the tamoxifen metabolites, except for 4-OHT (CV=20.7%).

Covariate information

Information on participant characteristics, including demographics, menopausal, and reproductive history, was collected via a standard health questionnaire. Prior to and 12 months after tamoxifen initiation, data on patient experience of endocrine symptoms (hot flashes and joint pain) in the past two weeks prior to the visit (yes/no), alcohol intake in the past 24 hours (yes/no), and smoking status were collected. Current smoking status (yes/no) was determined via responses to two questions, whether they were smokers (yes/no) and the date when last smoked. Height, measured at baseline, and weight, measured at each visit, were used to compute body mass index (BMI, kg/m^2). Women were considered postmenopausal if periods stopped for 12 months or longer due to natural causes, or surgical procedures, such as bilateral oophorectomy. Women aged 55 years or older who had a hysterectomy but not oophorectomy, and whose periods stopped due to endometriosis, uterine ablation, irregular perimenopausal cycles, novasure procedure, medication/drug therapy, or any other reason were classified as postmenopausal. After 12 months following tamoxifen therapy, if in addition to the above, periods stopped for 12 months or longer due to depo shots or tamoxifen use, women were classified as postmenopausal if aged ≥55 years. Four women changed menopausal status (premenopausal to postmenopausal) from the baseline to the 12-month follow-up visit.

Statistical analysis

Distributions of participant characteristics by menopausal status were assessed by estimating frequencies and percentages (categorical variables) as well as means and standard deviations (continuous variables). Among postmenopausal women, paired t-tests (continuous variables) and McNemar's tests (categorical variables) were used to evaluate differences in select characteristics between baseline and 12-month follow-up visits. Parent estrogens and their metabolites were natural-log transformed to account for non-normal right-skewed data. Estrogen metabolites were analyzed individually, and as the sum of all 15 EMs (total EMs), parent estrogens (E1 and E2), and based on metabolic pathways (2-OH, 4-OH, and 16-OH). The metabolic pathways were created by summing the individual metabolites within respective pathways.

Paired t-tests were used to assess the mean change in circulating levels of EMs from baseline to 12 months after tamoxifen initiation. Percent change in circulating EMs, 12-months following treatment initiation, was estimated by the ratio of geometric mean (GM) difference between baseline and 12-month EM levels to baseline EM levels, multiplied by 100. GMs and 95% confidence intervals (CIs) were estimated for the metabolites of estrogen and tamoxifen at 12 months following tamoxifen initiation, by menopausal status. Spearman rank correlation coefficients were used to evaluate the association between EMs, and tamoxifen metabolites, by menopausal status. In cross-sectional analyses, multivariable linear regression was used to estimate slope parameters ("beta coefficients", presented in 100 ng/ml.) and 95% confidence intervals (CIs) for the association between tamoxifen and estrogen metabolites, by menopausal status. The participant characteristics that were associated with both tamoxifen and EM in simple linear regression analyses at significance level of 0.05 were identified as confounders.

Additionally, to account for baseline concentrations of parent estrogens, the sum of estrone and estradiol was added as an adjustment factor. Therefore, age (quadratic), BMI (normal/overweight/obese) at the 12-month follow-up visit, and baseline parent estrogen levels (continuous) were identified as confounders in the relationship between estrogen and tamoxifen metabolites at 12-months following tamoxifen initiation, and were included in the final models. Statistical significance was assessed at α=0.05. As this was an exploratory analysis, multiple testing corrections were not applied. All analyses were conducted using SAS 9.4 (Cary, North Carolina).

Results

Participant characteristics

The demographic and reproductive characteristics of the analytic populations are presented in Tables 4.1 and 4.2. At baseline, postmenopausal women (n=23) were primarily African American (≥60%) breast cancer patients (≥70%), and had a mean (SD) age of 60.7 (6.2) years and BMI of 31.1 (6.9) kg/m^2 (Table 4.1). (Table 4.1). Participant characteristics at 12 months following tamoxifen initiation are presented by menopausal status in Table 4.2. Premenopausal women (n=33) had a mean (SD) age of 47.3 (5.9) years and a BMI of 29.9 (6.6) kg/m^2 , while the average (SD) age and BMI of postmenopausal women (n=27) were 60.5 (6.6) years and 31.2 (7.6) Kg/m^2, respectively (Table 4.2).

Longitudinal change in estrogen and estrogen metabolites (EMs) among postmenopausal women

Among postmenopausal women, the highest concentrations of EMs prior to tamoxifen treatment were observed for circulating estradiol (GM=190.4, 95% CI: 146.2, 248.0) followed by 2-OHE1

(GM=95.0, 95% CI: 78.1, 115.7) and E3 (GM=72.3, 95% CI: 60.2, 86.8). In the 12 months following tamoxifen initiation, serum levels of select EMs either decreased or remained stable among postmenopausal women as compared to baseline measures pre-tamoxifen initiation (n=23). Although most EMs decreased from baseline to 12 months following tamoxifen initiation, statistically significant decreases ranging from 13.8-27% were only observed in the mean concentrations of total EMs (p= 0.03), 2-ME1 (p= 0.001), 2-ME2 (p= 0.02), E3 (p= 0.02), and the 2-OH (p= 0.03) and 16-OH pathway (p= 0.004) EMs from baseline to 12 months after tamoxifen initiation (Table 4.3).

Distribution of estrogen and tamoxifen metabolites at 12 months following tamoxifen initiation

Geometric means (GMs) of the circulating EMs (pmol/L) at 12 months following tamoxifen initiation are presented in Table 4.4. On average, GMs of total estrogens were nearly three-fold higher among pre- (GM=1361.6, 95% CI: 944.1, 1963.8) as compared with postmenopausal (GM=550.7, 95% CI: 487.4, 622.2) women. Among premenopausal women, the GMs were highest for the parent estrogens, E2 (GM=478.5, 95% CI: 314.1, 729.1) and E1 (GM=183.3, 95% CI: 109.2, 307.5), followed by 2-OHE1 (GM=142.7, 95% CI: 101.3, 201.2) and E3 (GM=120.0, 95% CI: 81.4, 176.8). Among postmenopausal women, the highest EM concentrations were observed for E2 (GM=166.4, 95% CI: 133.8, 207.0) followed by 2-OHE1 (GM=81.0, 95% CI: 67.7, 96.9), and E3 (GM=57.9, 95% CI: 47.2, 71.0). The GMs of the concentration of tamoxifen metabolites (ng/ml) among both pre-and postmenopausal women were highest for NDT followed by tamoxifen (Table 4.4). The levels of all tamoxifen metabolites were higher among postmenopausal women compared to premenopausal women (Table 4.4).

Statistically significant positive correlations were observed between most of the EMs among premenopausal women. The strongest correlation was observed between E1 and E2 (r=0.95); of the metabolite pathways, strong correlations were observed between the parent estrogens, E1 and E2 with most metabolites of the 16-OH pathway (16α-OH-E1, E3, 16-keto-E2, and 16-epiE3) ($r \geq 0.70$) and select metabolites of the 2-OH pathway (2-OHE1, 2-ME1) ($r \geq 0.75$) (Table S4.1). Among postmenopausal women, however, fewer and more modest correlations were observed. The strongest correlation was observed between E1 and E2 (r=0.75), followed by those between E2 and 2-OHE1 and E2 (r=0.62), E1 and 2-ME1 (r=0.58), and 2-OHE1 and 2-OHE2 (r=0.58) (Table S4.2). Of the tamoxifen metabolites, the strongest correlation was observed between tamoxifen and NDT among both premenopausal (r= 0.80) and postmenopausal women (r=0.71) (Table S4.3).

Cross-sectional association between the metabolites of tamoxifen and estrogen at 12 months following tamoxifen initiation:

Premenopausal women

Serum tamoxifen concentrations were positively associated with levels of total EM (β=1.02, p= 0.006), parent estrogens (β=1.29, p=0.004), E1 (β=1.24, p=0.004), and E2 (β=1.39, p=0.01) (Figure 4.2, Table S4.4). Significant positive associations were also observed between circulating endoxifen with levels of total EM (β=8.9, p= 0.01), parent estrogens (β=10.6, p=0.01), E1 (β=10.1, p=0.01), and E2 (β=12.4, p=0.01) (Figure 4.3, Table S4.4).

Of the 2-OH pathway metabolites, tamoxifen was positively associated with the levels of 2-OHE1 (β=0.72, p=0.04) and 2-ME1 (β=1.15, p=0.01), while endoxifen was positively associated with the levels of 2-OHE1 (β=6.57, p= 0.04), 2-OHE2 (β=5.82, p= 0.02) and 2-ME1

(β=8.58, p=0.04) (Figure 4.2 and 4.3, Table S4.5a). 4-OHT, a tamoxifen metabolite, was positively associated with the levels of 2-OHE1 (β=37.1, p=0.03), 2-ME1 (β=42.5, p=0.04) and the 2-OH pathway (β=32.8, p=0.04). (Table S4.5a). Among the 4-OH pathway metabolites, a statistically significant association was only observed between the tamoxifen metabolite, 4-OHT and levels of 4ME1 (β=27.9, p=0.01) (Table S4.6). Tamoxifen was also statistically significantly associated with all the 16-OH pathway metabolites. Specifically, tamoxifen was positively associated with the levels of 16α-OH-E1 (β=1.10, p=0.003), E3 (β=0.9, p=0.04), 16-keto-E2 (β=0.84, p=0.02), 16-epiE3 (β=0.78, p=0.04), 17-epiE3 (β=0.74, p=0.04), and the 16-OH pathway (β=0.9, p=0.01) (Figure 4.2, Table S4.7a). Positive associations were also observed between endoxifen and the levels of 16α-OH-E1 (β=7.96, p=0.02)**,** E3 (β=12.6, p=0.0007), 16-epiE3 (β=10.3, p=0.002), and the 16-OH pathway (β=9.0, p=0.003) (Figure 4.3, Table S4.7a). The metabolite 4-OHT was positively associated with levels of E3 (β=50.8, p=0.01) and the 16-OH pathway (β=33.8, p=0.04) (Table S4.7a). Finally, the tamoxifen metabolite, NDT was positively associated with levels of 16α-OH-E1 (β=0.56, p=0.04) only (Table S4.7a).

Postmenopausal women

Among postmenopausal women, positive associations were observed between NDT and levels of 3ME1 (β=0.18, p=0.02) (Table S4.5b). No other cross-sectional associations between the metabolites of tamoxifen and estrogen were detected among postmenopausal women.

Discussion

In this exploratory analysis of the effect of tamoxifen therapy on circulating estrogen metabolites, a decrease in the levels of select metabolites of the 2-OH and 16-OH EM pathways

was observed among postmenopausal women within the first year of treatment. Furthermore, circulating tamoxifen and endoxifen were positively associated with the parent estrogens and select metabolites of the 2-OH pathway (2-OHE1, 2-OHE2, 2-ME1) and all 16-OH pathway metabolites among premenopausal women. No such pattern was noted among postmenopausal women. The findings provide etiologic insight into the biomechanism of tamoxifen's effect on estrogen metabolism, which emphasizes the significance of estrogen metabolism pathways and further highlights a crucial role of tamoxifen metabolites in breast carcinogenesis that varies by menopausal status.

The effectiveness of tamoxifen on breast cancer incidence and progression is mediated via estrogen signaling, and previous studies have reported conflicting associations between tamoxifen use and circulating parent estrogens[212-216]. Among postmenopausal women in our study, we observed non-statistically significant decreases in parent estrogens, estrone and estradiol, 12 months after tamoxifen initiation. Consistent with our findings, Levin et al observed significant decreases in both estrone and estradiol levels[214], and Lønning et al reported a significant decrease in mean estradiol levels (12.1%) but a non-significant decrease in mean estrone levels (6.9%), among postmenopausal breast cancer patients after 12 months of tamoxifen therapy[213]. In contrast, Lum et al. reported a significant increase in mean estradiol levels (239%), and a non-significant increase in mean estrone levels (264%) in circulation, after two years of tamoxifen treatment among breast cancer patients[212]. While our study utilized highly reliable and sensitive LC-MS/MS methods to assess serum EM, these prior studies assessed circulating parent estrogens using radioimmunoassay (RIA)[212-214], lacking both sensitivity and specificity to measure low levels of endogenous estrogens among postmenopausal women[243].

This study explored the association between tamoxifen therapy and a comprehensive panel of fifteen EM in circulation. The LC-MS/MS assay allows concurrent measurement of even low concentrations of EMs across three different pathways (2-OH, 4-OH, 16-OH)[55]. Using this novel and advanced method, it was observed that levels of select circulating EMs declined post-tamoxifen therapy. Although non-statistically significant decreases were observed for most of the individual EM concentrations, statistically significant reductions were observed for the metabolites of the 2-OH (2-ME1, 2-ME2, and sum of 2-OH pathway) and 16-OH (estriol and sum of 16-OH pathway) pathway, following 12 months of tamoxifen treatment. Our data suggests a 13-27% decrease in the mean circulating levels of EMs, with the greatest decrease observed in the 2-OH pathway metabolites, 2-ME1 and 2-ME2, and the 16-OH pathway metabolite, E3. A prior laboratory-based study had reported that of all EMs, 2-ME2 had the strongest inhibitory effect, while E3 had the strongest proliferative effect on breast cancer cells[282]. Hence, the observed influence of tamoxifen on the circulating levels of the metabolites 2-ME1, 2-ME2 and E3 as well as on the sum of their respective pathways lends support to the crucial role of these metabolites in breast cancer etiology. Given that this is an exploratory analysis, the decreases in levels of the EMs post-tamoxifen therapy support an inverse relationship between tamoxifen use and circulating EM among postmenopausal women.

This study contributes knowledge on the relationship between circulating tamoxifen metabolites and a comprehensive panel of 15 serum EMs. One prior study assessed correlations between tamoxifen metabolites and parent estrogens in serum, among postmenopausal women, and observed significant but weak positive correlations (Spearman rank r=0.29)[204]. We did not observe an association between the tamoxifen metabolites and parent estrogens among postmenopausal women. However, among premenopausal women, for whom tamoxifen is the

primary adjuvant endocrine therapy[283], statistically significant findings were noted, such that tamoxifen metabolites were positively associated with the parent estrogens, estrone and estradiol, and metabolites of the 2- and 16-OH pathway. Positive associations of tamoxifen and its main metabolite, endoxifen were observed with most metabolites of the 2-OH (2-OHE1, 2-ME1, 2-ME2), and all metabolites of the 16-OH pathway. Cytochrome P450 (CYP) enzymes metabolize both tamoxifen and estrogen into their respective metabolites[142,267-271], and differences in the activity of the CYP enzymes among breast cancer patients have been predicted to influence the therapeutic effect of tamoxifen on treatment outcomes[284]. These differences in enzymatic activity among individuals may explain the variability in the relationship between tamoxifen metabolites and EMs across different pathways and subsequent variations in outcomes among breast cancer patients. However, the lack of data on the CYP 450 enzymes and/or their genotypes in the Ultrasound Study of Tamoxifen, limited the possibility to account for or assess this potential explanation.

Of the tamoxifen metabolites, both endoxifen and 4-OHT have approximately a 30-100-fold binding affinity towards the estrogen receptors (ER), when compared to tamoxifen[199,205,206,268]. Though endoxifen has equally potent antiestrogenic activity as 4-OHT, the plasma concentration of endoxifen is 5-10-fold higher than that of the 4-OHT, making it the more clinically relevant tamoxifen metabolite[207-210,285]. This is consistent with our findings of stronger associations noted with endoxifen compared to tamoxifen. On the contrary, among postmenopausal women, the only positive associations was observed between NDT and the 2-OH pathway metabolite, 3-ME1. In comparison to cyclic estrogen production among premenopausal women across the menstrual cycle, endogenous estrogen levels among postmenopausal women are lower and more constant[130]. Although tamoxifen is a primary

treatment among premenopausal women, it is also clinically indicated among postmenopausal breast cancer patients[286]. Prior evidence suggests that hormonal status may be an important predictor of pharmacokinetics of tamoxifen and its metabolites, where levels of metabolites may vary based on menopausal status[287]. In this study, postmenopausal women had higher concentrations of tamoxifen metabolites compared to premenopausal women. However, the difference in association by menopausal status in our study is not easily explained.

The increased potency of the bioactive tamoxifen metabolites suggests stronger antiestrogenic activity that may indicate their importance in the etiologic pathway of ER-positive breast cancer[200,288]. Yet, little is known about the relationship between the metabolites of tamoxifen and estrogen in circulation. Recent evidence indicates that similar to parent estrogens, EMs are also associated with risk of breast cancer[17,125,289]. In addition to the ER-dependent pathway of tumor formation, the target of tamoxifen, the potential genotoxic role of the EMs may cause target cell DNA damage and stimulate subsequent tumor formation[15,16,19]. Prior literature suggests that the estrogenic properties of the EMs and their subsequent carcinogenic effect vary by the estrogen metabolism pathways[15,238,239]. While the metabolites of the 2-OH pathway have antiestrogenic properties and may have a protective role on breast cancer risk[237,238,282], the 16-OH pathway metabolites have high estrogenic activity and are possibly associated with increased breast cancer risk[239,280,282]. Epidemiologic studies, too, have reported that higher levels of the 2-OH pathway metabolites and the ratio of 2-OH:16-OH metabolites are inversely associated with risk of breast cancer among premenopausal[145] and postmenopausal women[12,17,143,241]. The effect of tamoxifen treatment in decreasing the concentrations of 16-OH pathway EMs in this study coupled with the prior evidence of a positive relationship between 16-OH pathway metabolites and breast cancer risk[239,280,282] may indicate a possible mechanism of

tamoxifen in reducing breast cancer risk via its effect on the 16-OH pathway EMs. However, as the 2-OH pathway metabolite levels are inversely associated with breast tumor proliferation[237,238,282], the observed tamoxifen-mediated decrease in 2-OH pathway metabolites needs to be explored further in future longitudinal studies. Evaluating the estrogen metabolism pathways in relation to tamoxifen treatment suggests that biomechanisms of tamoxifen may extend beyond estrogen signaling. The effect of tamoxifen mediated declines in circulating 2-OH and 16-OH pathway EMs, and the positive association between the circulating tamoxifen metabolites and the 2-OH and 16-OH pathway EMs, 12 months after treatment initiation, confirms the importance of estrogen metabolism pathways and additionally highlights a crucial role of tamoxifen metabolites in breast carcinogenesis.

This study has some limitations to be considered. The absence of menstrual cycle data among premenopausal women restricted some of the analyses to postmenopausal women, which further limited the small sample size. The timing of daily tamoxifen intake among women in the Ultrasound Study of Tamoxifen was not known and thus, differences in the timing of tamoxifen intake may have led to variability in estrogen metabolite levels. The half-life of tamoxifen is 5-7 days and that it reaches its highest concentration in serum approximately 5 hours after intake[200,263]. However, as patients are advised to take tamoxifen at the same time each day[266]. the within-individual variability in the EM concentrations after tamoxifen initiation is expected to be minimal. Due to the exploratory nature of the analysis, multiple testing corrections were not applied. Additionally, as this analysis was conducted among tamoxifen treated women only, it was not possible to ascertain if the longitudinal changes in estrogen levels among postmenopausal women were solely due to the effect of tamoxifen. Finally, the levels of EMs

measured in serum may not represent the absolute levels, as some of these metabolites may be excreted via urine.

This study has several important strengths. It is the first study to measure changes in levels of 15 circulating EMs and their summary measures across three different pathways (2-OH, 4-OH, and 16-OH) in relation to tamoxifen use. Furthermore, this is also the first study to explore the relationship between the circulating metabolites of tamoxifen and estrogen. Since circulating tamoxifen metabolites were measured 12 months after treatment initiation, a significant strength of this study was having a validated measure to confirm adherence to tamoxifen treatment among women in the analytic population. Evaluating these relationships highlighted the importance of estrogen metabolism and active tamoxifen metabolites in breast cancer etiology. Since EMs were measured among the same postmenopausal women before and after 12 months of tamoxifen initiation, this approach controlled for confounding arising from invariant personal factors, including age, race, BMI, reproductive factors, etc. Our study population is racially diverse, and more than 50% of the women were African American, making our results more generalizable. Liquid chromatography-tandem mass spectrometry (LC-MS/MS), a highly specific and sensitive assay, was used to measure estrogen metabolite levels in serum, allowing measurement of even low levels of estrogen, especially important for postmenopausal women with relatively low levels of circulating estrogens[55]. Finally, the longitudinal design allowed measuring any detectable changes in serum estrogen metabolite levels both at baseline and 12 months following tamoxifen initiation among postmenopausal women.

Given the importance of estrogen metabolism pathways in breast carcinogenesis, the influence of tamoxifen and/or its metabolites on specific EMs strengthen the significance of tamoxifen therapy in breast cancer prevention and treatment. In the future, larger prospective

studies with longer follow-up periods are needed to gain further etiologic insight on the relationship of tamoxifen and/or its metabolites with estrogen metabolism and assess differential effects by menopausal status.

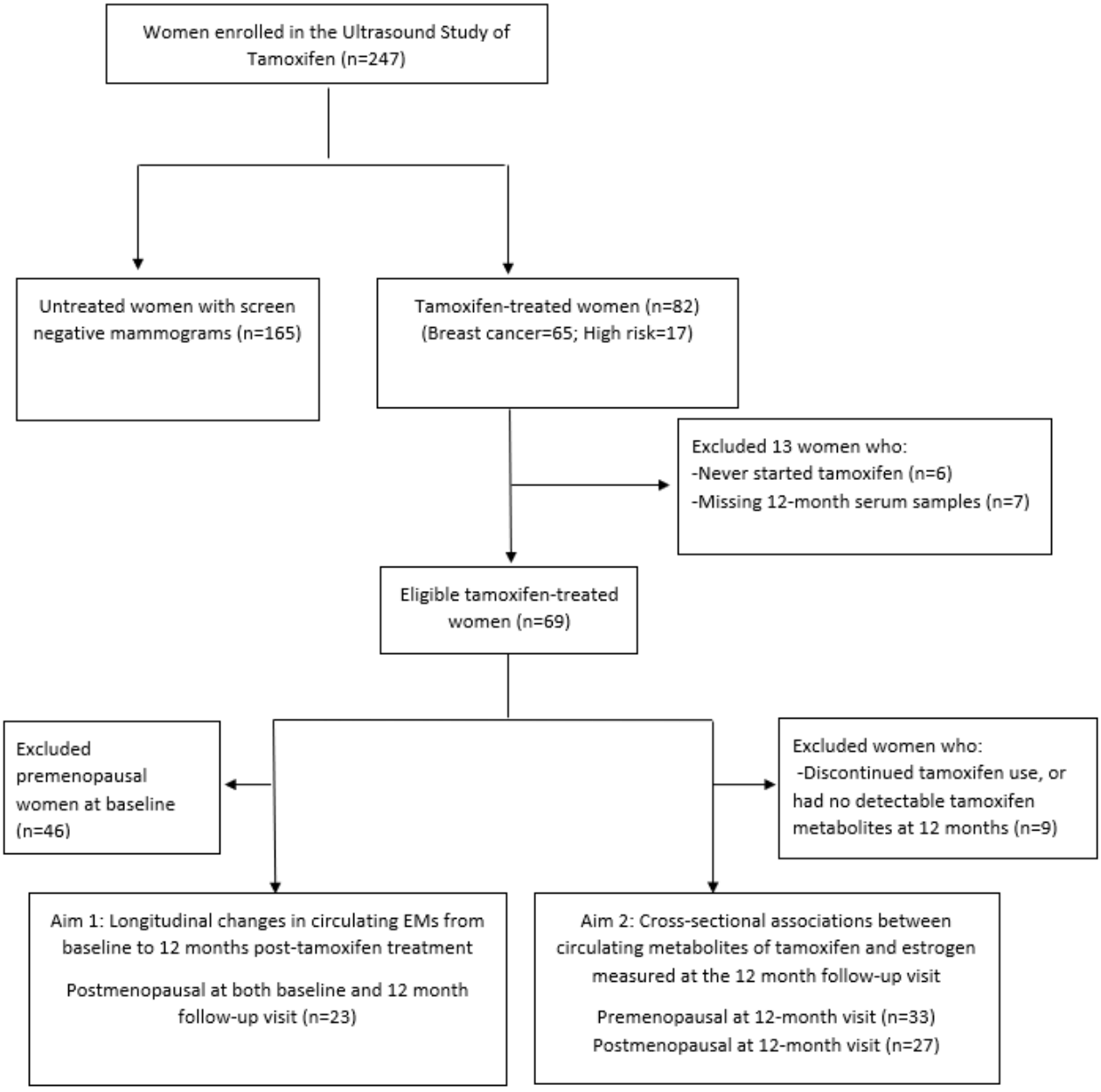

Figure 4.1: Flow diagram depicting the eligibility criteria and derivation of the analytic population

Table 4.1: Characteristics of postmenopausal women at baseline (pre-tamoxifen) and 12-months post-tamoxifen initiation (n=23)

Participant Characteristics	**Baseline visit (pre-tamoxifen)**	**12-month visit (post-tamoxifen)**	**p-value***
BMI (kg/m^2), mean (SD)	31.1 (6.9)	31.0 (7.6)	0.9
Alcohol intake in the last 24 hours, n (%)	4 (17.4)	5 (21.7)	0.3
Current smoker, n (%)	3 (13.0)	4 (17.4)	0.5
Endocrine symptoms in the past two weeks			
Hot flushes, n (%)	9 (39.1)	13 (56.5)	0.1
Joint pain, n (%)	12 (52.2)	10 (43.4)	0.3
		Baseline characteristics	
Age (years), mean (SD)		60.7 (6.2)	
Race, n (%)			
White		7 (30.4)	
Black/African American		15 (65.2)	
Other		1 (4.4)	
Education, n (%)			
High school or less		7 (30.4)	
Some college		9 (39.1)	
College or higher		7 (30.4)	
Tamoxifen indication, n (%)			
High risk		7 (30.4)	
Breast cancer		16 (69.6)	
First degree relative with breast cancer, n (%)		10 (43.5)	
Type of menopause, n (%)			
Natural		16 (69.6)	
Surgery		7 (30.4)	
Former use of menopausal hormone therapy, n (%)		7 (30.4)	
Age at menarche (years), n (%)			
≤ 12		10 (43.5)	
13-14		8 (34.8)	
≥ 15		5 (21.7)	
Ever pregnant, n (%)		22 (95.7)	
No. of live births, n (%)			
0		3 (13.0)	
1-2		12 (52.2)	
≥3		8 (34.8)	
Parous, n (%)		20 (87.0)	
Age at first birth (years), n (%)[a]			
<20		8 (40.0)	
20-24		4 (20.0)	
25-29		3 (15.0)	
≥30		5 (25.0)	
Ever breastfed, n (%)[a]		8 (40.0)	

*****p values generated from paired t-tests (continuous variables) and McNemar tests (categorical variables). [a]Among parous women (n=20)

Table 4.2: Characteristics of tamoxifen-treated pre- and postmenopausal women at 12 months post-tamoxifen initiation

Participant Characteristics	**Premenopausal women (n=33)**	**Postmenopausal women (n=27)**
Age (years), mean (SD)	47.3 (5.9)	60.5 (6.6)
BMI (kg/m^2), mean (SD)	29.9 (6.6)	31.2 (7.6)
Alcohol intake in last 24 hours, n (%)	7 (21.2)	6 (22.2)
Current smoker, n (%)	3 (9.1)	4 (14.8)
Endocrine symptoms in the past two weeks		
Hot flushes, n (%)	25 (75.8)	17 (63.0)
Joint pain, n (%)	17 (51.5)	10 (37.0)
Race, n (%)		
White	11 (33.3)	9 (33.3)
Black/African American	18 (54.6)	17 (63.0)
Other	4 (12.1)	1 (3.7)
Education, n (%)		
High school or less	11 (33.3)	8 (29.6)
Some college	8 (24.2)	11 (40.7)
College or higher	14 (42.4)	8 (29.6)
Tamoxifen indication, n (%)		
High risk	4 (12.1)	7 (25.9)
Breast cancer	29 (87.9)	20 (74.1)
First degree relative with breast cancer, n (%)	11 (33.3)	12 (44.4)
Former use of menopausal hormone therapy, n (%)	1 (3.0)	8 (29.6)
Age at menarche (years), n (%)		
≤ 12	23 (69.7)	13 (48.2)
13-14	5 (15.2)	8 (29.6)
≥ 15	5 (15.2)	6 (22.2)
Ever pregnant, n (%)	28 (84.9)	26 (96.3)
No. of live births, n (%)		
0	6 (18.2)	3 (11.1)
1-2	17 (51.5)	14 (51.9)
≥3	10 (30.3)	10 (37.0)
Parous, n (%)	27 (81.8)	24 (88.9)
Age at first birth (years), n (%)[a]		
<20	7 (25.9)	8 (33.3)
20-24	3 (11.1)	5 (20.8)
25-29	9 (33.3)	5 (20.8)
≥30	8 (29.6)	6 (25.0)
Ever breastfed, n (%)[a]	16 (59.3)	10 (41.7)

[a]Among parous women (premenopausal, n=27; postmenopausal, n=24). *n=4 premenopausal women changed menopausal status to postmenopausal from baseline to 12-month follow-up visit

Table 4.3: Change in estrogen metabolite concentrations between baseline and 12 months after tamoxifen initiation among postmenopausal women (n=23)

Estrogen and EMs (pmol/l)	**Baseline visit** Geometric mean (95% CI)	**12 months visit** Geometric mean (95% CI)	**p-value**[a]	**%Δ**[b] **(95% CI)**
Total estrogens	624.5 (542.3, 719.3)	538.5 (469.1, 618.3)	**0.02**	-13.8 (-30.2, -3.3)
Parent estrogens	237.0 (185.3, 303.1)	211.7 (167.3, 268.0)	0.2	-10.7 (-30.6, 4.1)
Estrone	44.3 (35.2, 55.8)	41.1 (34.0, 49.7)	0.1	-7.27(-32.3, 3.1)
Estradiol	190.4 (146.2, 248.0)	168.2 (129.6, 218.4)	0.4	-11.7 (-27.5, 9.1)
2-Hydroxylation pathway	161.6 (136.2, 191.7)	136.9 (116.2, 161.3)	**0.03**	-15.3 (-36.4, -2.2)
2-Hydroxyestrone	95.0 (78.1, 115.7)	80.1 (64.8, 99.1)	0.1	-15.7 (-45.0, 2.9)
2-Hydroxyestradiol	24.2 (20.6, 28.6)	21.9 (19.0, 25.1)	0.2	-9.5 (-29.7, 5.3)
2-Methoxyestrone	21.3 (17.0, 26.8)	17.0 (14.2, 20.4)	**0.001**	-20.2 (-41.4, -11.4)
2-Methoxyestradiol	14.8 (11.6, 18.9)	12.0 (9.7, 14.8)	**0.02**	-18.9 (-45.8, -4.5)
2-Hydroxyestrone-3methyl ether	1.4 (1.2, 1.7)	1.3 (1.1, 1.5)	0.3	-7.1 (-33.8, 8.8)
4-Hydroxylation pathway	9.7 (8.0, 11.8)	10.0 (8.1, 12.4)	0.8	3.1 (-27.5, 26.5)
4-Hydroxyestrone	7.0 (5.5, 8.9)	7.1 (5.3, 9.5)	0.9	1.4 (-37.6, 29.8)
4-Methoxyestrone	1.6 (1.4, 1.9)	1.6 (1.3, 1.8)	0.8	0 (-16.1, 11.2)
4-Methoxyestradiol	0.8 (0.7, 1.0)	0.7 (0.6, 0.9)	0.2	-12.5 (-31.9, 5.6)
16-Hydroxylation pathway	186.6 (160.1, 217.5)	154.5 (130.3, 183.1)	**0.003**	-17.2 (-35.2, -7.9)
16a-Hydroxyestrone	24.7 (19.9, 30.7)	23.5 (19.3, 28.7)	0.5	-4.9 (-22.4, 10.0)
Estriol	72.3 (60.2, 86.8)	52.8 (43.7, 63.9)	**0.001**	-27.0 (-60.8, -16.4)
16-Ketoestradiol	31.5 (19.5, 51.0)	30.9 (20.1, 47.3)	0.8	-1.9 (-27.7, 18.1)
16-Epiestriol	19.7 (16.2, 23.9)	18.2 (15.1, 22.0)	0.4	-7.6 (-26.6, 8.0)
17-Epiestriol	14.1 (11.6, 17.1)	12.8 (10.4, 15.8)	0.3	-9.2 (-29.4, 6.5)

$p < 0.05$ in bold. [a]p value estimated using paired t-tests. [b]%Δ indicates percent change in EM levels estimated by the ratio of the geometric mean difference between baseline and 12-month EM levels to the geometric mean of the baseline EM levels, multiplied by 100.
Total estrogens represent the sum of all estrogens and EM's: E1, E2, 2-OHE1, 2-OHE2, 2-ME1, 2-ME2, 3-ME1, 4-OHE1, 4-ME1, 4-ME2, 16α-OH-E1, E3, 16-keto-E2, 16-epiE3, and 17-epiE3
Parent estrogens= Sum of E1 and E2
2-Hydroxylation pathway= Sum of 2-OHE1, 2-OHE2, 2-ME1, 2-ME2, and 3-ME1
4-Hydroxylation pathway= Sum of 4-OHE1, 4-ME1, and 4-ME2
16-Hydroxylation pathway= Sum of 16α-OH-E1, E3, 16-keto-E2, 16-epiE3, and 17-epiE3

Table 4.4: Serum concentrations of estrogen metabolites (pmol/l) and tamoxifen metabolites at 12 months following tamoxifen initiation by menopausal status

Estrogen and EMs (pmol/l)	**Premenopausal women (n=33)**	**Postmenopausal women (n=27)**
	Geometric mean (95% CI)	Geometric mean (95% CI)
Total estrogens	1361.6 (944.1, 1963.8)	550.7 (487.4, 622.2)
Parent estrogens	679.6 (437.4, 1055.7)	209.3 (171.6, 255.3)
Estradiol	478.5 (314.1, 729.1)	166.4 (133.8, 207.0)
Estrone	183.3 (109.2, 307.5)	40.2 (33.5, 48.2)
2-Hydroxylation pathway	259.7 (187.4, 359.8)	139.2 (121.3, 159.8)
2-Hydroxyestrone	142.7 (101.3, 201.2)	81.0 (67.7, 96.9)
2-Hydroxyestradiol	35.5 (27.4, 46.0)	22.4 (19.9, 25.2)
2-Methoxyestrone	41.8 (27.1, 64.4)	17.7 (15.2, 20.7)
2-Methoxyestradiol	18.6 (14.0, 24.7)	12.5 (10.4, 14.9)
2-Hydroxyestrone-3-methyl ether	1.7 (1.4,2.2)	1.3 (1.1, 1.5)
4-Hydroxylation pathway	14.4 (10.9, 19.0)	10.0 (8.4, 12.0)
4-Hydroxyestrone	10.6 (7.6, 14.8)	7.1 (5.6, 9.0)
4-Methoxyestrone	2.1 (1.7, 2.6)	1.6 (1.4, 1.8)
4-Methoxyestradiol	0.8 (0.7, 1.0)	0.8 (0.6, 0.9)
16-Hydroxylation pathway	331.2 (240.3, 456.6)	165.1 (139.5, 195.5)
16a-Hydroxyestrone	58.7 (40.1, 86.1)	24.8 (20.0, 30.6)
Estriol	120.0 (81.4, 176.8)	57.9 (47.2, 71.0)
16-Ketoestradiol	57.4 (41.3, 79.7)	33.9 (23.4, 49.1)
16-Epiestriol	35.3 (25.3, 49.2)	18.5 (15.5, 22.1)
17-Epiestriol	22.0 (15.9, 30.6)	12.2 (10.1, 14.7)
Tamoxifen metabolites (ng/ml)		
Tamoxifen	107.8 (89.2-130.4)	140.3 (117.0-168.2)
Endoxifen	6.5 (5.0-8.5)	8.8 (6.4-12.0)
4-OHT	1.3 (1.0-1.7)	1.5 (1.1-2.1)
NDT	185.4 (158.5-216.9)	226.6 (192.3-267.0)

*n=4 premenopausal women changed menopausal status to postmenopausal from baseline to 12-month follow-up visit
Total estrogens represent the sum of all estrogen and EM's: E1, E2, 2-OHE1, 2-OHE2, 2-ME1, 2-ME2, 3-ME1, 4-OHE1, 4-ME1, 4-ME2, 16α-OH-E1, E3, 16-keto-E2, 16-epiE3, and 17-epiE3
Parent estrogens= Sum of E1 and E2
2-Hydroxylation pathway= Sum of 2-OHE1, 2-OHE2, 2-ME1, 2-ME2, and 3-ME1
4-Hydroxylation pathway= Sum of 4-OHE1, 4-ME1, and 4-ME2
16-Hydroxylation pathway= Sum of 16α-OH-E1, E3, 16-keto-E2, 16-epiE3, and 17-epiE3
Abbreviations: 4-OHT: 4-hydroxy-tamoxifen; NDT: N-desmethyl tamoxifen

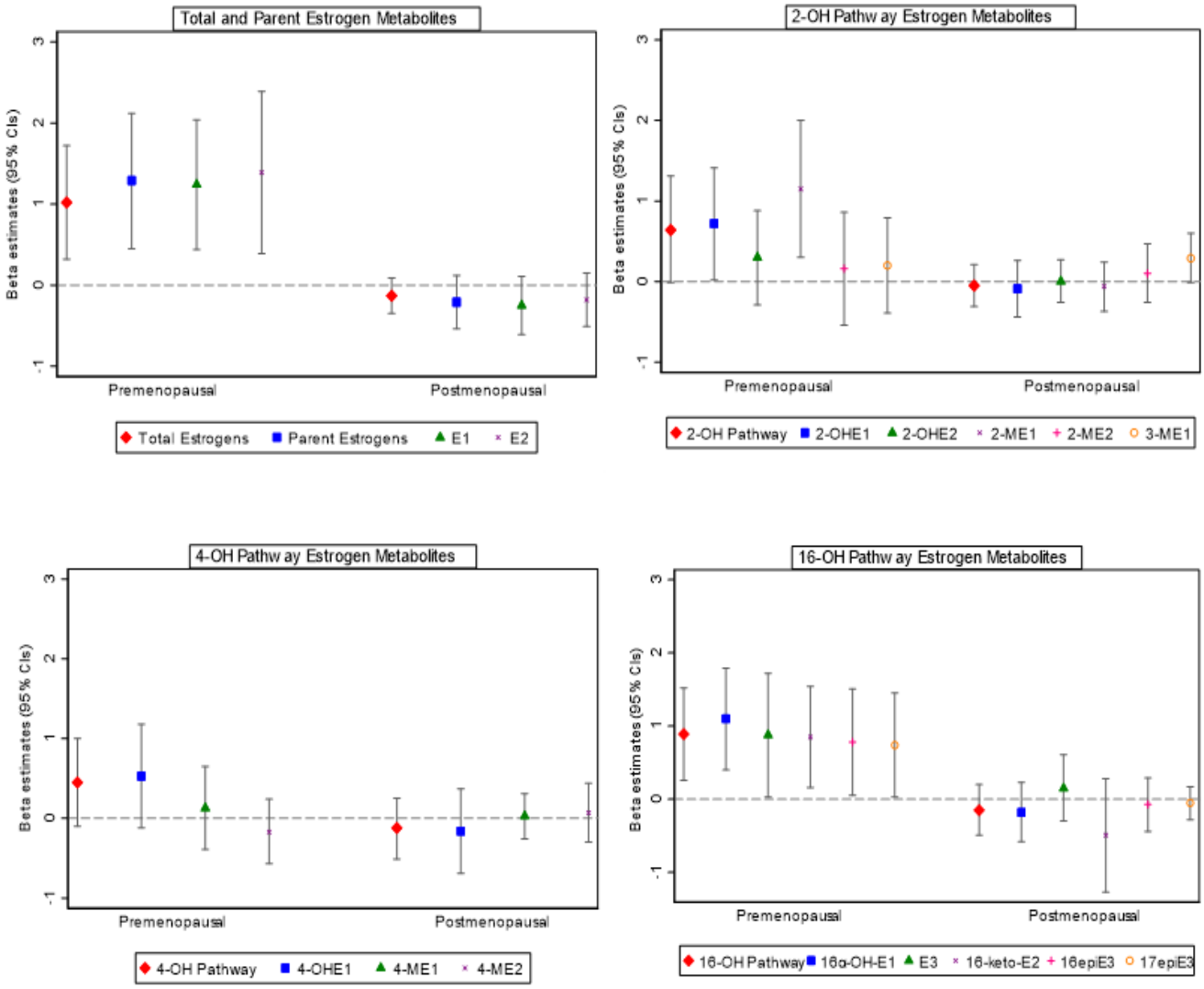

Figure 4.2: Beta estimates and 95% confidence intervals for the association between serum tamoxifen and estrogen metabolites at 12 months following tamoxifen initiation by menopausal status

Plots present results from multivariable linear regression analyses, adjusted for age, baseline parent estrogens, and BMI at 12 months following tamoxifen initiation, with each estrogen metabolite modeled individually.
Total estrogens represent the sum of all estrogens and EMs: E1, E2, 2-OHE1, 2-OHE2, 2-ME1, 2-ME2, 3-ME1, 4-OHE1, 4-ME1, 4-ME2, 16α-OH-E1, E3, 16-keto-E2, 16-epiE3, and 17-epiE3
Parent estrogens/EMs= Sum of E1 and E2
2-Hydroxylation pathway= Sum of 2-OHE1, 2-OHE2, 2-ME1, 2-ME2, and 3-ME1
4-Hydroxylation pathway= Sum of 4-OHE1, 4-ME1, and 4-ME2
16-Hydroxylation pathway= Sum of 16α-OH-E1, E3, 16-keto-E2, 16-epiE3, and 17-epiE3

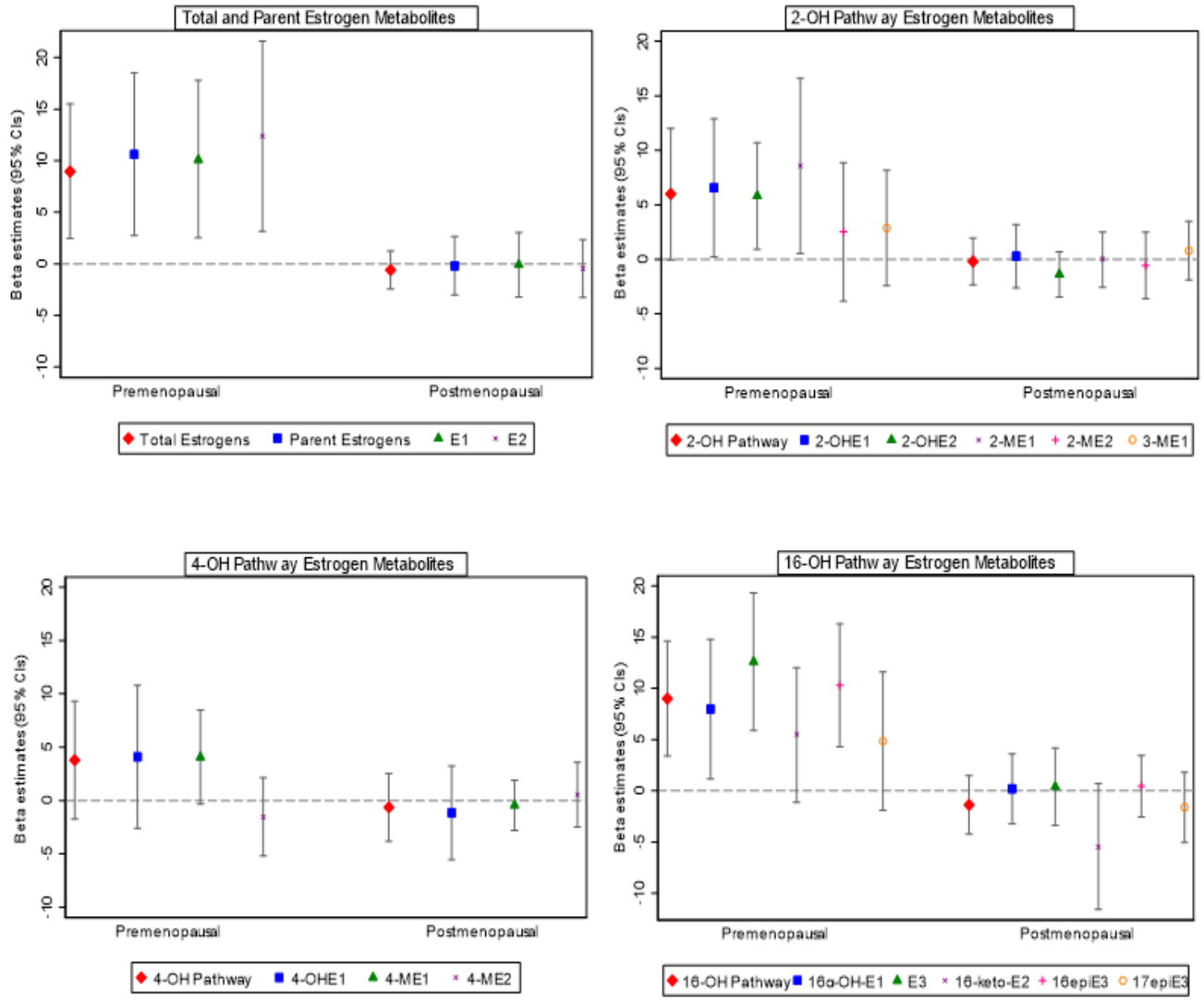

Figure 4.3: Beta estimates and 95% confidence intervals for the association between serum endoxifen and estrogen metabolites at 12 months following tamoxifen initiation by menopausal status

Plots present results from multivariable linear regression analyses, adjusted for age, baseline parent estrogens, and BMI at 12 months following tamoxifen initiation, with each estrogen metabolite modeled individually.
Total estrogens/EMs represent the sum of all estrogen and EM's: E1, E2, 2-OHE1, 2-OHE2, 2-ME1, 2-ME2, 3-ME1, 4-OHE1, 4-ME1, 4-ME2, 16α-OH-E1, E3, 16-keto-E2, 16-epiE3, and 17-epiE3
Parent estrogens/EMs= Sum of E1 and E2
2-Hydroxylation pathway= Sum of 2-OHE1, 2-OHE2, 2-ME1, 2-ME2, and 3-ME1
4-Hydroxylation pathway= Sum of 4-OHE1, 4-ME1, and 4-ME2
16-Hydroxylation pathway= Sum of 16α-OH-E1, E3, 16-keto-E2, 16-epiE3, and 17-epiE3

Supplemental Figures and Tables

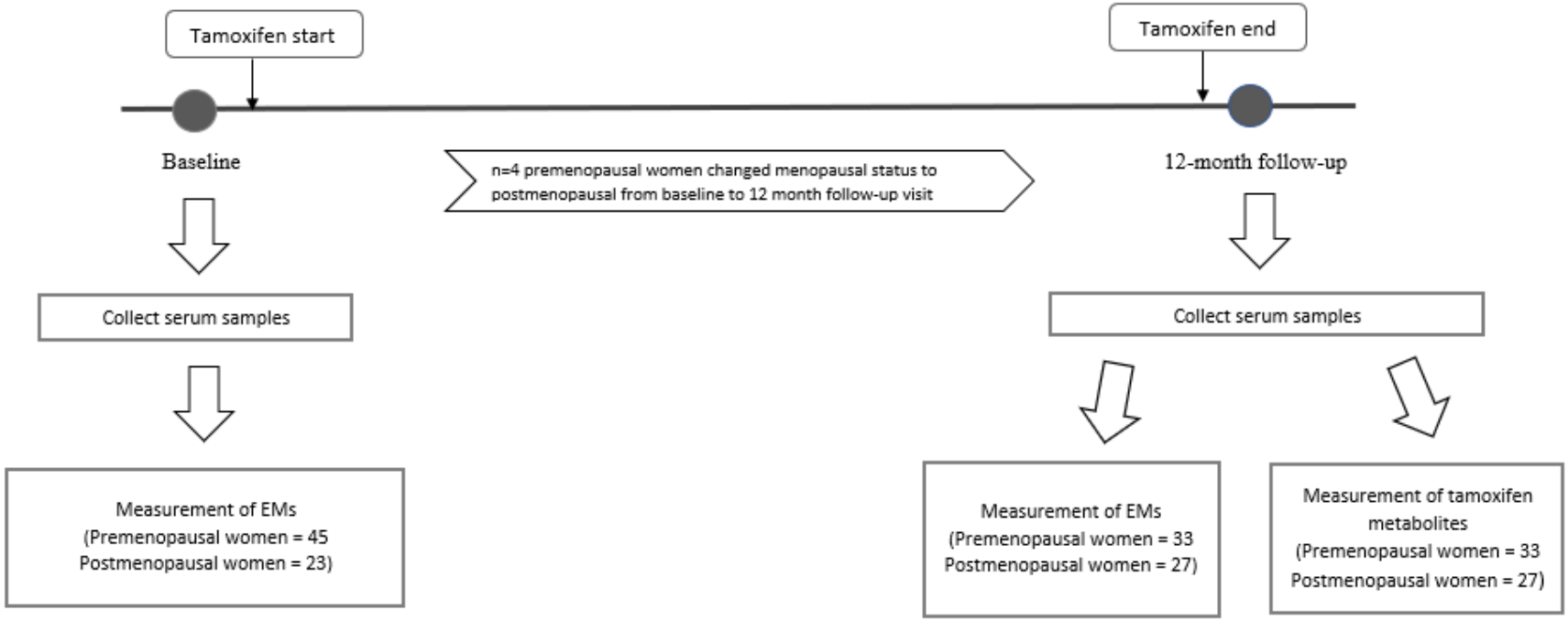

Figure S4.1: Measurement of circulating estrogen and tamoxifen metabolites among women in the Ultrasound Study of Tamoxifen

*EMs were measured in serial serum samples collected prior to and 12 months after tamoxifen initiation; Tamoxifen metabolites were measured 12 months post tamoxifen initiation

Table S4.1: Spearman's rank correlation coefficients between estrogens and estrogen metabolites among premenopausal women (n=33) at 12 months following tamoxifen initiation

Estrogen and EMs	Total estrogens	Parent estrogen	E1	E2	2-OH pathway	2-OHE1	2-OHE2	2-ME1	2-ME2	3-ME1	4-OH pathway	4-OHE1	4-ME1	4-ME2	16-OH pathway	16α-OH-E1	E3	16-keto-E2	16-epiE3	17-epiE3
Total estrogens	1.00																			
Parent estrogens	**0.98**‡	1.00																		
E1	**0.97**‡	**0.99**‡	1.00																	
E2	**0.97**‡	**0.98**‡	**0.95**‡	1.00																
2-OH pathway	**0.83**‡	**0.80**‡	**0.81**‡	**0.78**‡	1.00															
2-OHE1	**0.80**‡	**0.78**‡	**0.80**‡	**0.77**‡	**0.97**‡	1.00														
2-OHE2	**0.73**‡	**0.68**‡	**0.70**‡	**0.64**‡	**0.81**‡	**0.78**‡	1.00													
2-ME1	**0.77**‡	**0.80**‡	**0.81**‡	**0.75**‡	**0.83**‡	**0.78**‡	**0.59**§	1.00												
2-ME2	**0.48**‡	**0.47**§	**0.44**§	**0.47**§	**0.69**‡	**0.64**‡	**0.42**	**0.52**§	1.00											
3-ME1	**0.50**‡	**0.51**§	**0.49**§	**0.49**§	**0.54**§	**0.51**§	**0.38**	**0.56**§	**0.58**§	1.00										
4-OH pathway	**0.69**‡	**0.66**‡	**0.63**‡	**0.70**‡	**0.77**‡	**0.77**‡	**0.60**§	**0.65**‡	**0.61**§	**0.61**§	1.00									
4-OHE1	**0.70**‡	**0.67**‡	**0.64**‡	**0.72**‡	**0.75**‡	**0.76**‡	**0.59**§	**0.64**‡	**0.53**§	**0.58**§	**0.98**‡	1.00								
4-ME1	**0.49**§	**0.47**§	**0.49**§	**0.45**§	**0.59**§	**0.54**§	**0.48**§	**0.54**§	**0.59**§	**0.49**§	**0.53**§	**0.41**	1.00							
4-ME2	-0.01	0.04	0.01	0.05	0.16	0.14	-0.08	0.09	0.34	0.22	0.28	0.18	0.21	1.00						
16-OH pathway	**0.88**‡	**0.84**‡	**0.83**‡	**0.86**‡	**0.60**§	**0.57**§	**0.58**§	**0.63**‡	0.25	**0.42**	**0.52**§	**0.56**§	**0.42**	-0.14	1.00					
16α-OH-E1	**0.81**‡	**0.82**‡	**0.79**‡	**0.81**‡	**0.54**§	**0.51**§	**0.43**§	**0.65**‡	0.31	**0.37**	**0.51**§	**0.53**§	**0.47**§	-0.09	**0.87**‡	1.00				
E3	**0.84**‡	**0.81**‡	**0.82**‡	**0.83**‡	**0.61**§	**0.55**§	**0.59**§	**0.64**‡	0.28	**0.44**	**0.55**§	**0.55**§	**0.49**§	-0.01	**0.89**‡	**0.74**‡	1.00			
16-keto-E2	**0.74**‡	**0.72**‡	**0.73**‡	**0.70**‡	**0.58**§	**0.59**§	**0.64**‡	**0.53**§	0.20	**0.41**	**0.42**	**0.47**§	0.33	-0.26	**0.84**‡	**0.68**‡	**0.67**‡	1.00		
16-epiE3	**0.86**‡	**0.86**‡	**0.83**‡	**0.88**‡	**0.61**§	**0.61**§	**0.64**‡	**0.62**§	0.31	**0.37**	**0.60**§	**0.63**‡	**0.39**	-0.19	**0.82**‡	**0.77**‡	**0.78**‡	0.70	1.00	
17- epiE3	0.27	0.29	0.27	0.35	0.22	0.20	0.03	**0.36**	0.10	0.11	0.33	0.35	0.13	0.21	**0.36**§	0.34	**0.45**§	0.08	0.26	1.00

‡p < 0.0001; §p < 0.01; p < 0.05 in bold.

Total estrogens represent the sum of all estrogen and EM's: E1, E2, 2-OHE1, 2-OHE2, 2-ME1, 2-ME2, 3-ME1, 4-OHE1, 4-ME1, 4-ME2, 16α-OH-E1, E3, 16-keto-E2, 16-epiE3, and 17-epiE3
Parent estrogens= Sum of E1 and E2
2-Hydroxylation pathway= Sum of 2-OHE1, 2-OHE2, 2-ME1, 2-ME2, and 3-ME1
4-Hydroxylation pathway= Sum of 4-OHE1, 4-ME1, and 4-ME2
16-Hydroxylation pathway= Sum of 16α-OH-E1, E3, 16-keto-E2, 16-epiE3, and 17-epiE3

Table S4.2: Spearman's rank correlation coefficients between estrogens and estrogen metabolites among postmenopausal women (n=27) at 12 months following tamoxifen initiation

Estrogen and EMs	Total estrogens	Parent estrogens	E1	E2	2-OH Pathway	2-OHE1	2-OHE2	2-ME1	2-ME2	3-ME1	4-OH Pathway	4-OHE1	4-ME1	4-ME2	16-OH Pathway	16α-OH-E1	E3	16-keto-E2	16-epiE3	17-epiE3
Total estrogens	1.00																			
Parent estrogens	**0.79**‡	1.00																		
E1	**0.76**‡	**0.98**‡	1.00																	
E2	**0.82**‡	**0.81**‡	**0.75**‡	1.00																
2-OH Pathway	**0.66**§	**0.48**	**0.43**	**0.59**§	1.00															
2-OHE1	**0.65**§	**0.50**§	**0.44**	**0.62**§	**0.98**‡	1.00														
2-OHE2	0.33	0.10	0.07	0.16	**0.65**§	**0.58**§	1.00													
2-ME1	**0.49**	**0.60**§	**0.58**§	**0.47**	**0.55**§	**0.51**§	0.32	1.00												
2-ME2	0.14	0.09	0.09	0.14	0.28	0.17	0.22	0.21	1.00											
3-ME1	0.32	**0.48**	**0.49**§	0.34	0.33	0.30	0.06	0.34	0.31	1.00										
4-OH Pathway	0.39	0.16	0.15	0.16	**0.42**	**0.47**	**0.50**§	0.10	-0.20	-0.06	1.00									
4-OHE1	0.25	0.07	0.07	0.10	0.33	0.37	**0.53**§	0.03	-0.29	-0.10	**0.94**‡	1.00								
4-ME1	0.25	0.05	0.06	0.02	-0.04	-0.06	-0.26	0.02	0.40	0.001	-0.06	-0.29	1.00							
4-ME2	0.03	-0.08	-0.11	-0.03	0.06	0.003	0.02	-0.02	0.37	0.12	-0.24	**-0.39**	**0.56**§	1.00						
16-OH Pathway	**0.53**§	0.05	0.01	0.20	0.13	0.12	0.05	-0.07	0.10	-0.10	0.26	0.14	**0.41**	0.16	1.00					
16α-OH-E1	**0.54**§	0.27	0.22	**0.51**§	0.35	0.30	0.12	0.04	0.21	0.11	-0.05	-0.08	0.02	0.22	**0.50**§	1.00				
E3	0.08	-0.21	-0.23	0.002	0.06	0.02	-0.04	-0.22	0.32	-0.09	-0.004	-0.11	**0.39**	0.33	**0. 46**	0.30	1.00			
16-keto-E2	**0.44**	0.06	0.06	0.08	-0.04	-0.07	0.10	-0.03	0.04	-0.16	0.25	0.18	**0.39**	-0.001	**0.78**‡	0.18	0.04	1.00		
16-epiE3	0.25	0.10	0.10	0.36	0.12	0.14	-0.26	-0.13	0.13	0.13	-0.16	-0.12	0.06	-0.21	0.27	**0.57**§	0.33	-0.03	1.00	

Estrogen and EMs	Total estrogens	Parent estrogens	E1	E2	2-OH Pathway	2-OHE1	2-OHE2	2-ME1	2-ME2	3-ME1	4-OH Pathway	4-OHE1	4-ME1	4-ME2	16-OH Pathway	16α-OH-E1	E3	16-keto-E2	16-epiE3	17-epiE3
17-epiE3	**0.43**	0.16	0.16	0.28	-0.01	0.00	0.16	-0.20	0.08	-0.11	0.22	0.21	0.13	-0.14	**0.50**[§]	0.34	-0.04	**0.67**[§]	0.11	1.00

[‡]$p < 0.0001$; [§]$p < 0.01$; $p < 0.05$ in bold

Total estrogens represent the sum of all estrogen and EM's: E1, E2, 2-OHE1, 2-OHE2, 2-ME1, 2-ME2, 3-ME1, 4-OHE1, 4-ME1, 4-ME2, 16α-OH-E1, E3, 16-keto-E2, 16-epiE3, and 17-epiE3

Parent estrogens= Sum of E1 and E2

2-Hydroxylation pathway= Sum of 2-OHE1, 2-OHE2, 2-ME1, 2-ME2, and 3-ME1

4-Hydroxylation pathway= Sum of 4-OHE1, 4-ME1, and 4-ME2

16-Hydroxylation pathway= Sum of 16α-OH-E1, E3, 16-keto-E2, 16-epiE3, and 17-epiE3

Table S4.3: Spearman's rank correlation coefficients between tamoxifen and tamoxifen metabolites by menopausal status at 12 months following tamoxifen initiation

	Premenopausal Women (n=33)				**Postmenopausal Women n(27)**			
Tamoxifen Metabolites	Tamoxifen	Endoxifen	4-OHT	NDT	Tamoxifen	Endoxifen	4-OHT	NDT
Tamoxifen	1.00				1.00			
Endoxifen	**0.48**[§]	1.00			**0.40**	1.00		
4-OHT[a]	**0.54**[§]	**0.73**[‡]	1.00		**0.62**[§]	**0.68**[§]	1.00	
NDT	**0.80**[‡]	0.21	0.30	1.00	**0.71**[‡]	0.13	**0.45**	1.00

[‡]$p < 0.0001$; [§]$p < 0.01$; $p < 0.05$ in bold.
[a]For 4-OHT, N = 7 samples were below the lower limit of quantitation (LLOQ) and were reassigned as ½ of LLOQ (0.25)
4-OHT: 4-hydroxy-tamoxifen; NDT: N-desmethyltamoxifen

Table S4.4: Linear regression estimates for the associations between tamoxifen metabolites and parent estrogen metabolites at 12 months following tamoxifen initiation by menopausal status

	Premenopausal women (n=33)				**Postmenopausal women (n=27)**			
	Total estrogens	Parent estrogens	E1	E2	Total estrogens	Parent estrogens	E1	E2
	Beta estimates[a] (95% CI)							
Tamoxifen	**1.02**‡ **(0.32, 1.72)**	**1.29**‡ **(0.45, 2.12)**	**1.24**‡ **(0.44, 2.04)**	**1.39 (0.34, 2.30)**	-0.13 (-0.35, 0.09)	-0.21 (-0.46, 0.40)	-0.25 (-0.61, 0.11)	-0.18 (-0.51, 0.15)
Endoxifen	**8.96 (2.46, 15.5)**	**10.6 (2.76, 18.5)**	**10.1 (2.53, 17.8)**	**12.4 (3.17, 21.8)**	-0.59 (-2.43, 1.24)	0.19 (-0.03, 2.65)	-0.08 (-3.21, 3.04)	-0.45 (-3.27, 2.36)
4-OHT[b]	**37.1 (1.80, 72.4)**	41.5 (-1.65, 84.6)	**43.2 (2.18, 84.2)**	40.8 (-15.5, 88.8)	-3.17 (-14.7, 7.26)	-3.90 (-20.8, 13.0)	-3.12 (-21.8, 15.5)	-5.83 (-22.5, 10.8)
NDT	0.41 (-0.15, 0.98)	0.57 (-0.10, 1.23)	0.54 (-0.09, 1.19)	0.59 (-0. 15, 1.43)	0.02 (-0.10, 0.13)	-0.01 (-0.19, 0.17)	-0.05 (-0.26, 0.15)	0.07 (-0.11, 0.25)

[a]Adjusted for age, baseline parent estrogens, and BMI at 12 months following tamoxifen initiation. $p < 0.05$ in bold; ‡$p < 0.01$

[b]For 4-OHT, N = 7 samples were below the lower limit of quantitation (LLOQ) and were reassigned as ½ of LLOQ (0.25)

Total estrogens represent the sum of all estrogen and EM's: E1, E2, 2-OHE1, 2-OHE2, 2-ME1, 2-ME2, 3-ME1, 4-OHE1, 4-ME1, 4-ME2, 16α-OH-E1, E3, 16-keto-E2, 16-epiE3, and 17-epiE3

Parent estrogens= Sum of E1 and E2

Table S4.5a: Linear regression estimates for the associations between tamoxifen metabolites and 2-OH pathway estrogen metabolites at 12 months following tamoxifen initiation among premenopausal women (n=33)

	2-OH pathway	2-OHE1	2-OHE2	2-ME1	2-ME2	3-ME1
			Beta estimates[a] (95% CI)			
Tamoxifen	0.64 (-0.01, 1.31)	**0.72 (0.02, 1.41)**	0.29 (-0.29, 0.88)	**1.14 (0.30, 1.99)**	0.16 (-0.54, 0.86)	0.21 (-0.39, 0.79)
Endoxifen	6.0 (-0.03, 12.0)	**6.57 (0.21, 12.9)**	**5.82 (0.90, 10.7)**	**8.58 (0.52, 16.6)**	2.51 (-3.85, 8.87)	2.87 (-2.41, 8.15)
4-OHT[b]	**32.8 (2.04, 64.8)**	**37.1 (4.92, 69.3)**	24.8 (-1.5, 51.0)	**42.5 (0.61, 84.4)**	14.8 (-17.8, 47.5)	19.5 (-7.25, 46.3)
NDT	0.24 (-0.26, 0.74)	0.30 (-0.22, 0.83)	0.04 (-0.39, 0.47)	0.48 (-0.18, 1.14)	-0.03 (-0.54, 0.47)	0.10 (-0.33, 0.52)

[a]Adjusted for age, baseline parent estrogens, and BMI at 12 months following tamoxifen initiation. $p < 0.05$ in bold; ‡$p < 0.01$
[b]For 4-OHT, N = 7 samples were below the lower limit of quantitation (LLOQ) and were reassigned as ½ of LLOQ (0.25)
2-Hydroxylation pathway= Sum of 2-OHE1, 2-OHE2, 2-ME1, 2-ME2, and 3-ME1

Table S4.5b: Linear regression estimates for the associations between tamoxifen metabolites and 2-OH pathway estrogen metabolites at 12 months following tamoxifen initiation among postmenopausal women (n=27)

	2-OH pathway	2-OHE1	2-OHE2	2-ME1	2-ME2	3-ME1
			Beta estimates[a] (95% CI)			
Tamoxifen	-0.05 (-0.31, 0. 21)	-0.09 (-0.44, 0.26)	0.004 (-0.26, 0.27)	-0.06 (-0.37, 0.24)	0.10 (-0.26, 0.47)	0.29 (-0.01, 0.60)
Endoxifen	-0.19 (-2.34, 1.95)	0.28 (-2.63, 3.18)	-1.38 (-3.45, 0.68)	-0.04 (-2.58, 2.49)	-0.58 (-3.63, 2.47)	0.80 (-1.90, 3.50)
4-OHT[b]	-5.84 (-18.4, 6.7)	-2.23 (-19.6, 15.1)	-11.4 (-23.2, 0.44)	-12.4 (-26.4, 1.64)	-7.71 (-25.7, 10.3)	-5.57 (-21.7, 10.5)
NDT	0.10 (-0.03, 0.24)	0.10 (-0.08, 0.28)	0.09 (-0.04, 0.23)	0.09 (-0.06, 0.25)	0.11 (-0.08, 0. 31)	**0.18 (0.03, 0.34)**

[a]Adjusted for age, baseline parent estrogens, and BMI at 12 months following tamoxifen initiation. $p < 0.05$ in bold; ‡$p < 0.01$
[b]For 4-OHT, N = 7 samples were below the lower limit of quantitation (LLOQ) and were reassigned as ½ of LLOQ (0.25)
2-Hydroxylation pathway= Sum of 2-OHE1, 2-OHE2, 2-ME1, 2-ME2, and 3-ME1

Table S4.6: Linear regression estimates for the associations between tamoxifen metabolites and 4-OH pathway estrogen metabolites at 12 months following tamoxifen initiation by menopausal status

	Premenopausal women (n=33)				**Postmenopausal women (n=27)**			
	4-OH pathway	4-OHE1	4-ME1	4-ME2	4-OH pathway	4-OHE1	4-ME1	4-ME2
	Beta estimates[a] (95% CI)							
Tamoxifen	0.45 (-0.10, 1.0)	0.53 (-0.12, 1.18)	0.13 (-0.39, 0.65)	-0.17 (-0.57, 0.24)	-0.12 (-0.51, 0.25)	-0.16 (-0.69, 0.37)	0.03 (-0.26, 0.31)	0.7 (-0.30, 0.44)
Endoxifen	3.67 (-1.40, 8.75)	3.95 (-2.09, 9.98)	4.6 (-0.42, 8.54)	-1.53 (-5.26, 2.21)	-0.63 (-3.80, 2.54)	-1.16 (-5.54, 3.23)	-0.44 (-2.78, 1.89)	0.55 (-2.49, 3.59)
4-OHT[b]	19.0 (-7.14, 45.2)	17.0 (-14.4, 48.4)	**27.9 (6.05, 49.8)**	-2.0 (-21.5, 17.5)	-2.54 (-21.5, 16.4)	-5.69 (-32,0, 20.6)	3.6 (-10.4, 17.6)	1.57 (-16.7, 19.8)
NDT	0.16 (-0.24, 0.58)	0.23 (-0.25, 0.72)	-0.12 (-0.49, 0.25)	-0.15 (-0.44, 0.14)	-0.02 (-0.23, 0.18)	-0.03 (-0.32, 0.26)	0.03 (-0.12, 0.18)	0.04 (-0.15, 0.24)

[a]Adjusted for age, baseline parent estrogens, and BMI at 12 months following tamoxifen initiation. $p < 0.05$ in bold; ‡$p < 0.01$

[b]For 4-OHT, N = 7 samples were below the lower limit of quantitation (LLOQ) and were reassigned as ½ of LLOQ (0.25)

4-Hydroxylation pathway= Sum of 4-OHE1, 4-ME1, and 4-ME2

Table S4.7a: Linear regression estimates for the associations between tamoxifen metabolites and 16-OH pathway estrogen metabolites at 12 months following tamoxifen initiation among premenopausal women (n=33)

	16-OH pathway	16α-OH-E1	E3	16-keto-E2	16-epiE3	17-epiE3
	Beta estimates[a] (95% CI)					
Tamoxifen	**0.89 (0.26, 1.52)**	**1.10‡ (0.40, 1.79)**	**0.89‡ (0.03, 1.72)**	**0.85 (0.16, 1.54)**	**0.78 (0.05, 1.51)**	**0.74 (0.03, 1.45)**
Endoxifen	**9.0‡ (3.40, 14.6)**	**7.96 (1.15, 14.8)**	**12.6‡ (5.87, 19.3)**	5.51 (-1.15, 12.0)	**10.3‡ (4.30, 16.3)**	4.85 (-1.90, 11.6)
4-OHT[b]	33.8 (-2.20, 65.3)	29.1 (--7.89, 66.1)	**50.8 (12.5, 89.0)**	27.8 (-6.71, 62.2)	30.1 (-5.38, 65.6)	9.98 (-26.0, 46.0)
NDT	0.29 (-0.22, 0.80)	**0.56 (0.01, 1.11)**	0.10 (-0.55, 0.77)	0.33 (-0.21, 0.87)	0.27 (-0.29, 0.83)	0.31 (-0.22, 0.85)

[a]Adjusted for age, baseline parent estrogens, and BMI at 12 months following tamoxifen initiation. $p < 0.05$ in bold; ‡$p < 0.01$
[b]For 4-OHT, N = 7 samples were below the lower limit of quantitation (LLOQ) and were reassigned as ½ of LLOQ (0.25)
16-Hydroxylation pathway= Sum of 16α-OH-E1, E3, 16-keto-E2, 16-epiE3, and 17-epiE3

Table S4.7b: Linear regression estimates for the associations between tamoxifen metabolites and 16-OH pathway estrogen metabolites at 12 months following tamoxifen initiation among postmenopausal women (n=27)

	16-OH pathway	16α-OH-E1	E3	16-keto-E2	16-epiE3	17-epiE3
			Beta estimates[a] (95% CI)			
Tamoxifen	-0.15 (-0.49, 0.20)	-0.18 (-0.58, 0.23)	0.15 (-0.30, 0.61)	-0.49 (-1.27, 0.28)	-0.07 (-0.44, 0.29)	-0.05 (-0.47, 0.37)
Endoxifen	1.39 (-4.24, 1.46)	0.19 (-3.21, 3.60)	0.38 (-3.4, 4.17)	-5.49 (-11.6, 0.68)	0.44 (-2.55, 3.44)	-1.62 (-5.04, 1.79)
4-OHT[b]	-3.31 (-20.7, 14.1)	1.04 (-19.4, 21.4)	7.01 (-15.4, 29.4	-22.2 (-60.7, 16.4)	7.41 (-10.2, 25.1)	3.92 (-16.9, 24.8)
NDT	-0.05 (-0.24, 0.14)	-0.0001 (-0.22, 0.22)	0.08 (-0.16, 0.32)	-0.20 (-0.63, 0.22)	-0.003 (-0.20, 0.19)	-0.05 (-0.28, 0.17)

[a]Adjusted for age, baseline parent estrogens, and BMI at 12 months following tamoxifen initiation. $p < 0.05$ in bold; ‡$p < 0.01$
[b]For 4-OHT, N = 7 samples were below the lower limit of quantitation (LLOQ) and were reassigned as ½ of LLOQ (0.25)
16-Hydroxylation pathway= Sum of 16α-OH-E1, E3, 16-keto-E2, 16-epiE3, and 17-epiE3

Chapter 5: The effect of adjuvant tamoxifen treatment on longitudinal changes in circulating IGFs and breast density

Abstract

Background: Tamoxifen-mediated decline in breast density is a strong indicator of its therapeutic efficacy. Tamoxifen's role in the modulation of circulating insulin-like growth factors (IGFs) among breast cancer patients is hypothesized to further contribute to its treatment benefits. Although an interplay between tamoxifen, IGFs, breast density, and breast cancer is suggested, data is limited regarding the relationship between concomitant changes in IGFs and breast density in relation to tamoxifen therapy. This study assessed the influence of tamoxifen treatment on the association between serial measures of IGFs and breast density among women diagnosed with breast cancer.

Methods: In the Ultrasound Study of Tamoxifen, volume averaged sound speed (VASS) measures of breast density (m/s) were assessed using a novel ultrasound tomography (UST) scanner among tamoxifen-treated women breast cancer patients (n=53) prior to and 12 months after treatment initiation. Serum concentrations of IGFs (IGF-I, and its binding protein, IGFBP-3) (ng/ml) were measured via an enzyme-linked immunosorbent assay at baseline and 12 months after tamoxifen therapy. Bioavailable IGF-I was estimated by calculating the ratio of IGF-I: IGFBP-3. In longitudinal analyses, changes in IGFs and VASS were assessed using paired t-tests, and the association between changes in IGFs and concomitant changes in VASS from baseline to 12-months was estimated using multivariable linear regression adjusted for age, menopausal status, change in BMI, and baseline VASS measures.

Results: At 12 months, mean circulating IGF-I and the IGF-I: IGFBP3 ratio statistically significantly decreased ($p < 0.0001$), with a mean (SD) decrease of -37.3 (30.9) ng/ml and -0.01

(0.006) ng/ml, respectively. Although a decline in VASS measures was observed from baseline to 12 months after tamoxifen treatment, the mean (SD) decrease of -3.9 (8.7) m/s did not reach statistical significance (p=0.18). No statistically significant associations were observed between changes in IGFs and concomitant changes in VASS, following 12 months of tamoxifen treatment.

Conclusion: Our findings suggest tamoxifen-mediated declines in circulating IGF-I after 12 months of treatment. However, mechanistic pathway of tamoxifen-associated changes in breast density needs to be further explored. Future longitudinal studies with larger sample sizes are needed to elucidate the biomechanisms of tamoxifen-associated breast density declines in breast risk and progression.

Keywords: Tamoxifen, insulin-like growth factors, breast density, volume averaged sound speed.

Introduction

Tamoxifen, one of the most widely used adjuvant endocrine therapies, decreases breast cancer recurrence and mortality by approximately 30%[41,290]. Elevated breast density is one of the strongest breast cancer risk factors; women with high breast density (≥60%) are at a 4-6-fold increased risk of breast cancer compared to women with less dense (≤5%) breasts[22,99]. A possible intermediate marker of breast cancer risk[20,22,99], breast density is also modifiable; a reduction in the same is associated with a lower risk of breast cancer[23]. Evidence from prior studies suggests that anti-estrogenic therapy such as tamoxifen decreases breast density[24,25,291,292], and the tamoxifen-mediated declines translate to lower breast cancer risk[24-26] and improved outcomes[28,29,291,292] among breast cancer patients. However, little is known about the determinants that influence declines in breast density and the subsequent breast cancer risk.

Previous literature indicates that insulin-like growth factors (IGFs) play a significant role in both breast carcinogenesis[30,32,158,159,293] and elevated breast density[34-36]. IGFs, peptide hormones that play a critical role in cell differentiation and proliferation[30,31] have been linked to tumorigenesis by stimulating the proliferation of cancerous cells and inhibiting apoptosis[152,157]. This association may partially explain the relationship of IGFs with high mammographic density as dense breast tissues likely reflect increased breast cell proliferation[294]. In cross-sectional associations, positive relationship between circulating IGF-I and breast density has been primarily observed among premenopausal women, whereas its binding protein, IGFBP-3 has been inversely associated[34-36]. Additionally, in longitudinal analysis, changes in IGFs (IGF-I and IGF-I: IGFBP-3 ratio) have been positively associated with changes in breast density in observational study[295] and randomized clinical trial[296], after 12 months of exercise intervention and vitamin D supplementation, respectively.

Prior studies also indicate that adjuvant tamoxifen treatment reduces circulating IGF-I levels and increases the concentrations of its main binding protein, IGFBP3, among breast cancer patients[33,37-39], suggesting that the protective effect of tamoxifen on breast cancer progression may be mediated via its influence on circulating IGF-I and IGFBP3 levels. The tamoxifen-mediated declines in breast density[24,25,291,292] and tamoxifen's effect in modulating circulating IGFs[33,37-39] support the significance of tamoxifen in the etiologic pathway of breast carcinogenesis. However, tamoxifen's effect on the relationship between changes in IGFs and breast density remains unclear. Since breast density is a strong determinant of breast cancer and changes in density influence breast cancer risk, understanding how IGFs influence breast density before and after tamoxifen initiation will be of crucial importance in elucidating breast cancer etiology. The objective of this study was to examine the association between changes in circulating IGFs and concomitant changes in breast density from baseline to 12 months following tamoxifen initiation.

Methods

Study design and population

The study population for this analysis was drawn from the Ultrasound Study of Tamoxifen, a longitudinal study that assessed changes in breast density measured by ultrasound tomography (UST). Details have been previously described[53,54]. In brief, the participants included women aged 30-70 years who were at high risk or diagnosed with breast cancer and referred to tamoxifen therapy (tamoxifen-treated), and an age, race, and menopausal status frequency matched comparison group who screened negative for breast cancer (untreated). The

participants were recruited from the Henry Ford Health Systems (HFHS) and Barbara Ann Karmanos Cancer Institute (KCI) in Detroit, MI after routine screening.

Derivation of the analytic population is summarized in Figure S5.1. In longitudinal analyses, the associations between changes in IGFs and changes in breast density from baseline (prior to tamoxifen initiation) to 12 months following treatment initiation were assessed among tamoxifen-treated women. From the initial analytic population of tamoxifen-treated patients (n=82), the following women were excluded from the analysis: high-risk patients referred to tamoxifen for chemoprevention (n=15), never started tamoxifen (n=6), did not complete the baseline ultrasound tomography (UST) scan (n=1), or had missing serum samples at the 12-month visit (n=7). The final analytic population for the longitudinal analyses consisted of n=53 tamoxifen-treated breast cancer patients.

As a supplemental analysis, cross-sectional associations between circulating IGFs and breast density at baseline (prior to tamoxifen initiation) were assessed among breast cancer cases and the comparison group. Of the women in the comparison group (n=165), n=24 women who were missing IGF information were excluded leading to the analytic population of n=53 breast cancer cases and n=141 women in the comparison group for these supplemental baseline analyses (Figure S5.1). Informed consent was obtained from all participants and study procedures were approved by the Institutional Review Boards of KCI, HFHS, and the National Cancer Institute (NCI).

Measurement of IGFs

Blood samples were collected and processed as serum at KCI using standard operating procedures and were stored at -80°C at the KCI pharmacy core laboratory until shipment to the

NCI biorepository, where vials were stored in liquid nitrogen. Serum concentrations (ng/ml) of IGFs, IGF-I, and its binding protein, IGFBP-3 were measured using an enzyme-linked immunosorbent assay (ELISA), prior to, and 12 months after tamoxifen initiation using previously described techniques[35]. Bioavailable IGF-I was estimated by calculating the ratio of IGF-I: IGFBP-3. Blinded duplicate quality control (QC) samples were included within and across batches. The within and between batch coefficients of variation (CV) were 1.96% and 2.14% for IGF-I, and 0.86% and 3.42% for IGFBP-3, respectively.

Assessment of sound speed measures of breast density

Breast density was assessed prior to and 12 months following tamoxifen initiation, using the UST scanner, a novel imaging technique. Whole-breast three-dimensional UST calculates volume averaged sound speed (VASS), which is a quantitative surrogate measure of volumetric breast density[53,57,104,297]. Details of the method are described elsewhere[54,57]. UST-measured VASS estimates have been shown to have high reliability for single timepoint estimates (ICC=93.3%) and for changes in VASS measures over time (ICC=70.4%)[54].

Covariate information

Information on participant characteristics, including demographics, reproductive history, and menopausal status was collected using a standard health questionnaire administered by a research nurse. Height, measured at baseline, and weight at each UST scan were used to compute body mass index (BMI, kg/m^2). Women were considered postmenopausal if periods stopped for 12 months or longer due to natural causes or surgical procedures such as bilateral oophorectomy. Women aged 55 years or more who had a hysterectomy but not oophorectomy, and whose periods stopped due to endometriosis, uterine ablation, irregular perimenopausal cycles,

novasure procedure, or medication/drug therapy, or any other reason were classified as postmenopausal.

Statistical analyses

In the longitudinal analyses, changes in IGFs (IGF-I, its binding protein, IGFBP-3 and the ratio of IGF-I: IGFBP-3) (ng/ml) and changes in VASS (m/s) were calculated by subtracting the baseline measures from the measures estimated at 12-month follow-up, among the tamoxifen-treated women. The changes in VASS between baseline and 12-month follow-up were approximately normally distributed, and were used in the analyses without any transformation. Paired t-tests were used to assess changes in circulating IGFs, VASS measures, and BMI from baseline to 12 months following tamoxifen treatment. Mean changes in VASS and IGFs were evaluated by participant characteristics using t-tests or ANOVA, as appropriate. Beta estimates and 95% confidence intervals (CIs) for the association between changes in IGFs and concomitant changes in breast density were assessed using multivariable linear regression models. Changes in IGFs were assessed on a continuous scale (per 100 ng/ml), and in categories based on tertile of change among the tamoxifen treated patients. The decrease in IGF was set as the referent category for the tertile-specific associations. Using t-tests and ANOVA, age (categories, assessed as an ordinal trend (<45, 45–<50, 50–<55, ≥55 years), menopausal status (premenopausal, postmenopausal), and BMI change at 12 months follow-up visit (tertiles, as an ordinal trend), and baseline VASS measures (tertiles, as an ordinal trend) were identified as potential confounders in the relationship between changes in IGFs and changes in VASS at a significance level of 0.1, and were included in the final models. In sensitivity analyses, race was added to the models. Sensitivity analyses were further conducted to assess the association between

concomitant changes in IGFs and breast density, after excluding women who either discontinued tamoxifen use or had no detectable tamoxifen metabolites in serum, 12 months after tamoxifen initiation. Heterogeneity between tertiles of IGFs for this analysis and the one described below was assessed by type III F tests arising from linear regression models.

For the supplemental analyses at baseline, prior to tamoxifen initiation, mean VASS and concentrations of IGFs (IGF-I, IGFBP3, and its ratio) by participant characteristics were assessed using t-tests and Kruskal-Wallis tests among breast cancer cases. Beta estimates and 95% confidence intervals (CIs) for the association between circulating IGFs and VASS, prior to tamoxifen initiation, were estimated using multivariable linear regression models among breast cancer cases and comparison group. IGFs concentrations were assessed on a continuous scale (per 100 ng/ml) and in tertiles; the percentiles were estimated based on their distribution among the comparison group. Given the heterogeneity of the associations between IGFs and breast density by menopausal status[34-36], the analyses were further restricted to premenopausal women as there was insufficient statistical power to evaluate these associations among postmenopausal women. Using t-tests and Kruskal-Wallis tests, age (categories, assessed as an ordinal trend (<45, 45–<50, 50–<55, ≥55 years), menopausal status (premenopausal, postmenopausal), and BMI at baseline (normal/overweight/obese, assessed as an ordinal trend) were identified as potential confounders in the relationship between circulating IGFs and baseline VASS at a significance level of 0.1, and were included in the final models. Evidence from prior literature indicates that race is associated with both IGFs (the exposure) [272-274] and breast density(outcome) [275,276], and therefore race was also included in the final models. Statistical significance was assessed at α=0.05. All analyses were conducted using SAS 9.4 (Cary, North Carolina).

Results

Participant characteristics

Tamoxifen-treated breast cancer patients consisted of slightly older and obese women with mean (SD) age and BMI of 51.6 (8.8) years and 30.4 (7.1) kg/m^2, respectively. The population was ≥ 60% African American and primarily premenopausal (69.8%) (Appendix E).

Longitudinal associations between changes in circulating IGFs and changes in VASS, 12 months following tamoxifen initiation

Circulating IGF-I and the ratio of IGF-I: IGFBP-3 statistically significantly decreased (p <0.0001) from baseline to 12 months following tamoxifen initiation. The mean (SD) decrease of IGF-I and the IGF-I: IGFBP-3 ratio was -37.3 (30.9) ng/ml and -0.01 (0.006) ng/ml, respectively (Table 5.1). Although VASS measures decreased from baseline to 12 months following tamoxifen initiation with a mean (SD) of -3.9 (8.7) m/s, the reduction was not statistically significant (p= 0.18) (Table 5.1). The associations of participant characteristics with changes in VASS (m/s) and changes in IGFs (ng/ml), among the tamoxifen-treated women are presented in Table 5.2. An increase in BMI from baseline to 12 months following treatment initiation was associated with a statistically significant reduction in VASS measures over the same time period (p =0.004). Higher VASS measures at baseline were associated with a greater decrease in VASS (p= 0.04) from baseline to 12 months after tamoxifen therapy (Table 5.2). While postmenopausal status was statistically significantly associated with a decrease in IGFBP-3 levels (mean (SD)= -197.4 (443.3) ng/ml, p= 0.01), only a borderline decrease was observed with the IGF-I levels (mean (SD)= -49.4 (27.1) ng/ml, p=0.06), 12 months following tamoxifen treatment (Table 5.2).

No statistically significant associations were observed between changes in IGFs and concomitant changes in VASS, from baseline to 12 months following tamoxifen initiation (Table 5.3, Figure S5.2). After excluding tamoxifen-treated women who either discontinued tamoxifen or had no detectable tamoxifen metabolites in serum (n=4), 12 months after treatment initiation, the relationship did not change (Appendix G).

Cross-sectional association between circulating IGF concentrations and VASS, prior to tamoxifen treatment

Associations of participant characteristics with baseline VASS and circulating IGFs among breast cancer patients are presented in Table S5.1. BMI was inversely associated with both VASS and circulating IGF-I (p= 0.03), prior to tamoxifen initiation. Baseline measures of VASS (p= 0.006) and circulating IGF-I (p= 0.04) levels were higher among premenopausal women compared to postmenopausal women (Table S5.1). Among the comparison group, elevated VASS measures at baseline (mean (SD)= 142.3 (47.5) m/s) were positively associated with circulating IGF-I levels (p =0.005) (Table S5.2).

The supplemental cross-sectional associations between IGFs and VASS are presented in Table S5.3. Among the breast cancer patients, both higher continuous IGF-I (β= -11.1, 95% CI: -20.5, -1.63) and higher continuous IGFBP-3 (β= -0.52, 95% CI: -0.98, -0.06) were associated with lower VASS measures at baseline (Table S5.3). In contrast, among women in the comparison group, higher levels of the IGF-I: IGFBP-3 ratio were positively associated with VASS measures at baseline both in the unadjusted (β_{T3vsT1}= 9.69, 95% CI: 3.58, 15.79, p trend =0.002) and adjusted analyses (β_{T3vsT1}= 5.82, 95% CI: 0.04, 11.59, p trend =0.05) (Table S5.3). Although higher continuous IGF-I (β= 7.05, 95% CI: 1.29, 12.82) and the IGF-I: IGFBP-3 ratio

(β= 3.27, 95% CI: 0.77, 5.78), and elevated IGF-I levels (β_{T3vsT1}= 6.61, 95% CI: 0.38, 12.83, p trend= 0.04) were associated with higher baseline VASS measures among comparison group women in the unadjusted analyses, these associations were not statistically significant in the multivariable models (Table S5.3). The association between IGF-I levels and VASS measures at baseline differed significantly among breast cancer cases and the comparison group ($p_{int}< 0.05$) (Table S5.3). Upon restricting the analyses to premenopausal women, the findings were similar to the overall associations reported above (Appendix H).

Discussion

In this analysis of tamoxifen's effect on the relationship between changes in IGFs and concomitant changes in breast density, tamoxifen decreased concentrations of circulating IGF-I among women diagnosed with breast cancer within the first year of treatment. Although VASS, a highly reliable surrogate marker of volumetric breast density[57,104,297] decreased from baseline to 12 months following tamoxifen treatment, the observed reduction was not statistically significant. No associations were noted between the changes in IGFs and concomitant changes in VASS, 12 months after treatment initiation among the tamoxifen-treated breast cancer patients. The observed declines in IGF-I among breast cancer patients may provide mechanistic insights into the biomechanisms of tamoxifen in breast carcinogenesis.

One prior observational study assessed the relationship between longitudinal changes in IGFs and changes in breast density, however, the authors evaluated the effect of exercise intervention among postmenopausal women[295]. Hence, the reported decrease in IGFs (IGF-I, IGFBP-3, and the IGF-I: IGFBP-3 ratio) and percent dense volume after 12 months of follow-up was not comparable to the present study that assessed the effect of tamoxifen treatment[295].

Similarly, the non-significant decreases in both IGFs (IGF-I and IGF-I: IGFBP-3 ratio) and breast density reported by the randomized clinical trial (RCT) that assessed the effect of vitamin D supplementation on changes in IGFs and breast density after 12-months follow-up among premenopausal women at high risk of breast cancer[296], cannot be compared to the tamoxifen mediated declines in IGF-I observed in the present study. The authors of this prior analyses also observed statistically significant relationship between longitudinal changes in IGFs and breast density[295,296]. In the observational study, changes in both IGF-I and IGFBP-3 were positively associated with changes in dense and percent dense area of area-based mammographic density, after 12 months of exercise intervention[295]. Statistically significant positive associations were also reported between changes in IGF-I (IGF-I and IGF-I: IGFBP-3 ratio) with changes in mammographic density at 12 months after vitamin D treatment in the RCT[296]. Although the present study was conducted among tamoxifen-treated breast cancer patients, and the changes in IGF-I had a non-statistically significant positive relationship with concomitant changes in VASS. The small sample size of this analysis may explain the lack of statistical significance observed in the association between concomitant changes in IGFs and VASS after 12 months of tamoxifen treatment. Future longitudinal studies with a larger sample size are needed to evaluate the influence of tamoxifen on the relationship between IGFs and breast density, and may provide insight into the underlying biological mechanisms of tamoxifen-associated declines in IGFs and/or breast density in breast cancer risk and progression.

Prior studies have demonstrated that tamoxifen alters circulating IGFs, reducing the levels of IGF-I and increasing the concentration of IGFBP-3, among breast cancer patients[33,37-39]. In this study, the circulating IGF-I, including bioavailable IGF-I (the IGF-I: IGFBP-3 ratio) also decreased among tamoxifen-treated women, 12 months following treatment initiation. Boyd et

al. has suggested that IGF-I has stronger mitogenic properties on breast tissues compared to estradiol, and the effect of tamoxifen in lowering IGF-I levels may contribute to its cytostatic effect on breast tumor cells[37]. Although the mechanism of action of tamoxifen on IGF levels is not fully understood, evidence suggests that this reduction may be caused by a decrease in the production of growth hormones causing lower IGF-I in circulation[33,39]. Previous literature has also indicated that the anti-estrogenic effect of tamoxifen on breast cancer cells may directly alter the levels of IGF-I and its binding proteins released by the tumor cells[33,37,298], which suggests that the tamoxifen mediated change in IGF levels may be a marker of treatment response. The observed tamoxifen-associated declines in circulating IGF-I may serve as a prognostic marker of improved breast cancer outcomes that may be important in clinical settings to improve adherence to tamoxifen.

Evidence indicates that tamoxifen is associated with a reduction in breast density, especially within the first 12-18 months of treatment[24,25,291,292], where the tamoxifen mediated decline is a strong indicator of therapeutic efficacy in both preventative[24-26] as well as prognostic settings[28,29,291]. Although a prior study conducted within the Ultrasound Study of Tamoxifen among 224 tamoxifen-treated and untreated patients reported statistically significant reduction in VASS within first year of tamoxifen therapy[234], in the present analyses that additionally included serial measures of IGFs, the observed decrease in the volumetric breast density (VASS) after 12 months of treatment initiation was not statistically significant. The limited sample size of 53 tamoxifen treated patients have possibly contributed to the absence of statistical significance observed in the tamoxifen mediated decrease of VASS.

Existing literature that assessed cross-sectional associations between IGFs and breast density suggest that IGF-I and IGF-I: IGFBP3 ratio are positively associated with premenopausal

breast density, while inverse relationships have been observed with IGFBP-3 [34-36]. However, it should be noted that these prior studies were conducted among women who had no history of breast cancer. In the present analyses, differential associations were observed in the relationship between IGFs and breast density by case-comparison group status, prior to tamoxifen initiation. Among the comparison group, the bioavailable IGF-I (IGF-I: IGFBP3 ratio) was positively associated with VASS measures at baseline. These findings were in concert with the prior literature where circulating IGF-I was positively associated with breast density among women without any history of breast cancer[34-36]. On the contrary, inverse associations were observed between circulating IGF-I and IGFBP3 in relation to VASS, among breast cancer patients, prior to tamoxifen treatment. In previous literature, differential associations between IGFs and breast density have been reported, by and within menopausal status. Both positive[34-36] and null associations[168-170] were reported among premenopausal women, while the evidence of an association was rather limited among postmenopausal women[35,36,168-170]. In the studies by Byrne et al.[34] and Diorio et al.[35], IGF-I and the IGF-I: IGFBP3 ratio were positively while IGFBP3 was inversely associated with breast density, among premenopausal women. In the study by Boyd et al., while IGF-I was positively associated with breast density among premenopausal women, the inverse association between IGFBP3 and breast density was not significant[36]. However, no statistically significant relationships were observed among postmenopausal women in any of the prior studies[35,36,168-170]. The current study had insufficient statistical power to evaluate the associations by menopausal status. However, when the analytic population was restricted to premenopausal women, it was observed that relationships between IGFs and breast density mirrored those observed in the overall associations, among both breast cancer patients and the

comparison group, prior to tamoxifen initiation. Future research is needed to explore this association by case-comparison group and menopausal status.

Limitations to this study include small sample size which may have affected the statistical significance of the findings. Since the length of follow-up was 12 months, an increased duration of treatment and follow-up may be needed to ascertain the long-term effects of tamoxifen on IGFs and/or VASS. The timing of daily tamoxifen intake among women in the Ultrasound Study of Tamoxifen was not known and thus, differences in the timing of tamoxifen intake may have led to variability in IGF levels. The half-life of tamoxifen is 5-7 days and that it reaches its highest concentration in serum approximately 5 hours after intake[200,263]. However, as patients are advised to take tamoxifen at the same time each day[266]. the within-individual variability in the IGF concentrations after tamoxifen initiation is expected to be minimal. Additionally, the changes in IGFs and changes in VASS from baseline to 12-month follow-up were assessed among tamoxifen-treated patients only. It was not possible to ascertain if the observed longitudinal changes were solely due to the effect of tamoxifen. However, one prior study conducted within the Ultrasound Study of Tamoxifen observed tamoxifen mediated declines in VASS, 4-6 months after treatment initiation[234]. Despite these limitations, this study has several important strengths. Findings from this analysis highlight tamoxifen-associated changes in IGF-I, a proliferative marker of breast cancer risk. The longitudinal design using serial measurements allowed assessment of changes in IGFs and VASS from baseline to 12 months following tamoxifen treatment. The use of the UST scanner, a novel imaging technique, to assess VASS, a quantitative measure of volumetric breast density[53,54,57,233,297] is a significant strength of this study. In contrast to mammography, the most widely used method to assess breast density, the UST technique objectively measures physical breast density using sound

speed (VASS) measures without any exposure to harmful ionizing radiation. This allowed for repeated breast imaging to monitor changes in breast density over time [60]. Additionally, VASS has high reliability for measuring changes in density over time[54]. The assessment of within-woman changes in IGFs and changes in VASS reduced the potential for confounding. Finally, our study population is racially diverse and more than 60% of the population was African-American.

In conclusion, the tamoxifen mediated declines in circulating IGF-I among breast cancer patients may provide etiologic insight into the underlying mechanisms that may influence breast cancer progression. Future well-powered longitudinal studies using repeated measurements of IGFs and breast density in relation to tamoxifen therapy are warranted to elucidate the biological underpinnings of tamoxifen mediated breast density declines in breast cancer risk and progression.

Table 5.1: Changes in circulating IGFs, volume averaged sound speed (VASS) measures, and BMI between baseline and 12-month follow-up among tamoxifen-treated women (n=53)

	Baseline visit (pre-tamoxifen)		**12-months visit (post-tamoxifen)**		**Change from baseline to 12-month**		**p-value***
	Mean (SD)	Range	Mean (SD)	Range	Mean (SD)	Range	
Breast density							
VASS (m/s)	1456.7 (15.1)	1434.4-1499.6	1452.8 (14.6)	1432.6-1515.6	-3.9 (8.7)	-28.6-4.9	0.18
IGFs (ng/ml)							
IGF-I	137.0 (42.1)	55.1-258.5	99.6 (31.1)	39.8-172.5	-37.3 (30.9)	-104.9-19.0	<0.0001
IGFBP-3	4058.6 (822.6)	2114.4-6091.5	4118.1 (791.8)	2590.0-6169.6	59.5 (498.1)	-949.7-1148.4	0.39
IGF-I: IGFBP-3 Ratio	0.03 (0.01)	0.01-0.05	0.02 (0.01)	0.01-0.04	-0.01 (0.006)	-0.02-0.005	<0.0001
BMI (kg/m^2)	30.4 (7.1)	18.8-54.2	30.5 (6.9)	19.9-53.8	0.07 (1.5)	-4.1-2.7	0.45

*p value estimated using paired t-tests. Change calculated by subtracting the measures at baseline from the measures at follow-up. VASS= volume averaged sound speed, IGFs= insulin-like growth factors, IGF-I= insulin-like growth factor-I, IGFBP-3= insulin like growth factor binding protein 3, BMI: body mass index

Table 5.2: Mean change in volume averaged sound speed (VASS) measures and circulating IGFs from baseline to 12-month follow-up by participant characteristics, among tamoxifen-treated women (n=53)

Participant characteristics	N (%)	Change in VASS (m/s)		Change in IGF-I		Change in IGFBP-3	
		Mean (SD)	p-value*	Mean (SD)	p-value*	Mean (SD)	p-value*
Age at baseline (years)			0.9		0.12		0.02
<45	11 (20.8)	-1.5 (12.8)		-30.9 (39.4)		201.7 (402.5)	
45-<50	13 (24.5)	-5.8 (7.3)		-43.5 (30.9)		-9.0 (383.6)	
50-<55	14 (26.4)	-4.9 (8.1)		-23.9 (24.2)		312.0 (574.6)	
≥55	15 (28.3)	-2.9 (6.9)		-49.2 (26.2)		-221.1 (452.6)	
BMI at baseline (kg/m^2)			0.22		0.77		0.19
<25	13 (24.5)	-7.2 (10.1)		-42.2 (41.5)		235.2 (442.9)	
25-30	14 (26.4)	-4.0 (6.4)		-33.6 (30.1)		120.1 (464.9)	
>30	26 (49.1)	-2.1 (8.8)		-36.9 (25.9)		-61.0 (525.6)	
BMI at 12 months (kg/m^2)			0.36		0.97		0.03
<25	14 (26.4)	-6.2 (10.1)		-38.7 (40.4)		209.3 (440.0)	
25-30	15 (28.3)	-4.6 (6.3)		-35.9 (29.7)		239.2 (518.3)	
>30	24 (45.3)	-2.1 (9.1)		-37.4 (26.5)		-140.3 (460.1)	
Change in BMI (kg/m^2)			0.004		0.09		0.34
< -0.6	18 (34.0)	0.93 (8.5)		-46.8 (29.3)		20.8 (570.2)	
-0.6 - <0.7	17 (32.0)	-4.34 (7.5)		-24.8 (29.2)		202.8 (352.1)	
≥ 0.7	18 (34.0)	-8.21 (7.7)		-39.7 (31.8)		-37.2 (534.0)	
Race			0.58		0.41		0.42
White	17 (32.1)	-5.5 (9.4)		-38.3 (37.0)		181.6 (599.9)	
Black/African American	32 (60.4)	-2.9 (8.7)		-34.5 (27.6)		-13.9 (433.3)	
Other	4 (7.5)	-4.9 (5.7)		-56.4 (29.1)		127.4 (541.8)	
Education			0.65		0.55		0.84
High school or less	17 (32.1)	-3.9 (6.8)		-40.0 (32.3)		70.0 (377.5)	
Some college	15 (28.3)	-2.2 (10.3)		-29.9 (29.2)		-3.6 (671.2)	
College or higher	21 (39.6)	-5.0 (9.1)		-40.5 (31.6)		96.0 (459.6)	
First degree relative with breast cancer			0.53		0.56		0.007
No	37 (69.8)	-3.4 (8.7)		-35.7 (33.2)		177.4 (492.2)	
Yes	16 (30.2)	-5.0 (8.7)		-41.2 (25.6)		-213.2 (406.4)	

Participant characteristics	N (%)	Change in VASS (m/s)		Change in IGF-I		Change in IGFBP-3	
		Mean (SD)	p-value*	Mean (SD)	p-value*	Mean (SD)	p-value*
Age at menarche (years)			0.72		0.22		0.002
≤ 12	30 (56.6)	-3.7 (9.8)		-35.5 (31.9)		-14.7 (472.0)	
13	8 (15.1)	-2.1 (5.2)		-54.4 (30.3)		-296.7 (307.6)	
≥ 14	15 (28.3)	-5.2 (8.1)		-31.8 (27.8)		397.8 (453.8)	
No. of live births			0.31		0.65		0.60
0	7 (13.2)	-7.9 (7.7)		-27.2 (34.6)		226.7 (412.5)	
1-2	27 (50.9)	-4.1 (10.2)		-39.1 (32.9)		11.6 (441.8)	
≥3	19 (35.9)	-2.0 (6.3)		-38.6 (27.5)		65.8 (603.1)	
Age at first birth (years)[a]			0.41		0.34		0.88
<20	11 (23.4)	-1.2 (6.7)		-41.7 (28.8)		-20.7 (446.0)	
20-24	9 (19.2)	-2.9 (5.7)		-28.6 (32.7)		-11.9 (287.0)	
25-29	15 (31.9)	-1.9 (10.8)		-33.6 (27.1)		16.3 (616.9)	
≥30	12 (25.5)	-6.9 (9.2)		-50.8 (33.4)		136.3 (580.0)	
Menopausal status			0.24		0.06		0.01
Premenopausal	37 (69.8)	-4.6 (9.7)		-32.1 (31.4)		170.6 (484.3)	
Postmenopausal	16 (30.2)	-2.1 (5.7)		-49.4 (27.1)		-197.4 (443.3)	
Former use of menopausal hormone therapy[b]			0.17		0.94		0.61
No	11 (31.3)	-3.4 (5.5)		-49.1 (22.1)		-236.9 (364.6)	
Yes	5 (68.8)	0.9 (5.7)		-50.1 (39.0)		-110.6 (625.2)	
Tertiles of baseline sound speed (m/s)			0.04		0.0005		0.04
<1446.83	18 (34.0)	-0.20 (3.2)		-43.8 (24.4)		-28.5 (574.2)	
1446.83-<1460.46	18 (34.0)	-4.07 (6.5)		-52.1 (32.6)		-83.3 (328.5)	
≥1460.46	17 (32.0)	-7.52 (12.6)		-14.9 (22.9)		303.8 (496.4)	

*p-values for differences in breast density, IGF-I, and IGFBP-3 across categories of each covariate. t-tests for covariates with two categories; ANOVA for covariates with 3 or more categories. [a]Among parous women (n=46). [b]Among postmenopausal women (n=16). VASS= volume averaged sound speed, IGF-I= insulin-like growth factor-I, IGFBP-3= insulin like growth factor binding protein 3

Table 5.3: Association between changes in circulating IGFs and changes in volume averaged sound speed (VASS) measures from baseline to 12-month follow-up, among tamoxifen-treated women (n=53)

Change in IGFs (ng/ml)		**Change in VASS (m/s)**		
	N	**Model 1*** β (95% CI)	**Model 2**** β (95% CI)	**Model 3**** β (95% CI)
Change in IGF-I[a]				
< -51.7	18	Ref	Ref	Ref
-51.7 - <-17.6	18	3.47 (-2.27, 9.22)	4.30 (-1.03, 9.62)	3.93 (-1.80, 9.67)
≥ -17.6	17	-1.78 (-7.61, 4.05)	0.85 (-5.43, 7.12)	1.26 (-5.40, 7.93)
p trend (p-het)		0.57 (0.19)	0.57 (0.25)	0.57 (0.38)
Change in IGF-I (continuous)[b]		-1.80 (-9.68, 6.08)	2.14 (-5.65, 9.92)	1.68 (-6.50, 9.87)
Change in IGFBP-3[a]				
< -177.9	18	Ref	Ref	Ref
-177.9 - < 269.7	17	-4.32 (-10.13, 1.50)	-4.15 (-9.81, 1.51)	-3.61 (-9.42, 2.20)
≥ 269.7	18	-5.04 (-10.77, 0.69)	-3.67 (-9.35, 2.02)	-3.17 (-8.99, 2.64)
p trend (p-het)		0.08 (0.17)	0.16 (0.26)	0.24 (0.38)
Change in IGFBP-3 (continuous)[b]		-0.35 (-0.83, 0.13)	-0.32 (-0.80, 0.16)	-0.27 (-0.76, 0.21)
Change in IGF-I: IGFBP-3 Ratio[a]				
< -0.01	18	Ref	Ref	Ref
-0.01 - < -0.007	17	1.28 (-4.59, 7.15)	2.13 (-3.38, 7.64)	1.73 (-3.90, 7.36)
≥ -0.007	18	3.15 (-2.81, 9.10)	0.65 (-5.13, 6.44)	-0.05 (-5.97, 5.88)
p trend (p-het)		0.29 (0.57)	0.77 (0.73)	0.97 (0.78)
Change in IGF-I: IGFBP-3 Ratio (continuous)		0.08 (-3.69, 3.84)	1.53 (-1.96, 5.02)	1.52 (-2.16, 5.20)

Beta estimates and 95% CI were estimated from linear regression analyses. Model 1* Unadjusted; Model 2**adjusted for age (ordinal), BMI change at 12 months (ordinal), menopausal status (categories), baseline VASS (ordinal); Model 3***Adjusted for age(ordinal), menopausal status (categories), BMI change at 12 months (ordinal), baseline VASS (ordinal), and race (categories). [a]The categories are based on tertile cut points. Tertile values (ng/mL): IGF-I: T1 = -104.9 – -51.7, T2 = -50.0 – -17.6, T3 = - 16.5 – 19.0; IGFBP-3: T1 = -949.7 – -177.9, T2 = -163.9 – 252.3, T3: 269.7-1148.4; IGF-I: IGFBP-3 Ratio: T1 = -0.03 – -0.01, T2 = -0.01 – -0.007, T3 = -0.007–0.005. [b]Concentrations per 100 ng/ml. VASS= volume averaged sound speed, IGFs= insulin-like growth factors, IGF-I= insulin-like growth factor-I, IGFBP-3= insulin like growth factor binding protein 3

Supplemental Tables

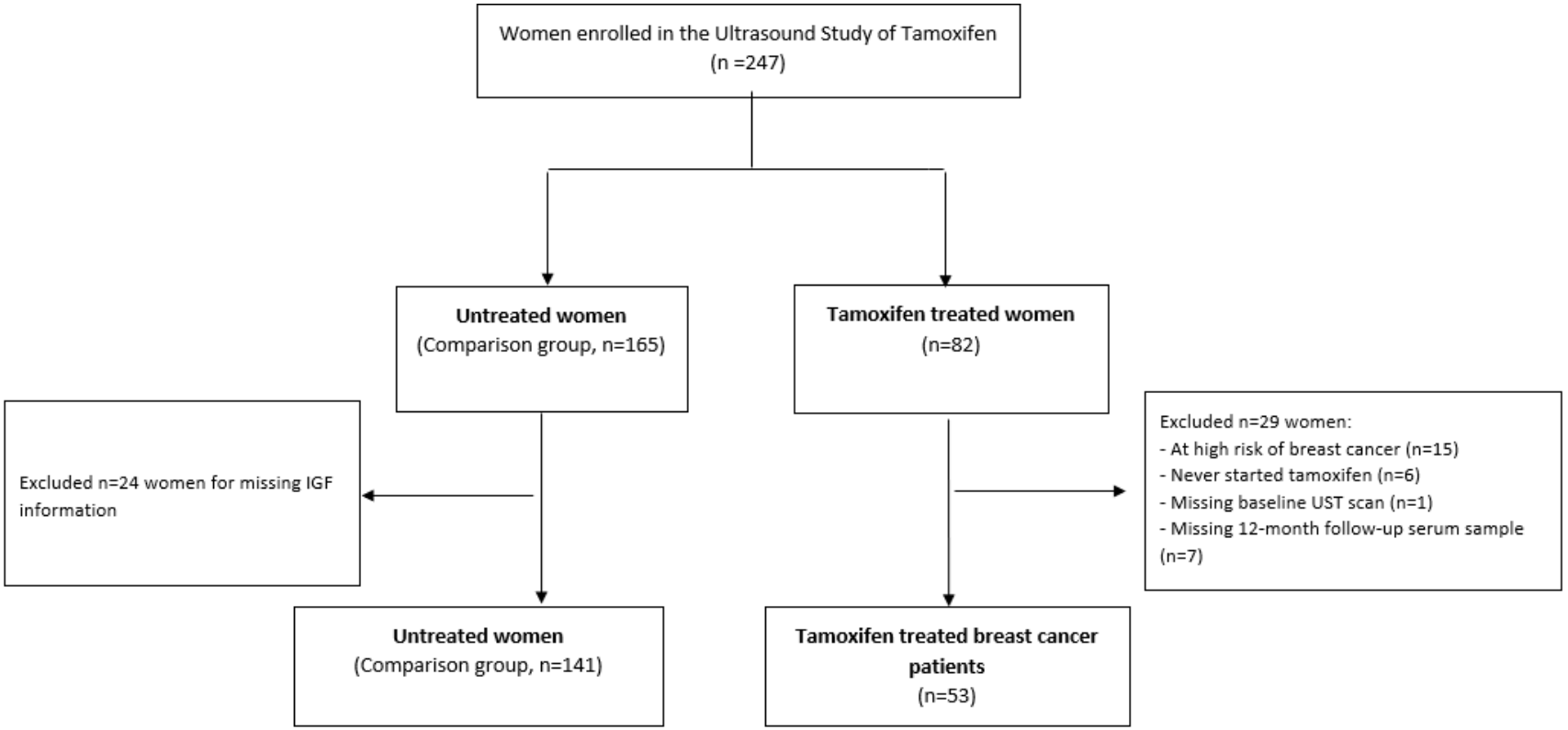

Figure S5.1: Flow diagram depicting the eligibility criteria and derivation of the analytic population

Table S5.1: Association of participant characteristics with volume averaged sound speed (VASS) measures and circulating IGFs at baseline among tamoxifen-treated women (n=53)

Participant characteristics	**N (%)**	**VASS (m/s)**		**IGF-I (ng/ml)**		**IGFBP-3 (ng/ml)**	
		Mean (SD)	p-value*	Mean (SD)	p-value*	Mean (SD)	p-value*
Age at Baseline (years)			0.16		0.11		0.6
<45	11 (20.8)	1458.5 (16.6)		154.6 (51.8)		3843.5 (1086.7)	
45-<50	13 (24.5)	1457.6 (12.5)		150.5 (39.2)		4113.0 (572.7)	
50-<55	14 (26.4)	1462.8 (18.6)		129.3 (34.3)		4220.5 (937.7)	
≥55	15 (28.3)	1449.0 (9.7)		119.5 (38.1)		4018.2 (710.6)	
BMI at Baseline (kg/m^2)			0.03		0.03		0.79
<25	13 (24.5)	1465.5 (18.9)		160.0 (44.8)		3993.5 (806.4)	
25-30	14 (26.4)	1456.8 (12.0)		125.7 (48.3)		3999.2 (837.9)	
>30	26 (49.1)	1452.3 (13.1)		131.5 (33.7)		4123.2 (849.5)	
Race			0.86		0.46		0.07
White	17 (32.1)	1456.0 (17.5)		133.6 (45.3)		4369.7 (1071.5)	
Black/African American	32 (60.4)	1457.1 (14.7)		136.2 (42.0)		3895.0 (651.2)	
Other	4 (7.5)	1457.0 (10.6)		157.5 (30.9)		4045.7 (624.2)	
Education			0.52		0.05		0.88
High School or Less	17 (32.1)	1459.4 (15.1)		145.0 (46.3)		4065.3 (775.8)	
Some College	15 (28.3)	1454.8 (17.3)		113.8 (37.3)		3915.6 (994.6)	
College or Higher	21 (39.6)	1455.9 (14.0)		147.0 (36.8)		4155.4 (748.4)	
First Degree Relative with Breast Cancer			0.41		0.61		0.31
No	37 (69.8)	1455.6 (15.2)		138.9 (44.8)		3982.5 (872.7)	
Yes	16 (30.2)	1459.3 (15.1)		132.5 (36.0)		4234.7 (686.0)	
Age at Menarche (years)			0.58		0.68		0.19
≤ 12	30 (56.6)	1456.0 (15.2)		141.0 (40.2)		4111.3 (812.9)	
13	8 (15.1)	1452.6 (8.5)		135.4 (46.4)		4429.3 (1022.7)	
≥ 14	15 (28.3)	1460.3 (17.7)		129.7 (45.5)		3755.6 (662.3)	
No. of Live Births			0.08		0.92		0.91
0	7 (13.2)	1464.1 (13.7)		141.0 (53.3)		4015.9 (849.5)	

Participant characteristics	N (%)	VASS (m/s)		IGF-I (ng/ml)		IGFBP-3 (ng/ml)	
		Mean (SD)	p-value*	Mean (SD)	p-value*	Mean (SD)	p-value*
1-2	27 (50.9)	1457.5 (14.4)		135.7 (37.6)		4018.7 (785.5)	
≥3	19 (35.9)	1452.8 (16.2)		137.3 (46.1)		4131.0 (902.5)	
Age at First Birth (years)[a]			0.64		0.28		0.39
<20	11 (23.4)	1451.2 (12.2)		124.8 (49.2)		4147.6 (852.6)	
20-24	9 (19.2)	1459.7 (16.3)		133.9 (34.7)		3711.4 (533.8)	
25-29	15 (31.9)	1456.7 (16.2)		130.4 (36.7)		3975.0 (909.2)	
≥30	12 (25.5)	1454.8 (16.0)		155.3 (41.0)		4374.4 (851.9)	
Menopausal Status			0.006		0.04		0.93
Premenopausal	37 (69.8)	1460.4 (15.8)		144.8 (41.2)		4065.5 (869.4)	
Postmenopausal	16 (30.2)	1448.3 (9.1)		118.8 (39.6)		4042.7 (728.9)	
Former Use of Menopausal Hormone Therapy[b]			0.18		0.83		0.76
No	11 (31.3)	1450.4 (9.4)		117.4 (37.0)		4082.9 (648.4)	
Yes	5 (68.8)	1443.7 (7.1)		122.0 (49.4)		3954.2 (963.0)	
Endocrine Symptoms in the past two weeks							
Hot Flushes	18 (34.0)		0.39		0.65		0.98
No	18 (34.0)	1455.3 (14.8)		138.8 (49.5)		4060.6 (875.5)	
Yes	17 (32.0)	1459.1 (15.9)		134.0 (26.8)		4055.4 (749.0)	
Joint pain	17 (32.1)		0.26		0.84		0.86
No	32 (60.4)	1459.0 (16.1)		138.1 (44.5)		4079.0 (936.3)	
Yes	4 (7.5)	1454.3 (13.9)		135.8 (40.3)		4037.4 (703.4)	
Tertiles of baseline sound speed (m/s)					0.24		0.05
<1446.83	17 (32.1)	-	-	133.9 (43.3)		4224.8 (807.6)	
1446.83-<1460.46	15 (28.3)	-	-	151.6 (44.8)		4275.9 (755.1)	
≥1460.46	21 (39.6)	-	-	124.8 (35.0)		3652.6 (800.6)	

*p values for differences in VASS and IGF levels across categories of each covariate. t-tests for covariates with two categories; Kruskal-Wallis test for covariates with 3 or more categories. [a]Among parous women (n=46). [b]Among postmenopausal women (n=16). VASS= volume averaged sound speed, IGF-I= insulin-like growth factor-I, IGFBP-3= insulin like growth factor binding protein 3

Table S5.2: Association of participant characteristics with circulating IGFs at baseline among untreated women in the comparison group (n=141)

Participant Characteristics	**N (%)**	**IGF-I**		**IGFBP-3**	
		Mean (SD)	p-value*	Mean (SD)	p-value*
Age at Baseline (years)			0.32		0.72
<45	29 (20.6)	131.6 (40.8)		3637.2 (912.7)	
45-<50	33 (23.4)	135.5 (53.6)		3833.0 (811.7)	
50-<55	31 (22.0)	123.9 (39.3)		3952.6 (898.0)	
≥55	48 (34.0)	118.9 (40.7)		3791.6 (987.2)	
BMI at Baseline (kg/m^2)			0.07		0.15
<25	33 (23.4)	139.5 (51.4)		3873.5 (689.7)	
25-30	40 (28.4)	127.8 (38.8)		3974.7 (707.4)	
>30	68 (48.2)	119.4 (41.7)		3671.8 (1083.7)	
Race			0.68		0.07
White	46 (32.6)	119.0 (30.0)		3936.6 (923.8)	
Black/African American	90 (63.8)	130.1 (49.9)		3699.4 (889.4)	
Other	5 (3.6)	130.5 (28.1)		4492.7 (860.2)	
Education			0.9		0.45
High School or Less	41 (29.1)	122.3 (39.0)		3632.9 (1068.0)	
Some College	45 (31.9)	132.5 (56.1)		3862.4 (846.6)	
College or Higher	55 (39.0)	124.7 (35.3)		3886.1 (828.3)	
First Degree Relative with Breast Cancer			0.83		0.61
No	111 (78.7)	126.9 (42.9)		3825.5 (924.6)	
Yes	30 (21.3)	125.0 (47.8)		3729.0 (846.7)	
Age at Menarche (years)			0.40		0.30
≤ 12	79 (56.0)	122.1 (40.6)		3730.2 (919.2)	
13	34 (24.1)	137.8 (54.9)		4017.2 (822.2)	
≥ 14	28 (19.9)	125.1 (36.0)		3758.1 (975.5)	
No. of Live Births			0.85		0.75
0	34 (24.1)	126.5 (39.1)		3871.5 (943.7)	

Participant Characteristics	N (%)	IGF-I		IGFBP-3	
		Mean (SD)	p-value*	Mean (SD)	p-value*
1-2	60 (42.6)	128.0 (49.1)		3824.4 (949.2)	
≥3	47 (33.3)	124.6 (40.6)		3731.9 (846.6)	
Age at First Birth (years)[a]			0.69		0.27
<20	40 (37.4)	131.9 (46.9)		3696.5 (962.4)	
20-24	30 (28.0)	123.3 (52.7)		4072.4 (946.6)	
25-29	21 (19.6)	118.3 (39.4)		3467.6 (808.4)	
≥30	16 (15.0)	129.4 (34.8)		3875.6 (639.3)	
Menopausal Status			0.06		0.4
Premenopausal	78 (55.3)	132.6 (42.7)		3863.7 (890.9)	
Postmenopausal	63 (44.7)	118.9 (44.4)		3732.2 (935.1)	
Former Use of Menopausal Hormone Therapy[b]			0.09		0.81
No	49 (77.8)	124.0 (47.0)		3679.7 (1009.6)	
Yes	14 (22.2)	100.9 (28.4)		3747.2 (923.2)	
Endocrine Symptoms in the past two weeks					
Hot Flushes			0.82		0.49
No	78 (55.3)	127.2 (50.0)		3756.8 (923.8)	
Yes	63 (44.7)	125.5 (50.0)		3864.5 (896.4)	
Joint pain			0.88		0.63
No	60 (42.6)	127.1 (37.9)		3761.5 (826.1)	
Yes	81 (57.4)	126.0 (48.0)		3837.1 (971.3)	
Tertiles of baseline sound speed (m/s)			0.005		0.36
<1446.83	46 (32.6)	122.1 (44.1)		3837.8 (1068.7)	
1446.83-<1460.46	48 (34.1)	115.2 (35.5)		3659.1 (847.7)	
≥1460.46	47 (33.3)	142.3 (47.5)		3921.7 (794.7)	

*p values for differences in IGF levels across categories of each covariate. t-tests for covariates with two categories; Kruskal-Wallis test for covariates with 3 or more categories. [a]Among parous women (n=107). [b]Among postmenopausal women (n=63). VASS= volume averaged sound speed, IGF-I= insulin-like growth factor-I, IGFBP-3= insulin like growth factor binding protein 3

Table S5.3: Associations between circulating IGFs and volume averaged sound speed (VASS), among tamoxifen-treated and untreated group, prior to treatment initiation

IGFs (ng/ml)	**Tamoxifen treated group (n=53)**			**Untreated group (n=141)**			**p-int**
		Model 1*	**Model 2****		**Model 1***	**Model 2****	
	N	β (95% CI)	β (95% CI)	N	β (95% CI)	β (95% CI)	
IGF-I							
T1	10	Ref	Ref	47	Ref	Ref	0.04
T2	26	8.69 (-2.44, 19.81)	1.51 (-9.15, 12.17)	47	2.84 (-3.38, 9.06)	1.82 (-4.12, 7.77)	
T3	17	1.31 (-10.6, 13.23)	-8.21 (-19.79, 3.36)	47	6.61 (0.38, 12.83)	2.52 (-3.31, 8.34)	
p trend (p-het)		0.89 (0.16)	0.09 (0.08)		0.04 (0.11)	0.39 (0.68)	
IGF-I (continuous)[a]		-2.24 (-12.33, 7.85)	-11.1 (-20.5, -1.63)		7.05 (1.29, 12.82)	2.83 (-2.60, 8.27)	0.03
IGFBP-3							
T1	8	Ref	Ref	46	Ref	Ref	0.88
T2	21	-2.12 (-14.90, 10.67)	-5.89 (-17.35, 5.57)	48	-1.53 (-7.84, 4.79)	-2.61 (-8.38, 3.16)	
T3	24	-4.87 (-17.43, 7.70)	-7.79 (-19.08, 3.49)	47	-1.94 (-8.29, 4.41)	-3.66 (-9.46, 2.14)	
p trend (p-het)		0.39 (0.69)	0.19 (0.39)		0.54 (0.81)	0.21 (0.44)	
IGFBP-3 (continuous)[a]		-0.60 (-1.09, -0.11)	-0.52 (-0.98, -0.06)		-0.01 (-0.29, 0.27)	-0.07 (-0.33, 0.19)	0.09
IGF-I: IGFBP-3 Ratio							
T1	17	Ref	Ref	47	Ref	Ref	0.71
T2	16	2.56 (-7.91, 13.03)	-4.06 (-14.39, 6.26)	47	4.43 (-1.67, 10.54)	1.29 (-4.41, 7.00)	
T3	20	8.53 (-1.38, 18.45)	-0.65 (-11.54, 10.24)	47	9.69 (3.58, 15.79)	5.82 (0.04, 11.59)	
p trend (p-het)		0.08 (0.21)	0.97 (0.66)		0.002 (0.009)	0.05 (0.11)	
IGF-I: IGFBP-3 Ratio (continuous)		3.15 (-1.32, 7.63)	-1.37 (-6.62, 3.88)		3.27 (0.77, 5.78)	2.11 (-0.30, 4.51)	0.36

Beta estimates and 95% CI were estimated from linear regression analyses. Model 1* Unadjusted; Model 2** Adjusted for age (ordinal), BMI (ordinal), menopausal status(categories), race (categories). p-het: p for heterogeneity. p-int: p for interaction between cases and controls (Model 2). [a]Concentrations per 100 ng/ml. Tertile values (ng/mL):- IGF-I: T1 <106.126, T2 = 106.126-<141.113, T3 ≥141.113; IGFBP-3: T1 <3408.10, T2 = 3408.10-<4141.89, T3: ≥4141.89; IGF-I: IGFBP-3 Ratio: T1 <0.029011, T2 = 0.029011-<0.035482, T3 ≥ 0.03548. VASS= volume averaged sound speed, IGFs= insulin-like growth factors, IGF-I= insulin-like growth factor-I, IGFBP-3= insulin like growth factor binding protein 3

Figure S5.2: Scatter plots of the association between changes in IGFs and changes in VASS, among tamoxifen-treated women, 12 months following treatment initiation

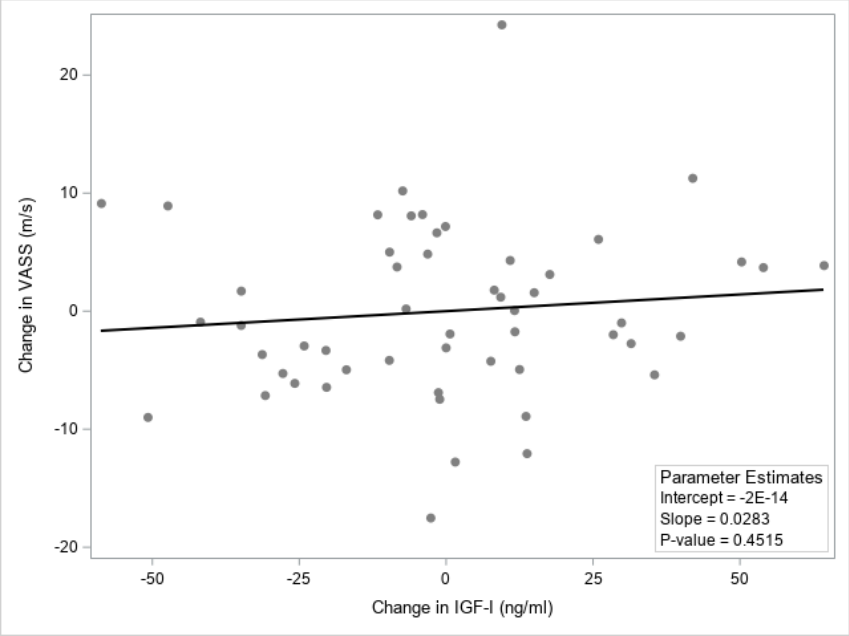

Figure S5.2a: Association between changes in IGF-I and changes in VASS

This scatter plot depicts the association between concomitant changes in IGF-I and VASS among tamoxifen-treated breast cancer patients.

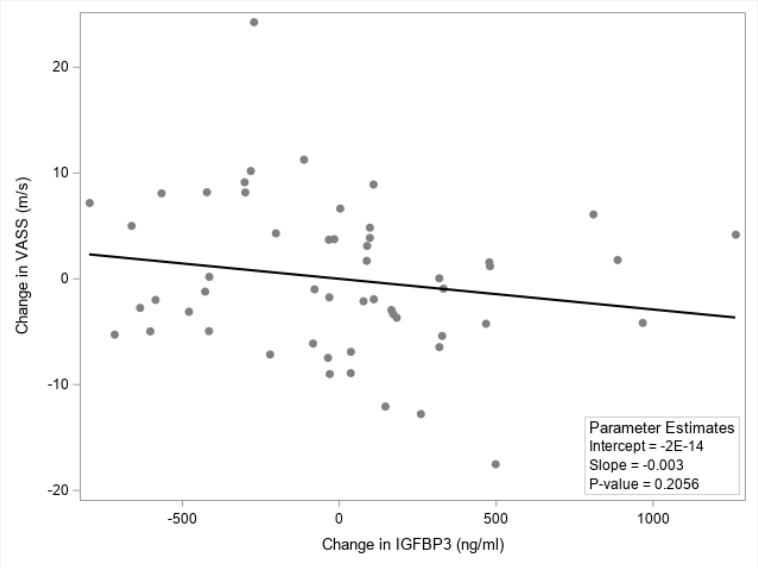

Figure S5.2b: Association between changes in IGFBP-3 and changes in VASS

This scatter plot depicts the association between concomitant changes in IGFBP-3 and VASS among tamoxifen-treated breast cancer patients.

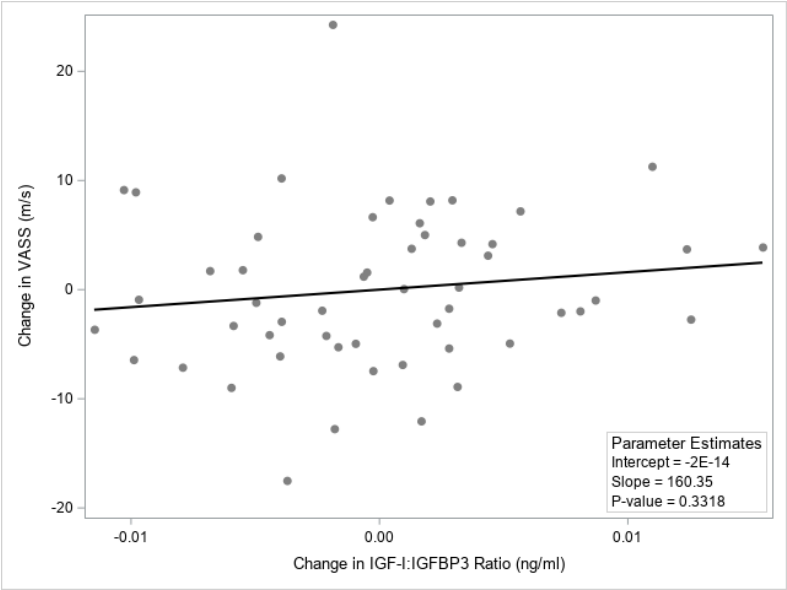

Figure S5.2b: Association between changes in IGF-I: IGFBP-3 ratio and changes in VASS

This scatter plot depicts the association between concomitant changes in IGF-I: IGFBP-3 ratio and VASS among tamoxifen-treated breast cancer patients.

*VASS= volume averaged sound speed, IGFs= insulin-like growth factors, IGF-I= insulin-like growth factor-I, IGFBP-3= insulin like growth factor binding protein 3

Chapter 6: Adjuvant endocrine therapy and risk of contralateral breast cancer: A systematic review and meta-analysis

Abstract

Background: Evidence synthesized from randomized clinical trials (RCTs) supports a reduction in contralateral breast cancer (CBC) risk with the use of adjuvant endocrine therapy. However, RCTs may not reflect real-world treatment scenarios given their controlled settings and highly selective populations. Population-based observational studies mirror treatment effects among the general population, but little is known about the effect of endocrine therapy on CBC risk among select subgroups of breast cancer patients with inconclusive findings reported. This systematic review and meta-analysis aimed to synthesize and re-analyze published findings from observational studies assessing the relationship between adjuvant endocrine therapy and CBC risk among women diagnosed with primary breast cancer.

Methods: PubMed and Embase databases were systematically searched for English language observational studies published by April 15, 2022, that reported risk estimates of the association between endocrine therapy use and CBC risk among primary breast cancer patients. A random effects meta-analysis was conducted to estimate summary relative risks (RRs) and 95% confidence intervals (CIs) for associations of endocrine therapy, defined as ever use of tamoxifen, aromatase inhibitors (AIs), or both, in relation to CBC risk. Heterogeneity across studies was assessed using the I^2 test. Subgroup analyses were conducted by study design (cohort, case-control), menopausal status (premenopausal, postmenopausal), and estrogen receptor (ER)-status of CBC (ER-positive, ER-negative).

Results: 17 eligible studies (retrospective cohort, n=13; case-control, n=4) were included in the systematic review and meta-analysis with a total sample size of 287,576 primary breast cancer

patients. Tamoxifen was the primary endocrine therapy assessed across the studies. Endocrine therapy use was associated with a reduced risk of CBC (RR: 0.62, 95% CI: 0.53, 0.73, I^2= 84.8%, p<0.0001), with no heterogeneity observed by study design (p_{het}= 0.9). The risk was similarly reduced when the meta-analysis was restricted to women with hormone receptor (HR)-positive primary breast cancer (RR: 0.62, 95% CI: 0.49, 0.78). In subgroup-specific analyses, endocrine therapy use reduced the risk of ER-positive CBC (RR: 0.55, 95% CI: 0.43, 0.70) but not ER-negative CBC (RR: 1.26, 95% CI: 0.95, 1.66), with significant heterogeneity observed between the groups (p_{het} <0.001). When stratified by menopausal status, a greater reduction in CBC risk was observed among premenopausal (RR: 0.58, 95% CI: 0.43, 0.78) compared to postmenopausal women (RR: 0.72, 95% CI: 0.60, 0.87).

Conclusion: The findings of this systematic review and meta-analysis of population based observational studies support that endocrine therapy reduces the risk of CBC among primary breast cancer survivors, in concert with the evidence synthesized from clinical trials, and further highlight the effectiveness of endocrine therapy in preventing ER-positive CBC, among both pre-and postmenopausal women.

Keywords: Endocrine therapy, tamoxifen, contralateral breast cancer, estrogen-receptor, menopausal status.

Introduction

The gradual increase in the incidence of breast cancer among women in the United States, in parallel with the steady rise in survival [2,3], places a growing population at high risk for second primary cancers. Breast cancer survivors have a greater than 2-fold increased risk of contralateral breast cancer (CBC), a second primary cancer occurring in the contralateral breast, compared to a women's risk of developing primary breast cancer, with a constant increase in risk over time[40,299,300]. Prior literature suggests a reduction in CBC risk with the use of adjuvant endocrine therapy[301], a treatment pivotal in decreasing mortality and improving prognosis among estrogen receptor (ER)-positive breast cancer patients[8,302]. The two most widely used endocrine therapies are tamoxifen and aromatase inhibitors (AIs) that inhibit tumor proliferation via their anti-estrogenic effects[8,288].

Evidence synthesized from randomized clinical trials (RCTs) suggests that endocrine therapy, including tamoxifen and AIs improves prognosis and reduces the risk of contralateral tumors among breast cancer patients[9,41,42,223]. Although clinical trials demonstrate treatment efficacy, they are conducted among a highly selective population under ideal conditions that may not reflect real-world treatment scenarios, limiting their generalizability. Population-based observational studies, however, mirror the effectiveness of these treatments in real-world settings. While observational studies, including cohort[43,45,47,48,85,303] and case-control[44,46,49,51,181,304,305] studies have primarily reported a reduction in CBC risk in relation endocrine therapy use, four cohort studies reported a null association between endocrine therapy use and CBC risk[50,306-308] and one cohort study reported an increased risk of CBC in relation to tamoxifen use among postmenopausal women who were diagnosed with breast cancer from 1977-1989[45]. Hence, it is crucial to summarize and re-analyze evidence of the efficacy of

endocrine therapies on CBC risk using observational studies and assess any differences by study designs.

Despite the suggested protective effect of endocrine therapy on CBC risk from RCTs[9,41,42,223], little is known about the effects of varying duration and gaps in use, factors crucial to real life treatments. Limited observational studies have assessed CBC risk in relation to duration and recency of use with inconsistent findings reported[44-47,49,51]. Additionally, few observational studies that have assessed the association between endocrine therapy use and CBC risk among subgroups of breast cancer patients, suggest differences in this relationship by menopausal status[43-47] and ER-status of CBC[47-52]. However, the differential effects of endocrine therapy on CBC risk among subgroups of breast cancer patients[43-52], or the influence of varying duration[44-47,49,51] are inconclusive and to date, these associations have not been synthesized using population-based studies.

This systematic review and a meta-analysis of observational studies aimed to assess the association between endocrine therapy use and the risk of developing CBC, by i) by study design, ii) menopausal status and iii) ER-subtype of CBC, and further summarized evidence of this relationship by varying duration and/or recency of use among women diagnosed with primary breast cancer.

Methods

This systematic review and meta-analysis was conducted following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines[309]. A systematic search of the PubMed and Embase databases was conducted on April 15, 2022. Original research articles that assessed the association between endocrine therapy, specifically, tamoxifen and AIs,

and the risk of contralateral breast cancer among primary breast cancer survivors were evaluated. The search was conducted using the combination of the following keywords: (*"tamoxifen" OR "aromatase inhibitors" OR "endocrine therapy"*) *AND ("Breast neoplasms" OR "breast cancer" OR "breast tumor" OR "breast carcinoma") AND "contralateral"* (Table S6.1).

Study selection

English language peer-reviewed original articles that met the following criteria were included: i) observational studies with comparative design (exposed vs unexposed) conducted among women diagnosed with either invasive or in-situ first primary breast cancer, ii) endocrine therapy use assessed as the exposure, iii) risk of CBC evaluated as the outcome, and iv) the study reported effect estimates (relative risks, RRs, hazard ratios, HRs, or odds ratios, ORs) and the corresponding 95% confidence intervals (CIs) for the relationship between endocrine therapy use and risk of CBC. There were no restrictions on the geographical location of the study population, publication date, or sample size. Eligible studies conducted using overlapping populations were reviewed and the study that included most comprehensive population with regards to a longer participant enrollment and/or follow-up period was selected for inclusion. The following types of studies were excluded from this analysis: meta-analyses, systematic or narrative reviews, randomized controlled trials, laboratory-based studies, editorials, reports, and conference abstracts. The study selection was conducted in two stages. First, the title and abstracts of all articles identified via the search strategy were screened to determine eligibility. Full text review was conducted for potentially eligible articles to confirm their inclusion. Reference lists of eligible articles were manually reviewed to identify other potentially relevant articles.

Data abstraction

Data were abstracted independently by two authors (R.G. and S.R) and reviewed for discrepancies. The following information was abstracted from the eligible articles: i) author name, publication year, country, study design and population, data source, participant characteristics, duration of follow-up (cohort studies), sample size, type of endocrine therapy received, prevalence of endocrine therapy use in the study population, prevalence of outcome (CBC) in the study population, statistical analyses, covariates assessed as confounders and/or sub-group analyses, and the reported effect estimates and 95% CIs.

Based on the recommendation of the Cochrane Handbook for Systematic Reviews, the Newcastle-Ottawa Scale (NOS) was used to assess the methodological quality of the observational studies included in this systematic review and meta-analysis[279]. This nine-star scale assesses the quality of the studies based on the following three aspects: selection of the groups (cases/control or exposed/unexposed), comparability of the groups, and exposure/outcome ascertainment. A score of 1-3 indicates low quality, 4-6 indicates moderate quality, and 7-9 indicates high quality [279].

Meta-analysis

A random effects meta-analysis was conducted to analyze the association between adjuvant endocrine therapy use and the risk of CBC using published effect estimates. Endocrine therapy use was summarized based on the following classification: ever use of any endocrine therapy defined as the use of tamoxifen, AIs, or both compared to never use of any endocrine therapy (reference group). Since CBC is a relatively rare outcome, the effect estimates (HRs, RRs, ORs) reported by the eligible studies approximate relative risk (RR) estimates and therefore, were

compared across the studies. The estimates and the corresponding 95% confidence intervals (CIs) of each study were log (natural) transformed and used to calculate standard errors (SEs). The pooled estimates and SEs across studies were used to generate summary relative risk (RR) estimates with 95% CIs. The overall and study-specific RRs and their corresponding 95% CIs were graphically presented using forest plots. Heterogeneity across studies was assessed using the I^2 test, where a I^2 value of >50% indicates the presence of high heterogeneity across the studies[310]. Since adjuvant endocrine therapy is primarily prescribed among hormone receptor (HR)-positive breast cancer patients[8,302], a meta-analysis was performed restricted to studies that reported effect estimates among HR-positive primary breast cancer survivors. Subgroup-specific analyses were conducted by study design (cohort, case-control), menopausal status (premenopausal, postmenopausal), and ER-status of CBC (ER-positive, ER-negative CBC). For subgroup analysis by menopausal status, age was used as a proxy for premenopausal (<50 years) and postmenopausal (≥ 50 years) status.

Studies that did not provide an overall risk estimate and only presented findings stratified by mutually exclusive groups, such as *BRCA1/2* mutation status[43,46,306,311], age[49,85,303,306], HR/HER2 status of primary breast cancer[48], menopausal status and calendar period of primary breast cancer diagnosis[45], were treated as separate studies in the meta-analyses. For the retrospective cohort study by Kramer et al., the risk estimates of ever use of tamoxifen were defined as endocrine therapy use in this meta-analysis, as there was a higher proportion of tamoxifen users (41%) compared to AI users (3%) in a population comprised of primarily postmenopausal (aged ≥50 years) breast cancer survivors[48]. The study by Gierach et al. also assessed both tamoxifen and AI use, however, the estimates of the former were reported only by duration and recency of treatment[47], hence the estimates of ever use of AIs were pooled for this

meta-analysis. Sensitivity analyses were conducted excluding studies that (1) reported risk estimates among BRCA mutation carriers[43,46,306,311] and (2) assessed low-dose tamoxifen use[308]. Publication bias was estimated by Egger's test[312] and visually examined by funnel plots. All analyses were conducted using Stata version 17.

Results

Systematic review

A total of 863 articles were identified from the initial database search (Figure 6.1). After excluding duplicate records (n=231), and screening for title and abstract, 33 studies were selected for inclusion. From the reference list of the 33 eligible studies, 3 additional articles were identified. The full text of 36 articles was reviewed for eligibility, of which 19 publications were excluded with the following reasons: exposure and/or outcome not relevant (n=11), same population included in the studies (n= 5), and non-observational design (n=3). In total, 17 studies that met the eligibility criteria were included in this systematic review and meta-analysis (Figure 6.1).

Study characteristics and quality

All observational studies eligible for inclusion (n=17) were published between 1995 and 2019 and included both retrospective cohort (n=13) and case-control studies (n=4) (Table 6.1). A total of 287,576 women diagnosed with primary invasive or in situ breast cancer were included in this systematic review and meta-analysis with the cohorts selected globally from Asia, Europe, and North America. The sample size of the retrospective cohort studies ranged from n=605 to n=85,411 with a total of 280,729 participants (CBC, n= 103,44), and of the case-control studies ranged from n=188 cases and n=328 controls to n=1521 cases and n=2212 controls, with a total of 6847 participants (n=2487 cases and n=4360 controls). The median(range) follow-up period

of the retrospective cohort studies ranged from 2.28 (0.45-8.89) to 7.7 (0.3-13.1) years (Table 6.1).

The analytic populations varied across the eligible studies. The majority of analyses were conducted among all breast cancer patients, irrespective of hormone receptor (HR) status, with the exception of two studies which restricted their analytic population to ER-positive breast cancer survivors[51,308]. Of the eligible studies, the analytic populations of four studies were comprised of *BRCA1/2* mutation carriers[43,46,306,311]. Information on endocrine therapy use was ascertained primarily from medical records[44,45,47-52,85,303,306,311,313], followed by hospital records[307], interviewer administered questionnaires[46], or self-reported data[43] (Table 6.1). Tamoxifen use was the primary endocrine therapy assessed (n=13)[43-50,306,308,311,313,314] and only two studies additionally assessed AIs[47,48]. Four studies reported associations with any endocrine therapy use[52,85,303,307], however, their analytic populations were primarily comprised of tamoxifen users. The quality of the studies assessed by the NOS scale ranged from 5 to 9, and n=13 articles scored ≥7, indicating an overall high quality of the published eligible studies (Table S6.2).

Association of endocrine therapy use and CBC risk

Findings from the meta-analysis of ever use of endocrine therapy support a statistically significant reduction in risk of CBC (RR: 0.62, 95% CI: 0.53, 0.73) compared to never use, with high heterogeneity observed between the studies (I^2= 84.8%, p<0.0001) (Figure 6.2). A similar inverse relationship was observed for retrospective cohort (RR: 0.62, 95% CI: 0.51, 0.75) and case-control (RR: 0.63, 95% CI: 0.52, 0.76) studies, with no observed heterogeneity by study design (p_{het}= 0.9) (Figure 6.2). When restricted to the studies that assessed tamoxifen as the endocrine therapy, ever use of tamoxifen was also associated with a similar reduction in the risk

of CBC in retrospective cohort (RR: 0.61, 95% CI: 0.47, 0.79) and case-control studies (RR: 0.63, 95% CI: 0.52, 0.76), compared to never use of tamoxifen (Figure S6.1). Since only two studies assessed AI use in relation to the risk of CBC[47,48], the estimates were not pooled for conducting a meta-analysis. The individual studies were conducted using the Kaiser Permanente (KP) Northwest/Colorado database[47] and the Netherlands Cancer Registry (NCR)[47], respectively. Though both studies reported a reduction in CBC risk in relation to AI use, the study using NCR conducted among breast cancer patients in the Netherlands reported a lower risk of CBC (RR: 0.32, 95% CI: 0.23, 0.44) compared to the study using KP database (RR: 0.48, 95% CI: 0.22, 0.97) among breast cancer survivors in the United States (Table 6.1).

Assessment of duration of endocrine therapy use and CBC risk

Duration and/or recency of endocrine therapy use was assessed by six studies (cohort, n=2; case-control, n=4) [44-47,49,51]. Due to differences in categories of treatment duration assessed across these few studies, it was not possible to quantitatively analyze the effects of varying duration of endocrine therapy on the risk of CBC. The findings of the effect of duration and recency of endocrine therapy on CBC risk from the six studies are summarized below.

<u>Duration of endocrine therapy use and CBC risk in case-control studies</u>

Among the eligible case-control studies, Cook et al. reported that ≥13 months of tamoxifen use was associated with a reduced risk of CBC (OR: 0.4, 95% CI: 0.2, 0.9), but not ≤12 months of use (OR: 0.7, 95% CI: 0.3, 1.5), when compared to never use of tamoxifen[44] (Table 6.1). Upon assessing the recency of use, the authors observed that ≤12 months since last use was inversely associated with CBC risk (OR= 0.4, 95% CI= 0.2, 0.8), however, no association was observed

between ≥13 months since last use of tamoxifen and the risk of CBC (OR= 1.0, 95% CI= 0.3, 3.1). In the study by Li et al., while <1 year of tamoxifen use (OR: 0.8, 95% CI: 0.5, 1.2) was not associated with the incidence of CBC, 1-4 years (OR: 0.6, 95% CI: 0.4, 0.8) and ≥ 5 years (OR: 0.6, 95% CI: 0.4, 0.8) of use were associated with a similar reduced risk of CBC, compared to never use of tamoxifen [51] (Table 6.1). Langballe et al. reported that both current use (OR: 0.73, 95% CI: 0.55, 0.97) and former use of ≤ 37 months (RR: 0.73, 95% CI: 0.53, 1.00) of tamoxifen was associated with a reduced risk of CBC compared to never use, but former use of ≥ 37 months had no association with CBC risk (RR: 0.98, 95% CI: 0.53, 1.00), compared to never users[49] (Table 6.1). In contrast to the aforementioned studies, Gronwald et al. reported that tamoxifen use for <1 year (OR: 0.37, 95% CI: 0.20, 0.67) and 1-4 years (OR: 0.53, 95% CI: 0.32, 0.87) was inversely associated with CBC risk, but not ≥ 4 years of use (OR: 0.83, 95% CI: 0.44, 1.55) of use, compared to never use of tamoxifen[46] (Table 6.1).

<u>Duration of endocrine therapy use and CBC risk in retrospective cohort studies:</u>

The cohort study by Gierach et al. reported findings by both duration and recency of tamoxifen use[47]. Among ER-positive breast cancer survivors, both current use (RR: 0.76, 95% CI: 0.64, 0.89) and former use was associated with the reduced risk of CBC, compared to never use. The reduction in risk was observed in relation to former use across all categories of recency of use: ≥ 30 days-<3 years (RR= 0.84, 95% CI= 0.74, 0.95), 3-<5 years (RR= 0.87, 95% CI= 0.75, 0.997), 5+ years (RR= 0.85, 95% CI= 0.71, 0.995) since last use [47] (Table 6.1). Mellemkjaer et al. demonstrated that current use of tamoxifen among premenopausal women diagnosed with primary breast cancer between 1999-2007 was inversely associated with the risk of developing CBC(HR= 0.34, 95% CI= 0.19, 0.58), but not past use of tamoxifen (1.04, 95% CI= 0.48, 2.23), when compared to never users[45] (Table 6.1). Among postmenopausal breast cancer survivors,

while current use of tamoxifen was associated with reduced risk of CBC among patients diagnosed in 1977-1989 (HR= 0.12, 95% CI= 0.02, 0.87), 1990-1998 (HR= 0.65, 95% CI= 0.43, 1.00), and 1999-2007 (HR= 0.45, 95% CI= 0.30, 0.67), past use of tamoxifen in the calendar period 1990-1998 and 1999-2007 was not associated the risk of developing CBC, compared to never users (Table 6.1). Past use of tamoxifen among postmenopausal women diagnosed with breast cancer from 1977-1989, in contrast, was associated with an increased risk of CBC (1.50, 95% CI= 1.20, 1.87) compared to never use[45]. In the same study, < 1 year (HR= 0.39, 95% CI= 0.18, 0.88) and 1-4 years (HR= 0.32, 95% CI= 0.17, 0.62) since last use of tamoxifen among premenopausal women diagnosed with primary breast cancer between 1999-2007, and 1-4 years since last use of tamoxifen among postmenopausal women diagnosed between 1990-1998 (HR= 0.68, 95% CI= 0.50, 0.94) and 1999-2007 (HR= 0.58, 95% CI= 0.39, 0.85) was inversely associated with CBC risk, compared to never use of tamoxifen (Table 6.1). However, among postmenopausal women diagnosed between 1977-1989, 1-4 years (HR= 1.52, 95% CI= 1.06, 2.18) and 5-9 years (HR= 1.68, 95% CI= 1.18, 2.41) since last tamoxifen use was associated with increased risk of CBC, when compared to never users[45] (Table 6.1).

Association of endocrine therapy use and CBC risk among HR-positive breast cancer patients

This meta-analysis demonstrated that ever use of endocrine therapy was associated with a reduced risk of CBC (RR: 0.62, 95% CI: 0.49, 0.78, I^2= 81.6%, p<0.0001), among HR-positive breast cancer survivors, compared to never use of endocrine therapy (Figure 6.3). Of the 17 eligible studies, six studies reported risk estimates of the association between endocrine therapy use and CBC risk among ER-positive breast cancer patients[43,45,47,50,51,308], and one study presented their findings by HR/HER2-status of the primary breast cancer[48].

Association of endocrine therapy use and CBC risk by ER-status of CBC

When stratified by ER-subtype of CBC, the findings of this meta-analysis indicate that endocrine therapy use reduced the risk of ER-positive CBC (RR: 0.55, 95% CI: 0.43, 0.70, I^2= 71.6%, p <0.0001), but not ER-negative CBC (RR: 1.26, 95% CI: 0.95, 1.66, I^2= 10.1%, p=0.22), compared to never use of endocrine therapy, with statistically significant heterogeneity observed between the groups (p_{het} <0.001) (Figure 6.4).

In this systematic review and meta-analyses, six studies reported risk estimates between endocrine therapy use and CBC risk by ER-status of CBC(Figure 6.4). Of these, the risk estimates of two studies were reported among ER-positive women[47,51], one study presented findings by HR/HER2 status of the primary tumor[48], and the remaining three were conducted among all breast cancer survivors irrespective of HR-subtype of primary cancer[49,50,52].

Association of endocrine therapy use and CBC risk by menopausal status

In the subgroup meta-analysis by menopausal status, use of endocrine therapy was associated with a greater reduction in risk of CBC among premenopausal breast cancer survivors (RR: 0.58, 95% CI: 0.43, 0.78, I^2= 77.6%, p <0.0001) compared to postmenopausal women (RR: 0.72, 95% CI: 0.60, 0.87, I^2= 71.6%, p <0.0001) (p_{het}= 0.22), when compared to never use of endocrine therapy (Figure 6.5). Of the eligible studies, eight studies assessed CBC risk among postmenopausal women, while seven studies assessed the same among premenopausal women in relation to endocrine therapy use (Figure 6.5).

Findings from the sensitivity analyses that meta-analyzed the efficacy of endocrine therapy on CBC risk after exclusion of studies conducted among *BRCA1/2* mutation carriers, or the study that assessed low-dose tamoxifen were similar to those presented above and are summarized in supplementary figures S6.2-S6.4**.**

Publication Bias

Results of the regression-based Egger's test (p= 0.06) suggested marginal evidence of publication bias in the articles assessing the relationship between endocrine therapy use and the risk of CBC (Figure S6.5). The same was confirmed by the nonparametric Begg test (p=0.07).

Discussion

Findings from this systematic review and meta-analysis of 17 published observational studies demonstrated that use of adjuvant endocrine therapy reduces the risk of CBC among primary breast cancer survivors, a finding which is in concert with those observed in RCTs. Significant heterogeneity in this relationship was observed in the subgroup-specific analyses by ER-subtype of CBC, where endocrine therapy use had a protective effect on the risk of ER-positive CBC, but not on ER-negative CBC. When stratified by menopausal status at primary breast cancer diagnosis, endocrine therapy reduced the risk among both pre-and postmenopausal women, where premenopausal breast cancer survivors had a greater reduction in risk of CBC, compared to postmenopausal women diagnosed with breast cancer.

Evidence from meta-analyses of RCTs reported that tamoxifen use was associated with an approximately 40% reduction in the risk of CBC among ER-positive breast cancer patients[9,41]. The findings of the present study also indicated that endocrine therapy, primarily tamoxifen use,

was associated with a 38% reduction in the risk of CBC among HR-positive breast cancer survivors. Thus, meta-analyzed findings from observational studies were remarkably consistent with the magnitudes of association observed in RCTs. A meta-analysis of clinical trials reported that treatment with AI use reduced CBC by approximately 54%, compared to never use of endocrine therapy[223]. In this meta-analysis, the lack of study power limited the ability to quantitatively examine the effect of AI use on the risk of developing CBC. However, the two observational studies that assessed AI use reported that AIs were associated with a 50-70% reduction in CBC risk[47,48], compared to never use of endocrine therapy. Future observational studies examining the relationship between AI use and CBC risk are warranted to explore this association further.

One of the most important aspects of assessing treatment outcomes in a real-world setting is evaluating the effects of varying duration and gaps in treatment. Although evidence from RCTs reflects the effectiveness of endocrine therapy in a controlled setting, clinical trials are severely limited in their ability to assess the magnitude and duration of the protective effects, which are pivotal in real world treatment scenarios. Since poor adherence is common among endocrine therapy users[315], information on the effects of duration and recency of use on contralateral tumor development is crucial in ascertaining the therapeutic efficacy and/or persistence of treatment effects during and after cessation of use. Upon synthesizing information from the limited population based observational studies that reported these associations[44-47,49,51], it was observed that current use of tamoxifen was consistently associated with a decreased CBC risk across the studies, compared to never use [45,47,49]. The relationship between former use of endocrine therapy and CBC risk varied across studies[45,47,49]. While a protective effect of former endocrine therapy use was reported by Gierach et al. across a varied period of recency of

treatment[47], and by Langballe et al. within 37 months of the treatment[49], no such protective effect was reported by Mellemkjaer et al[45]. Although Gierach et al. reported that both current and former tamoxifen use was associated with a reduced risk of CBC compared to never use, current use was associated with a greater reduction (25%) when compared to former use (15%)[47]. Studies that assessed the duration of endocrine therapy use, reported that >1 year of tamoxifen use is associated with a reduced risk of CBC[44,46,220], but not <1 year of use[44,51]. Given the importance of varying duration of treatment, further research is needed in this area to determine the maximum therapeutic benefits of endocrine therapy on CBC risk among primary breast cancer survivors.

Prior meta-analyses of clinical trials have synthesized evidence suggesting that endocrine therapy reduces the incidence of CBC, and improves prognosis and survival among breast cancer patients[9,41,223]. However, these studies do not reflect the heterogenous effect of endocrine treatment on select subgroups of breast cancer patients. The central finding of this meta-analysis is the differential association observed between endocrine therapy use and the risk of CBC by ER-subtype of CBC. While endocrine therapy was associated with a reduced risk of ER-positive CBC, no associations were observed for ER-negative CBC. These findings align with prior literature, specifically larger population-based studies suggesting that endocrine therapy is effective in reducing the risk of ER-positive CBC but not ER-negative CBC[47-49,220]. This is consistent with the evidence that endocrine therapy selectively inhibits the proliferation of estrogen-responsive ER-positive tumor cells, thereby being effective on ER-positive contralateral tumors only[41,316]. However, conflicting findings have been reported by a prior retrospective cohort study, which indicated a 5-fold increased risk of ER-negative CBC in relation to tamoxifen use[50]. Although the nested case-control study by Li et al., 2009 reported no

association between ever use of tamoxifen and the risk of ER-negative CBC, the findings differed by the duration of use, where breast cancer patients with ≥ 5 years of tamoxifen use had a 4.4-fold increased risk of ER-negative CBC, and approximately a 6-fold increased risk of developing ER/PR-negative CBC[220]. The same study, however, did not observe associations between <1 year or 1-4 years of tamoxifen use and the risk of either ER-negative or ER/PR-negative CBC. Future observational studies that assess subgroup-specific associations by ER-subtype of CBC and duration of endocrine therapy use in relation to CBC risk are warranted.

The menopausal status-specific findings of this meta-analysis support endocrine therapy mediated reductions in the risk of CBC among both pre-and postmenopausal women, where a greater magnitude of risk reduction was observed among premenopausal women (40%) compared to postmenopausal breast cancer survivors (30%). Among the studies that assessed this menopausal status-specific association, tamoxifen was the primary endocrine therapy used by the patients. Although clinically recommended for both pre-and postmenopausal women[286], tamoxifen is the primary treatment of choice among premenopausal women[283], while AIs are most common for postmenopausal women[317]. Prior literature suggests that compared to tamoxifen, AIs are a more effective treatment for postmenopausal women diagnosed with breast cancer[42]. Future studies that assesses menopausal status specific associations using both tamoxifen and AIs are needed to further elucidate this association.

Although the subgroup analysis by menopausal status demonstrated an inverse relationship between endocrine therapy and risk of CBC, one study conducted in Denmark using the Danish Breast Cancer Cooperative Group (DBCG) database, reported differential associations between tamoxifen use and risk of CBC, by menopausal status and the period of primary breast cancer diagnosis[45]. While tamoxifen use was associated with a reduced risk of

CBC among premenopausal women and postmenopausal women diagnosed between 1990-2007, an increased risk of CBC was reported among postmenopausal women diagnosed with primary breast cancer from 1977-1989[45]. Since tamoxifen was prescribed independent of HR-subtype of primary breast cancer in the earlier calendar periods in Denmark[45] and has no effect on ER-negative disease[41], authors posit that the observed increased risk of CBC may have been contributed by women with ER-negative breast cancer[45]. However, the null association between tamoxifen use and risk of CBC among ER/PR-negative postmenopausal primary breast cancer patients diagnosed between 1977-1989 did not support this rationale.

In addition to clarifying the important subgroup-specific associations of the effect of endocrine therapy on CBC risk, this systematic review and meta-analysis helped identify certain research gaps. Prior studies indicate differential effects of several key characteristics of the primary breast tumor, including histology, stage, and tumor size on the risk of CBC [91,98,300,318]. Evidence suggests that lobular and/or mixed histology[91,300,318], advanced stage[98], and increased tumor size (> 3 cm)[318] of primary breast cancer are associated with an increased risk of CBC. Despite these indications, limited data exists of the effect of tumor stage or size on the relationship between endocrine therapy and incidence of CBC. To date, only one observational study has assessed this relationship by the histology of the primary tumor and reported that endocrine therapy use reduced the risk of CBC among women diagnosed with lobular primary breast cancer compared to other histological types[49]. Given the importance of these determinants in tumorigenesis of the contralateral breast, exploring their effect on the relationship between endocrine therapy and risk of CBC is crucial in understanding any heterogeneity in the protective effects of endocrine therapy. Future population-based studies must explore the subgroup-specific associations by the histology, stage, and/or tumor size of primary breast cancer to provide much

needed insight into this relationship and determine whether therapeutic benefits of endocrine therapy vary by these factors.

This meta-analysis has some limitations to be considered. Due to the limited number of studies (n=2) that assessed AI use, it was not possible to pool the estimates to meta-analyze the effect of AI use on the risk of CBC. Differences in the categories of treatment duration across the studies limited the possibility of quantitatively examining the effects of duration of endocrine therapy use in a meta-analysis. Finally, the small sample size of the eligible studies (n=17) may have contributed to the high heterogeneity observed between the studies. Despite these limitations, there are several strengths of this systematic review and meta-analysis. The meta-analysis was conducted using published effect estimates of endocrine therapy on the incidence of CBC collected from over 200,000 breast cancer survivors and informed important subgroup-specific associations. The effect of endocrine therapy was quantitatively examined by study design, menopausal status, and ER-subtype of CBC which provided crucial evidence as to whether the therapeutic benefits of endocrine therapy vary among subgroups of breast cancer patients. The studies included in this analysis primarily collected data from medical records with linkage to population-based cancer registries, reducing information bias. The potential of bias in this meta-analysis, as determined by the NOS bias assessment scale was low. The eligible studies also controlled for important confounders in the relationship between endocrine therapy and CBC, including age and/or menopausal status, breast cancer treatment regimens (chemotherapy, radiotherapy), and primary breast cancer determinants (stage and histology). Finally, this meta-analysis highlighted the important gaps in research, specifically, the need for assessing the relationship between endocrine therapy and the risk of CBC, by types of endocrine therapy

(tamoxifen and AIs), duration of treatment, and crucial determinants of primary breast cancer, including, stage, histology, and tumor size.

This systematic review and meta-analysis of population-based observational studies successfully demonstrated the protective effect of endocrine therapy on ER-subtype of CBC, among pre-and postmenopausal breast cancer survivors in a real-world treatment setting. The limited studies that assessed AI use and duration of treatment, as well as other crucial subgroup-specific associations by important determinants of the primary tumor (stage/histology/tumor size) indicate a research gap that must addressed. Future research in this domain should aim to fully explore this relationship, focusing on types of treatment, duration and recency of use, and tumor characteristics of the primary breast cancer, using larger population-based observational studies. Such studies will provide valuable evidence as to whether the therapeutic benefits of endocrine therapy vary by any potential heterogeneity of these determinants; this work will be pivotal in moving toward more personalized treatment and to ensuring better care for breast cancer survivors.

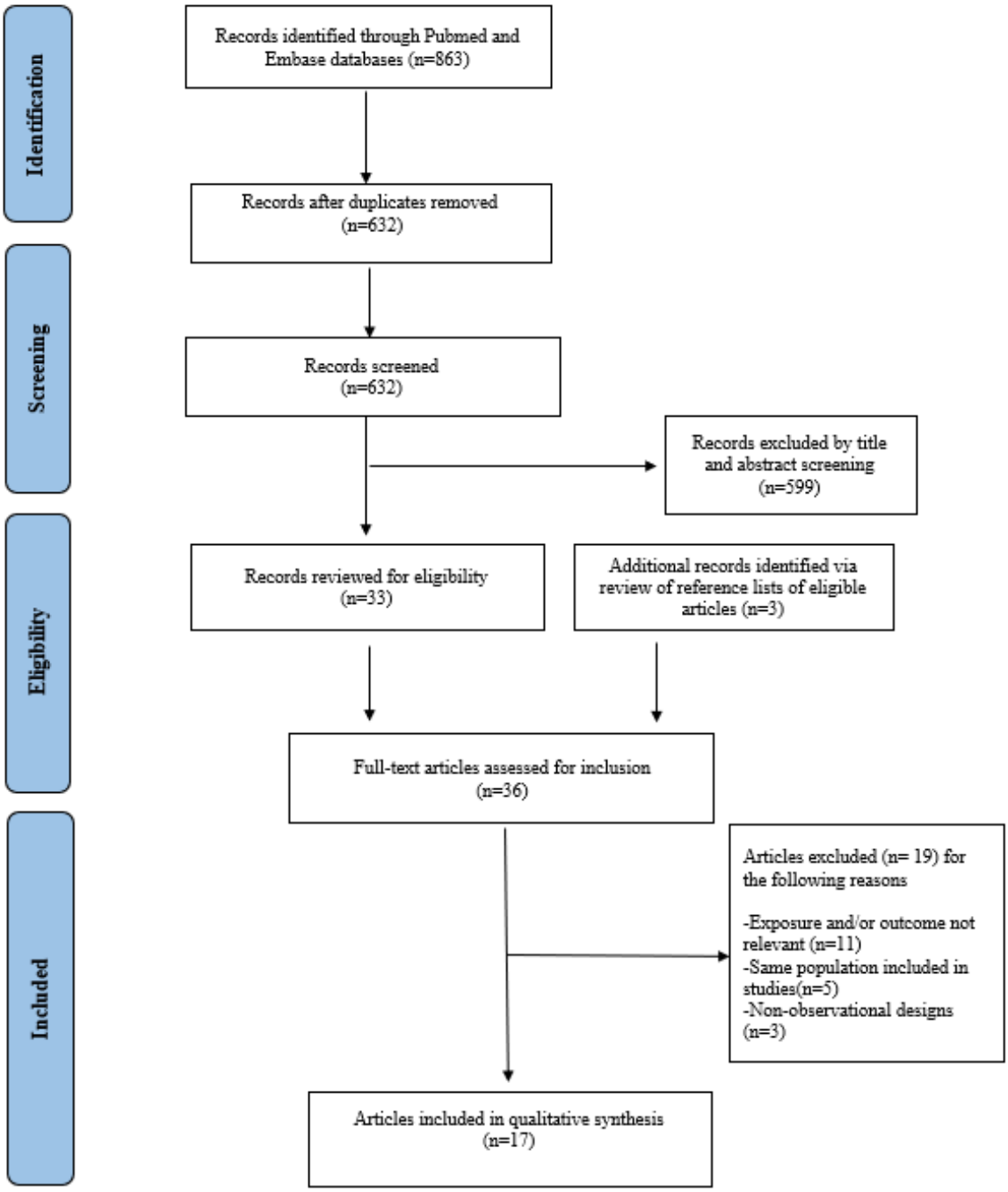

Figure 6.1: PRISMA flow diagram of studies assessing the association between endocrine therapy use and the risk of contralateral breast cancer

Table 6.1: Summary of the observational studies assessing the association of endocrine therapy use and risk of contralateral breast cancer included in this systematic review and meta-analysis (n=17)

Author, year	Data source, sample size, and country	Study population	Exposure definition and data Source	Statistical analyses	Association measures
Case-control studies					
L S Cook et al, 1995[44]	• Population based cancer registry, the Cancer Surveillance System (CSS) • Cases, n= 188, Controls, n= 328 • United States	• Women aged ≤ 85 years diagnosed with stage I-III unilateral breast cancer from January 1, 1978, through December 31, 1990 • Cases: Women diagnosed with ovarian, endometrial, or CBC before December 31, 1991 • Controls were matched to cases on year of initial breast cancer diagnosis, age, and stage of primary BC	• TAM use (ever/never) • Users: cases, n=18, controls, n=58 • Duration of use: ≤12 and ≥13 months • Time since last used: ≤12 and ≥13 months • Medical records and validated against a computerized pharmacy database in a subset of cases and controls	• MV Conditional logistic regression • Adjusted for: Surgical treatment, RT, and CT	• TAM use (ever vs never): OR= 0.5, 95% CI: 0.3, 0.9 • Duration of TAM use • ≤12 months: OR= 0.7, 95% CI= 0.3, 1.5 • ≥13 months: OR= 0.4, 95% CI= 0.2, 0.9 • Time since last use • ≤12 months: OR= 0.4, 95% CI= 0.2, 0.8 • ≥13 months: OR= 1.0, 95% CI= 0.3, 3.1
C Li et al, 2009[51]	• Population based cancer registry, the Cancer Surveillance System (CSS) • Cases, n= 367, Controls= 727 • United States	• Women diagnosed with first primary ER+ stage I-IIIB invasive breast cancer at age 40-79 years in the four county Seattle Puget Sound region from Jan 1990-September 30,2005 • Cases diagnosed with CBC from July 1, 1990, to March 31, 2007	• TAM use (ever/never) • Users: Cases, n= 214; controls, n=462 • HT (ever/never) use	• MV Conditional logistic regression • Adjusted for: RT	• TAM use (ever vs never): OR= 0.6, 95% CI: 0.5, 0.8 • Duration of TAM use • <1 year: OR= 0.8, 95% CI= 0.5, 1.2 • 1-4 years: OR= 0.6, 95% CI= 0.4, 0.8

Author, year	Data source, sample size, and country	Study population	Exposure definition and data Source	Statistical analyses	Association measures
		• Controls individually matched to cases in a 2:1 ratio on age, year of diagnosis, county at first diagnosis, race/ethnicity, SEER historic, stage of first breast cancer, and survival time	• Users: Cases, n= 223; controls, n=509 • Duration of use: <1 year, 1-4 years, and ≥5 years • Medical records and telephone interviews		• ≥5 years: OR= 0.6, 95% CI= 0.4, 0.8
Gronwald et al., 2014[46]	• Hereditary Breast Cancer Clinical Study • N= 1504 • Cases (CBC):411, Controls:1093 • 10 participating countries	• Women with BRCA1 or BRCA2 mutation diagnosed with bilateral breast cancer (cases) and unilateral breast cancer (controls) on or after January 1st, 1970 • Controls were matched to case on year of birth, age of diagnosis of primary breast cancer, BRCA mutation, and regional residence.	• TAM use (ever/never) • Ever use: Taken for a period equivalent to the period between first BC diagnosis and CBC diagnosis of the case • Users: • Cases: 14.8% • Controls: 24.7% • Duration of use: <1 year, 1-4 years, and ≥4 years	• MV Conditional logistic regression • Adjusted for: RT, CT	• TAM use (ever vs never) • All women: OR= 0.53, 95% CI: 0.37, 0.75) • All women: OR= 0.53, 95% CI: 0.37, 0.75) • BRCA2 mutation: OR= 0.39, 95% CI: 0.19, 0.83 • Duration of TAM use • <1 year: OR= 0.37, 95% CI= 0.20, 0.67 • 1-4 years: OR= 0.53, 95% CI= 0.32, 0.87 • ≥4 years: OR= 0.83, 95% CI= 0.44, 1.55

Author, year	Data source, sample size, and country	Study population	Exposure definition and data Source	Statistical analyses	Association measures
			• In-person questionnaire and telephone interviews		
Langballe et al, 2016[49]	• Eight population-based cancer registries • Cases, n= 1,521, Controls, n= 2,212 • United States, Canada, Denmark	Women aged < 55 years diagnosed with first primary invasive breast cancer between 1985-2008. • Cases diagnosed with CBC 1986-2011, and controls diagnosed with unilateral BC • Controls individually matched to cases by age and calendar-year of first diagnosis, cancer registry, and race/ethnicity.	• TAM use (ever/never) • Users: Cases, n= 467; controls, n=787 Duration of use • Current Use • Past use: <37 and ≥ 37 months since last use • Electronic medical records and self-reports	• MV Conditional logistic regression • Adjusted for: Age at primary BC diagnosis, first-degree family history of BC, BMI, histology, stage, ER/PR status of the first BC, RT, CT, and other ETs	• TAM use (ever vs never): OR= 0.76, 95% CI: 0.63, 0.92 • Duration of TAM use • Current use: OR= 0.73, 95% CI= 0.55, 0.97 • Past use (<37 months): OR= 0.73, 95% CI= 0.53, 1.00 • Past use (≥ 37 months): OR= 0.98, 95% CI= 0.73, 1.31
Retrospective cohort studies					
Newcomb et al, 1999[303]	• Nine cancer registries part of the Surveillance Epidemiology and End Results (SEER) • N= 85,411	• Women representing 10% of the US population diagnosed with local or regional primary BC from 1983-1990 from the states of Connecticut, Iowa, Hawaii, New Mexico, Utah, and the metropolitan areas of Atlanta,	• HT use (ever/never) • Users, n =14,984 • SEER medical records	• MV Cox proportional hazards regression • Adjusted for: Age, race, stage,	• HT use (ever/never): RR= 0.79, 95% CI: 0.70, 0.90

Author, year	Data source, sample size, and country	Study population	Exposure definition and data Source	Statistical analyses	Association measures
	• CBC, n=2,648 • United States	Detroit, and the Seattle–Puget Sound region • Mean follow-up duration= 76.6 months		registry, year of diagnosis, tumor size, and treatment (RT/CT)	
C Li et al, 2001[50]	• Population based cancer registry, the Cancer Surveillance System (CSS) • N= 8981 • CBC, n=189 • United States	• Women aged ≥ 50 years with early stage first primary invasive BC from January 1990 through December 1998 • Mean (SE) follow-up duration • TAM users= 3.9 (2.4) • TAM non-users= 4.2 (2.5)	• TAM use (ever/never) • Users, n =4,654 • Electronic medical records	• MV Cox proportional hazards regression • Adjusted for: Age, year of diagnosis, and tumor stage of the first breast cancer	• TAM use (ever/never): HR= 0.9, 95% CI: 0.7, 1.2
Kuo et al, 2006[313]	• National Taiwan University Hospital • N= 1,902 • CBC, n= 46 Taiwan	• Women with first primary, mainly invasive (85%) breast cancer diagnosed between 1990 and 1999 • Mean age (SD) at primary BC: 49.87 (12.25) years • Mean BMI (SD) at primary BC: 23.35 (3.62) Kg/m2 • Mean follow-up duration (SD): 3.59 (2.88) years	• TAM use (ever/never) • Users, n =1,475 • Medical records	• MV Cox proportional hazards regression • Adjusted for: Menopause, invasive lobular carcinoma of primary BC, CT, and/or RT	• TAM use (ever/never): HR= 0.50, 95% CI: 0.34, 0.74
Pierce et al., 2006[311]	• Institutional databases of 11 institutions	• Women diagnosed with stage I-II primary breast cancer by April 2001 and treated with breast conserving surgery	• TAM use (ever/never) • Medical records	• MV Cox proportional hazards regression	• TAM use (ever/never) : HR= 0.29 95% CI: 0.09, 0.97

Author, year	Data source, sample size, and country	Study population	Exposure definition and data Source	Statistical analyses	Association measures

	• N= 605 (BRCA mutation carriers= 160; sporadic controls =445)
	  • CBC= 48, (BRCA mutation carriers= 36; sporadic controls =12)
	• United States, Canada, Israel
		• BRCA1/2 mutation carriers were matched 1:3 by age and date of breast cancer diagnosis with women with < 5% probability of having a BRCA mutation (sporadic controls)
		• Median (range) age
		  • BRCA mutation carriers= 40.1 (21.9-74.3)
		  • Sporadic controls= 41.0 (22.6 – 75.1)
		• Median (range) follow-up duration
		  • BRCA mutation carriers= 7.9 (0.5-23.4)
		  • Sporadic controls= 6.7 (0.3 – 21.7)
				• Adjusted for: Age, stage, BRCA 1/2 mutation status, and CT	

M Schaapveld et al, 2007[85]	• Netherlands Cancer Registry: Comprehensive Cancer Centre North (CCCN), Amsterdam (CCCA) and South (CCCS) regions
	• N=45,229
	  • CBC, n=2,101
	• Netherlands
		• Women diagnosed with stage I-IIIA primary breast cancer between January 1989-January 2003 at the Comprehensive Cancer Centers of Netherlands
		• Mean follow-up duration (IQR): 5.8 (3.4-9.1)
			• HT use (ever/never)
			  • Users, n =11,698
			• Medical records
				• MV Cox proportional hazards regression
				• Adjusted for: Age, morphology, stage, and treatment at primary cancer diagnosis
					• HT (ever vs never): HR = 0.65, 95% CI: 0.48, 0.87)

Author, year	Data source, sample size, and country	Study population	Exposure definition and data Source	Statistical analyses	Association measures

Bouchardy et al, 2010[52]	• Geneva Cancer Registry
	• N=4,152
	  ◦ CBC, n= 63
	• Switzerland
		• Women diagnosed with unilateral first, primary invasive breast cancer between 1995 and 2007 in the Swiss canton of Geneva
		• Mean age (SD)
		  ◦ ER+= 60.4 (12.9) years
		  ◦ ER-= 56.8 (14.3) years
		  ◦ ER-unknown= 65.9 (15.9) years
		• Median follow-up duration= 5.2 years
			• Anti-estrogen use (ever/never)
			  ◦ Users
			  ◦ ER+ primary BC, n= 2836
			  ◦ ER- primary BC, n = 69
			  ◦ ER-unknown primary BC, n =78
			• Medical records
				• MV Cox proportional hazards regression
				• Adjusted for: Age, ER-status, period of diagnosis, and family history
					• Anti-estrogen use (ever vs never): HR= 0.51, 95% CI: 0.26, 0.99

Metcalfe et al, 2011[306]	• Medical records of 10 participating cancer genetics clinics
	• N=810
	  ◦ CBC, n= 149
	• United States, Canada
		• Women aged ≤ 65 years diagnosed with stage I-II invasive breast cancer between 1975 and 2008 from a family with a documented BRCA 1/2 mutation and at least 1 invasive BC case.
		• Mean age (range): 42.2 (21-65) years
		• Mean follow-up duration (range) = 11.5 (0.3-33.1) years
			• TAM use (ever/never)
			  ◦ Users, n =268
			• Medical records
				• MV Cox proportional hazards regression
				• Adjusted for: Age at first cancer diagnosis, mutation status (BRCA1 vs BRCA2), family history, and other treatments (ET, CT, oophorectomy)
					• TAM use (ever vs never)
					  ◦ All women: OR= 0.72, 95% CI: 0.47, 1.12)
					  ◦ BRCA1 mutation: OR= 0.61, 95% CI: 0.33, 1.13
					  ◦ BRCA2 mutation: OR= 0.86, 95% CI: 0.43, 1.73

Author, year	Data source, sample size, and country	Study population	Exposure definition and data Source	Statistical analyses	Association measures

T Aihara et al, 2012[307]	• Hospital data, n= 8 hospitals
	• N= 2074
	  • CBC, n=49
	• Japan
		• Women diagnosed with pTis, or pT1mic unilateral primary BC till December 2010, who underwent surgery
		• Mean age (SD) at primary BC: 54.1 (12.2) years
		• Mean follow-up duration= 5.2 years
			• ET use (ever/never)
			  • Users
			  • Overall, n=706
			  • TAM, n= 594
			  • AIs, n=112
			• Standardized electronic data collection form
				• MV Cox proportional hazards regression
				• Adjusted for: Age at surgery, type of surgery, histology (pTis/pT1mic)
					• ET use (ever vs never): HR= 0.64, 95% CI: 0.34, 1.19

KA Phillips et al, 2013[43]	• Enrolled in three cohort studies: IBCCS, kConFab, BCFR
	• N= 2464 (BRCA1= 1583; BRCA2=881)
	  • CBC, n=520
	• Australia, Canada, Europe, New Zealand, and the United States
		• Women with BRCA1/2 mutations and diagnosed with unilateral breast cancer since 1970 and enrolled in three cohort studies, IBCCS, kConFab, BCFR between September 1, 1993-December 2, 2009
		• Median (IQR):
		  • TAM users: 43 (37-49) years
		  • TAM non-users: 40 (34-47) years
		• Median follow-up duration (IQR)
		  • BRCA1 = 6.5(3.0-11.8) years
		  • BRCA2 = 6.6(3.0-11.2) years
			• TAM use (ever/never)
			  • Users:
			  • BRCA1 carriers, n= 383
			  • BRCA2 carriers, n= 454
			• Self-reported data
				• MV Cox proportional hazards regression
				Adjusted for: Age at primary BC diagnosis, year of diagnosis, bilateral oophorectomy, and country of residence
					• TAM use (ever vs never)
					  • BRCA1: OR= 0.38, 95% CI: 0.27, 0.55
					  • BRCA2: OR= 0.33, 95% CI: 0.22, 0.50

L Mellemkjaer et al, 2014[45]	• Danish Cancer Registry and Danish Breast Cancer	• Women aged >30 years diagnosed with invasive breast cancer from 1977-2007, who underwent surgery (BCS/mastectomy)	• TAM use
			  • Ever/never use
			  • Duration of use: Current/Past use
				• MV Cox proportional hazards regression	<u>Premenopausal (DBCG 1999-2007</u>
					• TAM use (ever vs never) : HR= 0.44, 95% CI: 0.27, 0.71

Author, year	Data source, sample size, and country	Study population	Exposure definition and data Source	Statistical analyses	Association measures

	Cooperative Group (DBCG) database
	• N=37,533
	  • CBC, n:
	  • Premenopausal =124
	  • Postmenopausal =1,281
	• Denmark
		• Median follow-up duration (5-95th pct)
		  • Premenopausal women, DBCG 1999-2007 = 2.83 (0.71-8.31) years
		  • Postmenopausal women, DBCG 1977-1989= 6.58 (0.54- 23.91); DBCG 1990-1998= 6.96 (0.22-16.87); DBCG 1999-2007 2.28 (0.45-8.89)
			• Time since start of use: <1, 1-4, 5-9, 10+ years)
			• Users, n= 15,863
			
			• DBCG database
				• Adjusted for: Age at primary BC diagnosis, histology of primary BC, RT, and CT (except postmenopausal women diagnosed between 1977-1989 and 1990-1998)	<u>Postmenopausal</u>
					• TAM use (ever vs never)
					  • DBCG 1977-1989: HR= 1.40, 95% CI: 1.12, 1.74)
					  • DBCG 1990-1998: HR= 0.82, 95% CI: 0.68, 0.99)
					  • DBCG 1999-2007: HR= 0.61, 95% CI: 0.44, 0.84)
					<u>Premenopausal (DBCG 1999-2007</u>
					• Duration of use
					  • Current use: HR= 0.34, 95% CI= 0.19, 0.58
					  • Past use: HR= 1.04, 95% CI= 0.48, 2.23
					
					• Time since last use
					  • < 1 years: HR= 0.39, 95% CI= 0.18, 0.88
					  • 1-4 years: HR= 0.32, 95% CI= 0.17, 0.62
					  • 5-9 years: HR= 1.45, 95% CI= 0.57, 3.70
					
					<u>Postmenopausal (DBCG 1977-1989)</u>
					• Duration of use

Author, year	Data source, sample size, and country	Study population	Exposure definition and data Source	Statistical analyses	Association measures
					• Current use: HR= 0.12, 95% CI= 0.02, 0.87 • Past use: HR= 1.50, 95% CI= 1.20, 1.87 • Time since last use • < 1 years: HR= 0.89, 95% CI= 0.36, 2.15 • 1-4 years: HR= 1.52, 95% CI= 1.06, 2.18 • 5-9 years: HR= 1.68, 95% CI= 1.18, 2.41 • ≥ 10 years: HR= 1.16, 95% CI= 0.80, 1.67 <u>Postmenopausal (DBCG 1990-1998)</u> • Duration of use • Current use: HR= 0.65, 95% CI= 0.43, 1.00 • Past use: HR= 0.87, 95% CI= 0.70, 1.06 • Time since last use • < 1 years: HR= 0.98, 95% CI= 0.45, 2.12 • 1-4 years: HR= 0.68, 95% CI= 0.50, 0.94 • 5-9 years: HR= 0.93, 95% CI= 0.68, 1.27 • ≥ 10 years: HR= 0.88, 95% CI= 0.58, 1.33

Author, year	Data source, sample size, and country	Study population	Exposure definition and data Source	Statistical analyses	Association measures

					<u>Postmenopausal (DBCG 1999-2007)</u>
					• Duration of use
					  • Current use: HR= 0.45, 95% CI= 0.30, 0.67
					  • Past use: HR= 1.05, 95% CI= 0.68, 1.63
					• Time since last use
					  • < 1 years: HR= 0.69, 95% CI= 0.34, 1.41
					  • 1-4 years: HR= 0.58, 95% CI= 0.39, 0.85
					  • 5-9 years: HR= 0.66, 95% CI= 0.33, 1.31

Guirreri-Gonzonga et al, 2016[308]	• European Institute of Oncology (IEO)
	• N=883
	  • CBC, n=62
	• Italy
		• Women diagnosed with ER+ primary DCIS between January 1997-December 2008, who underwent BCS
		• Median age (IQR) at primary BC: 53 (46-62) years
		• Median follow-up duration (IQR): 7.73 (5.81-9.67) years
			• Low dose TAM (user/non-users)
			  • Users, n= 467
			• European Institute of Oncology (IEO)
				• MV Cox proportional hazards regression
				• Adjusted for: Age, RT, BMI, margins, DCIS grade
					• Low dose TAM (users vs non-users): HR= 0.89, 95% CI: 0.51, 1.55)

G Gierach et al, 2017[47]	• Kaiser Permanente tumor registry
	  • Northwest (1990-2008), Colorado (1994-2008)
	• N=7,541
	  • CBC, n=248
		• Women aged ≥ 18 years and < 85 years diagnosed with unilateral invasive primary breast cancer from January 1, 1990- Dec 31, 2008, who survived at least 1 year	• AI use (ever/never)
			  • Users, n= 963
			• TAM use
			  • Users, n= 2,934
			  • Duration and recency of use
				• MV Poisson regression
				• Adjusted for: Study site, age, stage at diagnosis of primary breast
					• AI use (ever vs never): RR= 0.48, 95% CI: 0.22, 0.97
					• TAM use (duration)
					  • Current use: RR= 0.76, 95% CI= 0.64, 0.89

Author, year	Data source, sample size, and country	Study population	Exposure definition and data Source	Statistical analyses	Association measures
	• United States	following primary cancer diagnosis • Median age(range) at primary BC: 60.6 (24.9-84.9) years • Median follow-up duration (range): 6.3 (1.0-20.9) years	• Current Use • Former use: ≥ 30 days-<3 years, 3-<5 years, 5+ years since last use • Electronic KP prescription and medical records	cancer, ER status of the initial tumor, time since initial breast tumor diagnosis, year of diagnosis, AI use (with or without TAM), CT, and RT	• Former use (≥ 30 days-<3 years): RR= 0.84, 95% CI= 0.74, 0.95 • Former use (3-<5 years): RR= 0.87, 95% CI= 0.75, 0.997 • Former use (5+ years): RR= 0.85, 95% CI= 0.71, 0.995
I Kramer et al, 2019[48]	• Netherlands Cancer Registry • N=83,144 • CBC, n= 2,816 • Netherlands	• Women aged ≥ 18 years diagnosed with stage I-III invasive primary breast cancer, who underwent surgery between January 1, 2003- Dec 31, 2010 • Median age (range) at primary BC: 58.5 (19.4-101.3) years • Median follow-up duration (range): 7.7 (0.3-13.1) years	• TAM, AI: Ever use of TAM/AI vs never use of ET • Users: n= 40,283 • TAM, n= 33,862 • AIs, n= 2,393 • TAM+AIs, n=4,028 • Netherlands Cancer Registry	• MV Cox proportional hazards regression • Adjusted for: Age, stage, all other systemic therapies (CT, trastuzumab, other ET) at first BC diagnosis	• TAM use (ever vs never): (HR= 0.48, 95% CI: 0.44, 0.53) • AI use (ever vs never): (HR= 0.32, 95% CI: 0.23, 0.44) • TAM+AI use (ever vs never): (HR= 0.45, 95% CI= 0.36, 0.56)

TAM: Tamoxifen; AIs: Aromatase inhibitors; HT: Hormonal therapy; ET: Endocrine therapy; CT: Chemotherapy; RT: Radiotherapy; DCIS: Ductal carcinoma in situ; MV: Multivariable

*No details on TAM use was provided by Pierce 2006; Newcomb 1999 and Bouchardy 2010 defined hormone therapy and anti-estrogen use, respectively, primarily as tamoxifen use. Gierach 2017 defined TAM and AI use as treatment accumulated ≥90 days of therapy during any one treatment course following the first primary cancer diagnosis vs never use or used for <90 days. Guirreri-Gonzonga 2016 defined low dose tamoxifen use as 10 mg every other day, or 20 mg once a week for 5 years

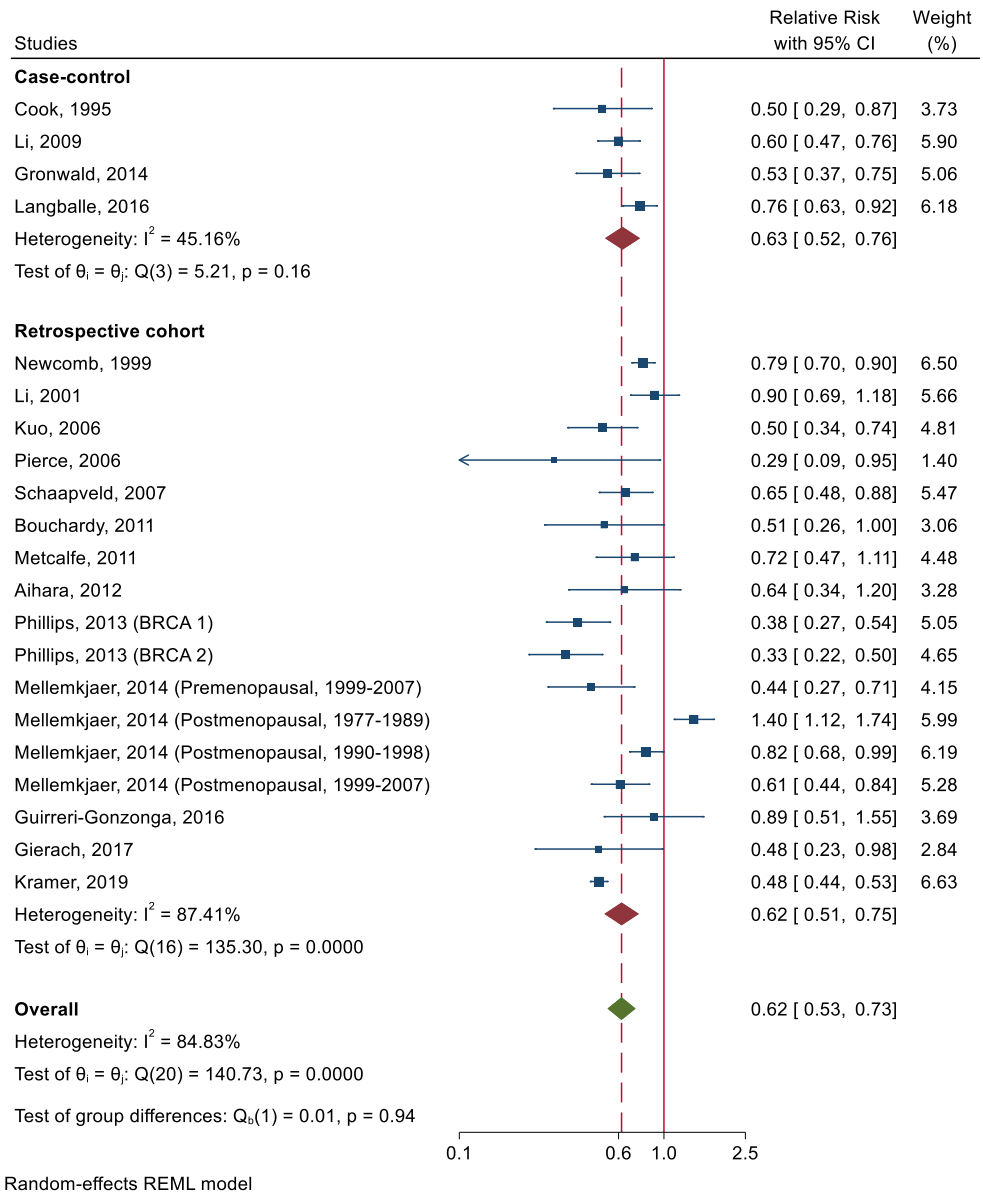

Figure 6.2: Forest plots of observational studies assessing the association between ever use of endocrine therapy and the risk of contralateral breast cancer by study design, among primary breast cancer survivors (n=17)

This forest plot depicts the relative risk of the association between any endocrine therapy and the risk of contralateral breast cancer by case-control and retrospective cohort study design. The studies were conducted among all primary breast cancer (irrespective of ER-subtype), except for Guirreri-Gonzaga 2016 and Li 2009, which were conducted among ER+ breast cancer survivors. Pierce 2006, Metcalfe 2011, Phillips 2013, and Gronwald 2014 were conducted among BRCA mutation careers. Phillips et al reported findings stratified by BRCA mutation status. Mellemkjaer 2014 reported estimates by menopausal status and calendar period of primary breast cancer diagnosis. All effect estimates are based on ever use of tamoxifen, except for Newcomb 1999, Schaapveld 2007, Bouchardy 2011 and Aihara 2012, which reported any endocrine therapy use, and Gierach 2017, which reported ever use of AIs. Guirreri-Gonzaga 2016 assessed low dose tamoxifen use. The studies are ordered in the plot by respective publication dates.

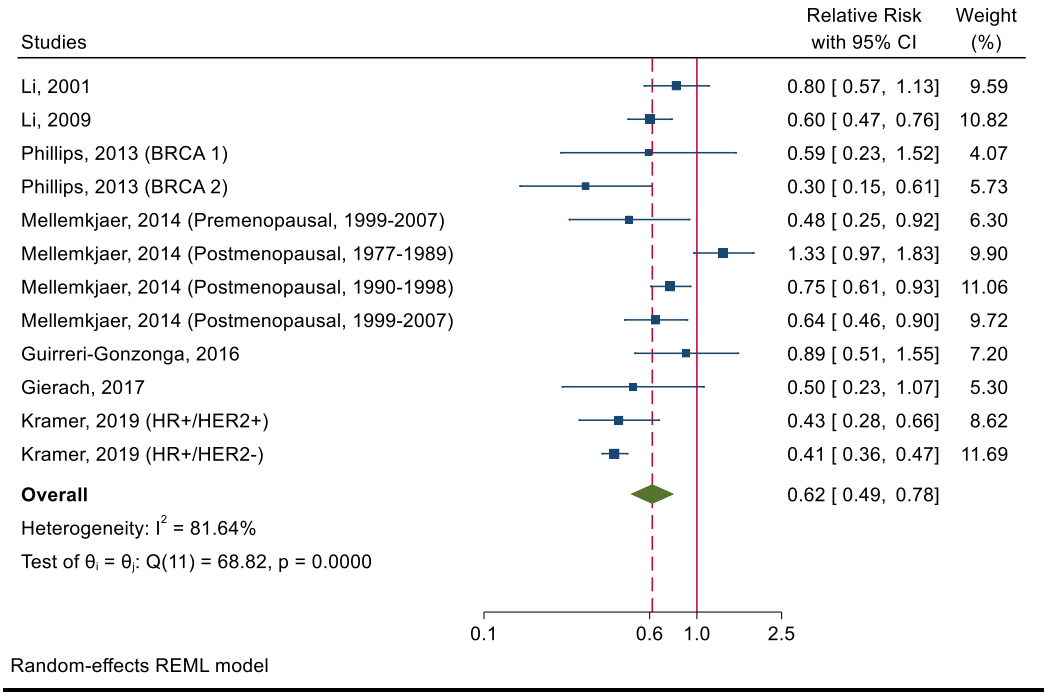

Figure 6.3: Forest plots of observational studies assessing the association between ever use of endocrine therapy and the risk of contralateral breast cancer among HR-positive primary breast cancer survivors (n=7)

This forest plot depicts the relative risk of the association between any endocrine therapy and the risk of contralateral breast cancer among HR-positive breast cancer patients. Phillips 2013 was conducted among BRCA mutation careers and reported findings stratified by BRCA mutation status. Mellemkjaer 2014 reported estimates by menopausal status and calendar period of primary breast cancer diagnosis. All effect estimates are based on ever use of tamoxifen, except Gierach 2017, which reported ever use of AIs. Guirreri-Gonzaga 2016 assessed low dose tamoxifen use. The studies are ordered in the plot by respective publication dates.

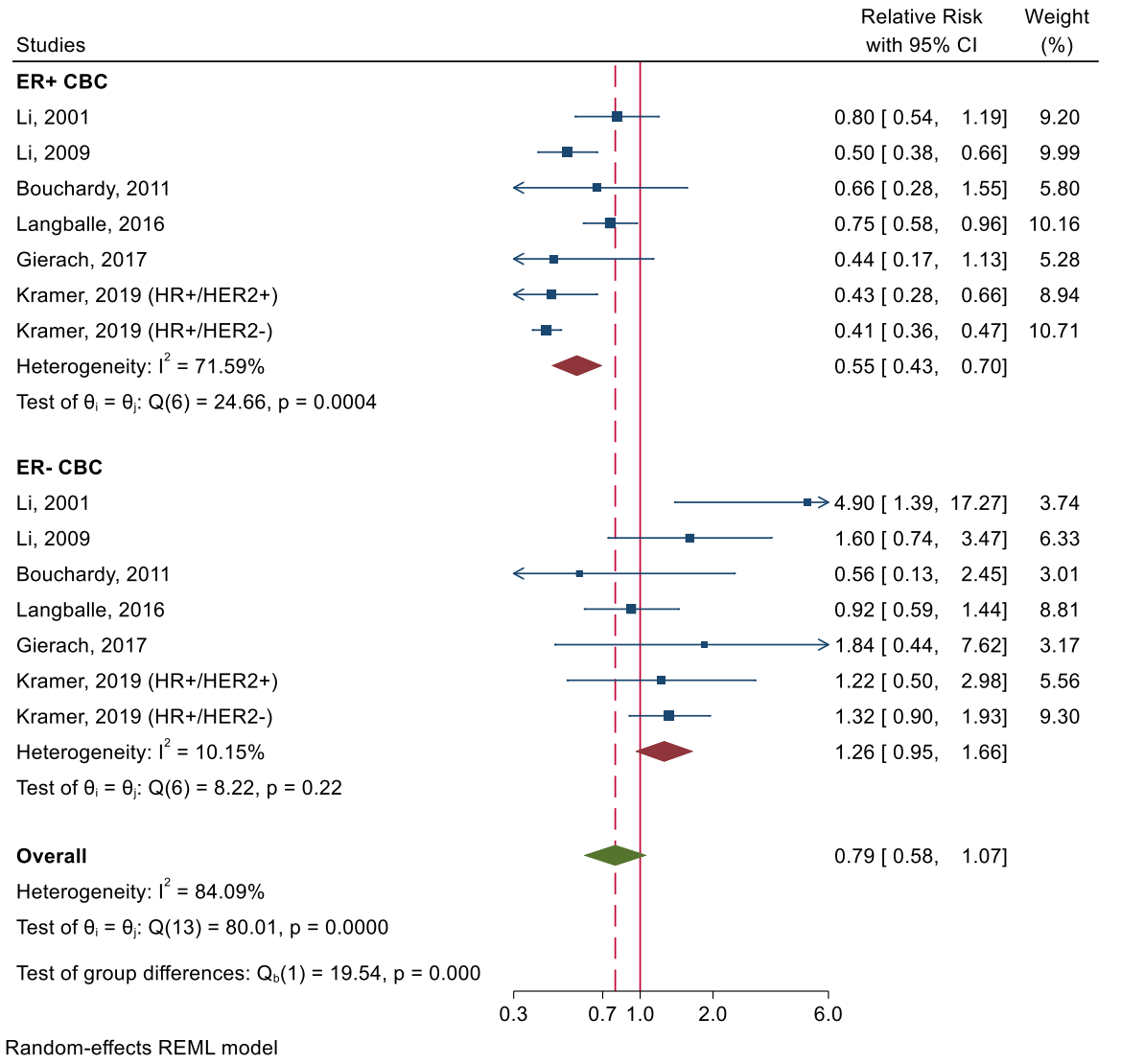

Figure 6.4: Forest plots of observational studies assessing the association between ever use of endocrine therapy and the risk of contralateral breast cancer by ER-subtype of CBC, among primary breast cancer survivors (n=6)

This forest plot depicts the relative risk of the association between any endocrine therapy and the risk of contralateral breast cancer among breast cancer survivors with ER-positive and ER-negative CBC. The studies were conducted among all primary breast cancer (irrespective of ER-subtype), except Li 2009, which was conducted among ER+ breast cancer survivors. Mellemkjaer 2014 reported estimates by menopausal status and calendar period of primary breast cancer diagnosis. All effect estimates are based on ever use of tamoxifen, except Bouchardy 2011, which reported any endocrine therapy use, and Gierach 2017, which reported ever use of AIs. The studies are ordered in the plot by respective publication dates.

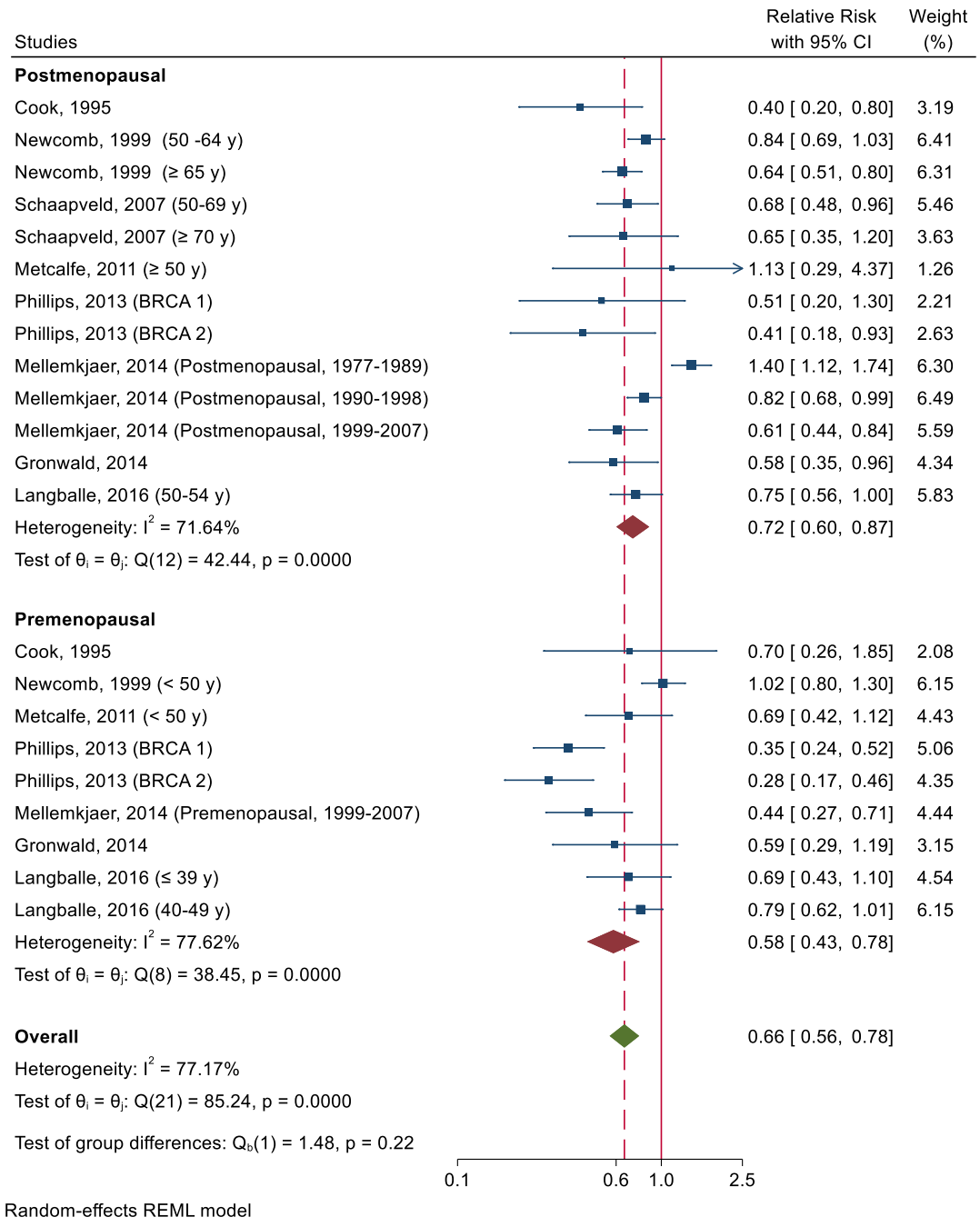

Figure 6.5: Forest plots of observational studies assessing the association between ever use of endocrine therapy and the risk of contralateral breast cancer, by menopausal status, among primary breast cancer survivors (n=6)

This forest plot depicts the relative risk of the association between any endocrine therapy and the risk of contralateral breast cancer among pre- and postmenopausal breast cancer survivors. Metcalfe 2011, Phillips 2013, and Gronwald 2014 were conducted among BRCA mutation careers. Phillips et al reported findings stratified by BRCA mutation status Mellemkjaer 2014 reported estimates by menopausal status and calendar period of primary breast cancer diagnosis. All effect estimates are based on ever use of tamoxifen, except for Newcomb 1999 and Schaapveld 2007, which reported any endocrine therapy use. The studies are ordered in the plot by respective publication dates.

Supplemental Tables and Figures

Table S6.1: Search string used to conduct the comprehensive search for the articles assessing endocrine therapy use and the risk of contralateral breast cancer on PubMed and Embase database

PubMed Search String
Tamoxifen[mh] OR tamoxifen[tiab] OR aromatase inhibitors[mh] OR endocrine therapy[mh] OR aromatase inhibitor*[tiab] OR anastrozole[tiab] OR Exemestane[tiab] OR letrozole[tiab] OR nolvadex[tiab] OR soltamox[tiab] OR endocrine therapy[tiab] AND (Breast neoplasms[mh] OR breast cancer[tiab] OR breast tumor*[tiab] OR breast carcinoma[tiab]) AND contralateral[tiab]

Embase Search String
'tamoxifen'/exp OR 'aromatase inhibitor'/exp OR 'aromatase inhibitor'/exp OR tamoxifen:ti,ab OR 'aromatase inhibitor*':ti,ab AND ('breast tumor'/exp OR 'breast neoplasm*':ti,ab OR 'breast cancer':ti,ab OR 'breast tumor*':ti,ab OR 'breast tumour*':ti,ab OR 'breast carcinoma':ti,ab) AND contralateral:ti,ab

Table S6.2: Quality assessment of the observational studies based on the Newcastle-Ottawa Scale

Author, year	Selection	Comparability	Exposure	Outcome	Total score
Case-control studies					
Cook, 1995	****	**	**	-	8
Li, 2009	****	**	***	-	9
Gronwald, 2014	**	**	*	-	5
Langballe, 2016	****	**	**	-	8
Retrospective cohort studies					
Newcomb, 1999	****	**	-	***	9
Li, 2001	****	**	-	***	9
Kuo, 2006	***	**	-	**	7
Pierce, 2006	**	**	-	**	6
Schaapveld, 2007	****	**	-	***	9
Bouchardy, 2011	****	**	-	***	9
Metcalfe, 2011	****	*	-	**	7
Aihara, 2012	***	*	-	**	6
Phillips, 2013	**	**	-	**	6
Mellemkjaer, 2014	****	**	-	***	9
Guirreri-Gonzonga, 2016	***	**	-	**	7
Gierach, 2017	****	**	-	***	9
Kramer, 2019	****	**	-	***	9

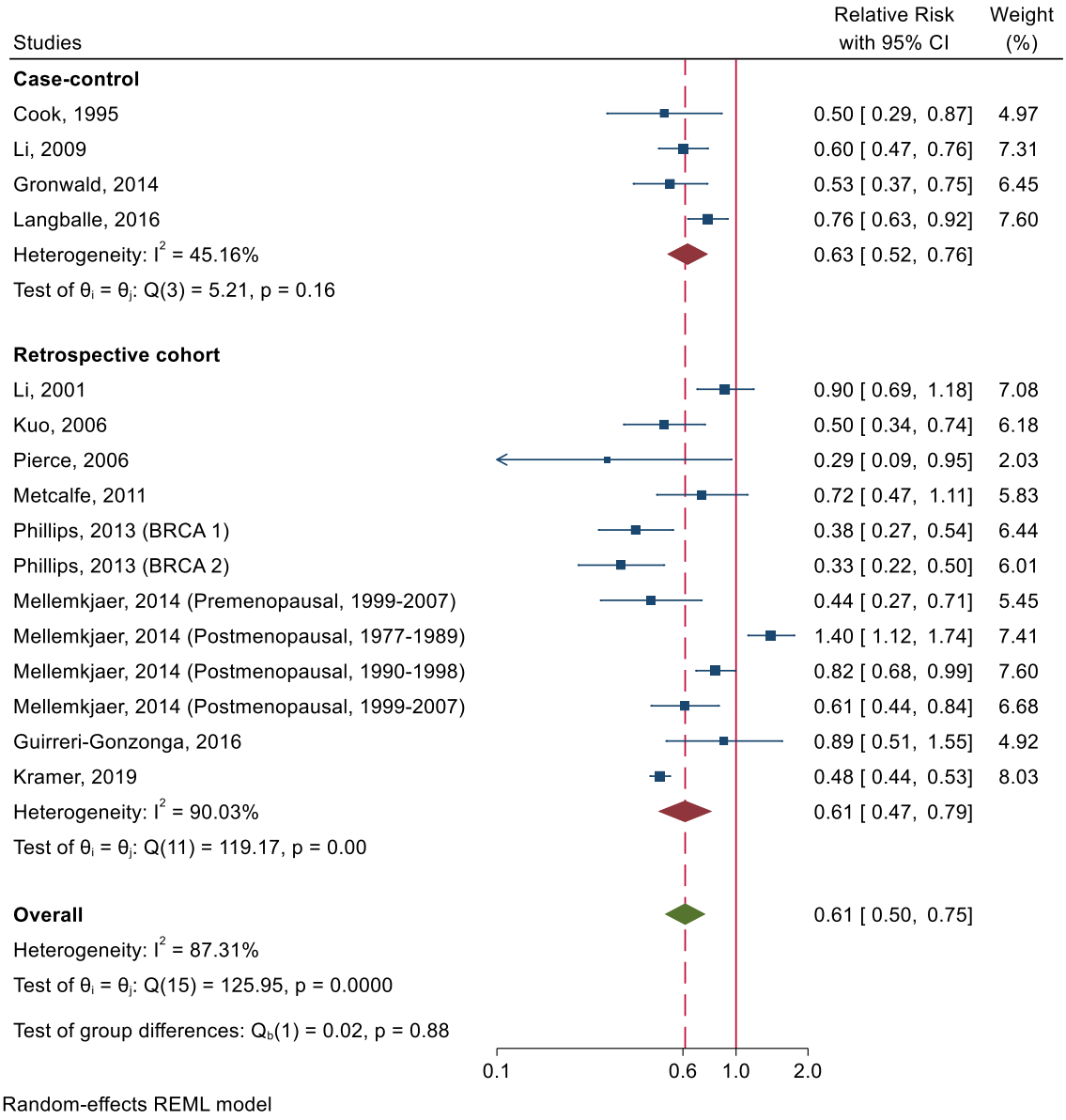

Figure S6.1: Forest plots of observational studies assessing the association between ever use of tamoxifen and the risk of contralateral breast cancer, by study design, among primary breast cancer survivors (n=12)

This forest plot shows the relative risk of the association between tamoxifen use and the risk of contralateral breast cancer by case-control and retrospective cohort study design. The studies were conducted among all primary breast cancer (irrespective of ER-subtype), except for Guirreri-Gonzaga 2016 and Li 2009, which were conducted among ER+ breast cancer survivors. Pierce 2006, Metcalfe 2011, Phillips 2013, and Gronwald 2014 were conducted among BRCA mutation careers. Phillips et al reported findings stratified by BRCA mutation status. Mellemkjaer 2014 reported estimates by menopausal status and calendar period. of primary breast cancer diagnosis. Guirreri-Gonzaga 2016 assessed low dose tamoxifen use. The studies are ordered in the plot by respective publication dates.

Sensitivity Analyses: Figure S6.2-S6.4

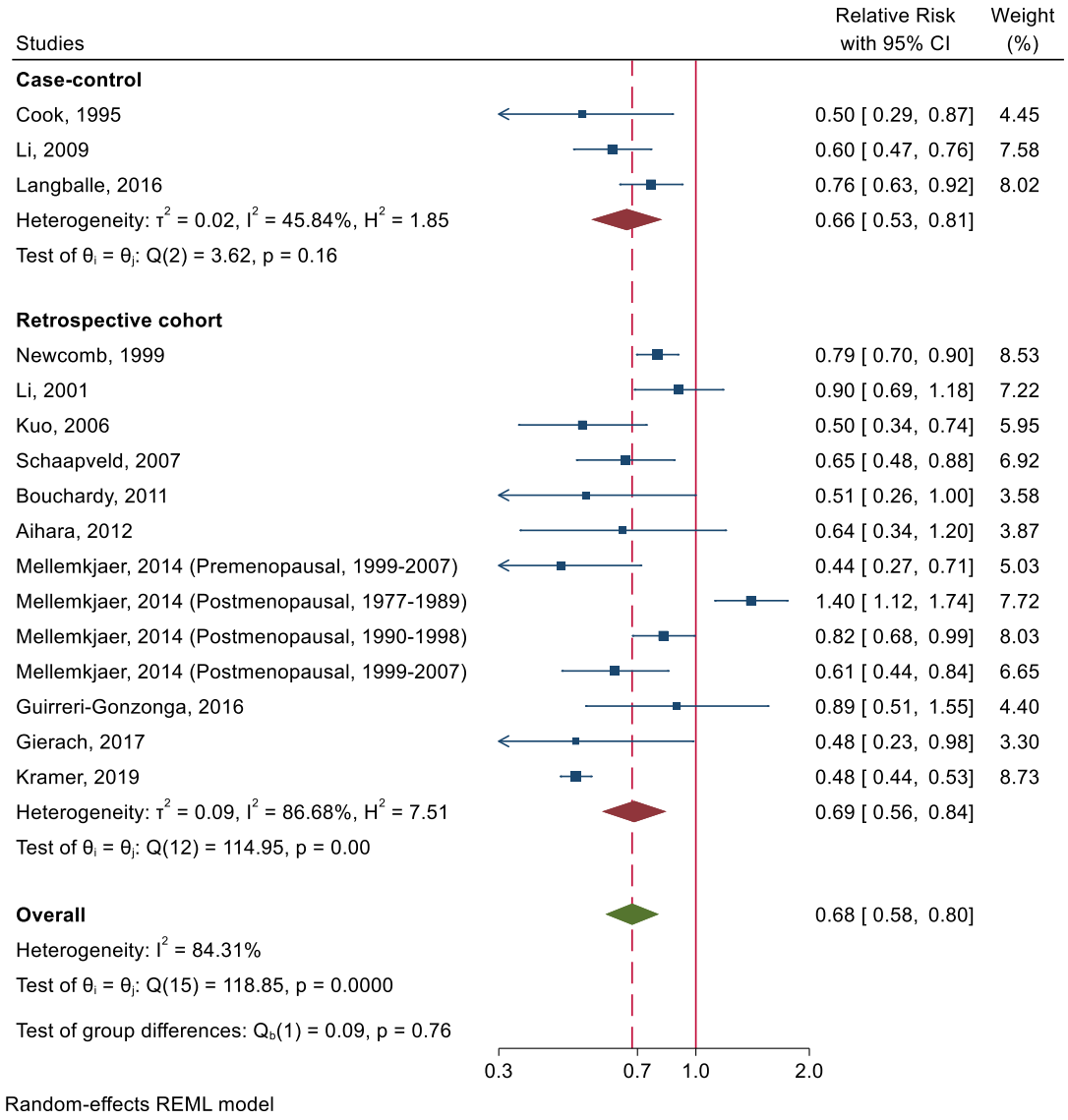

Figure S6.2: Forest plots of the observational studies assessing the association between ever use of endocrine therapy and the risk of contralateral breast cancer among primary breast cancer survivors, after excluding Pierce 2006, Metcalfe 2011, Phillips 2013, and Gronwald 2014 (BRCA mutation carriers) (n=13)

This forest plot depicts the relative risk of the association between any endocrine therapy and the risk of contralateral breast cancer by case-control and retrospective cohort study design, after excluding studies conducted among BRCA mutation carriers. The studies were conducted among all primary breast cancer (irrespective of ER-subtype), except for Guirreri-Gonzaga 2016 and Li 2009, which were conducted among ER+ breast cancer survivors. Mellemkjaer 2014 reported estimates by menopausal status and calendar period of primary breast cancer diagnosis. All effect estimates are based on ever use of tamoxifen, except for Newcomb 1999, Schaapveld 2007, Bouchardy 2011 and Aihara 2012, which reported any endocrine therapy use, and Gierach 2017, which reported ever use of AIs. Guirreri-Gonzaga 2016 assessed low dose tamoxifen use. The studies are ordered in the plot by respective publication dates.

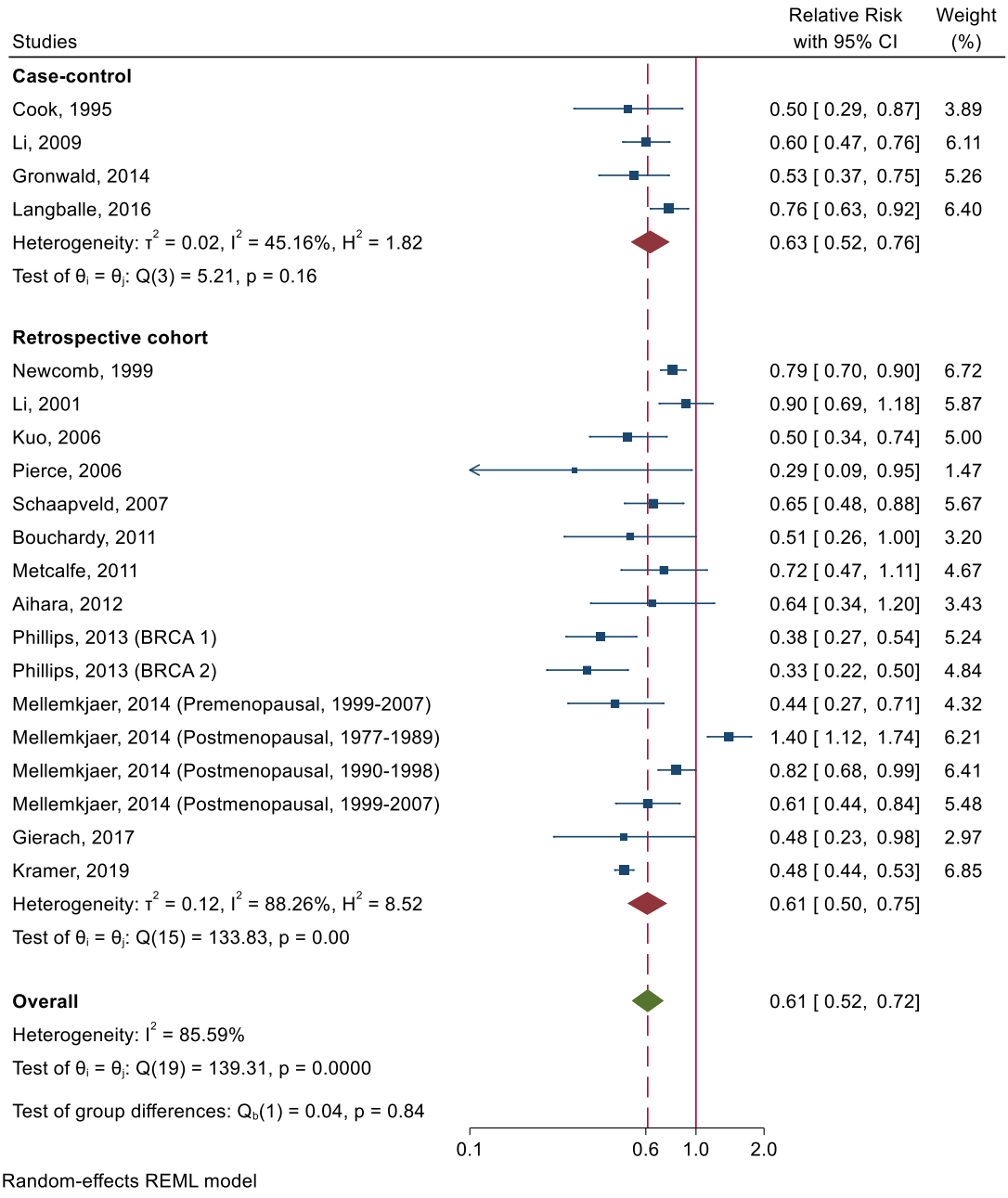

Figure S6.3: Forest plots of the observational studies assessing the association between ever use of endocrine therapy and the risk of contralateral breast cancer among primary breast cancer survivors, after excluding Guirreri-Gonzaga 2016 (low dose tamoxifen use) (n=16)

This forest plot depicts the relative risk of the association between any endocrine therapy and the risk of contralateral breast cancer by case-control and retrospective cohort study design, after excluding the study conducted among low-dose tamoxifen users. The studies were conducted among all primary breast cancer (irrespective of ER-subtype), except Li 2009, which was conducted among ER+ breast cancer survivors. Pierce 2006, Metcalfe 2011, Phillips 2013, and Gronwald 2014 were conducted among BRCA mutation careers. Mellemkjaer 2014 reported estimates by menopausal status and calendar period of primary breast cancer diagnosis. All effect estimates are based on ever use of tamoxifen, except for Newcomb 1999, Schaapveld 2007, Bouchardy 2011 and Aihara 2012, which reported any endocrine therapy use, and Gierach 2017, which reported ever use of AIs. The studies are ordered in the plot by respective publication dates.

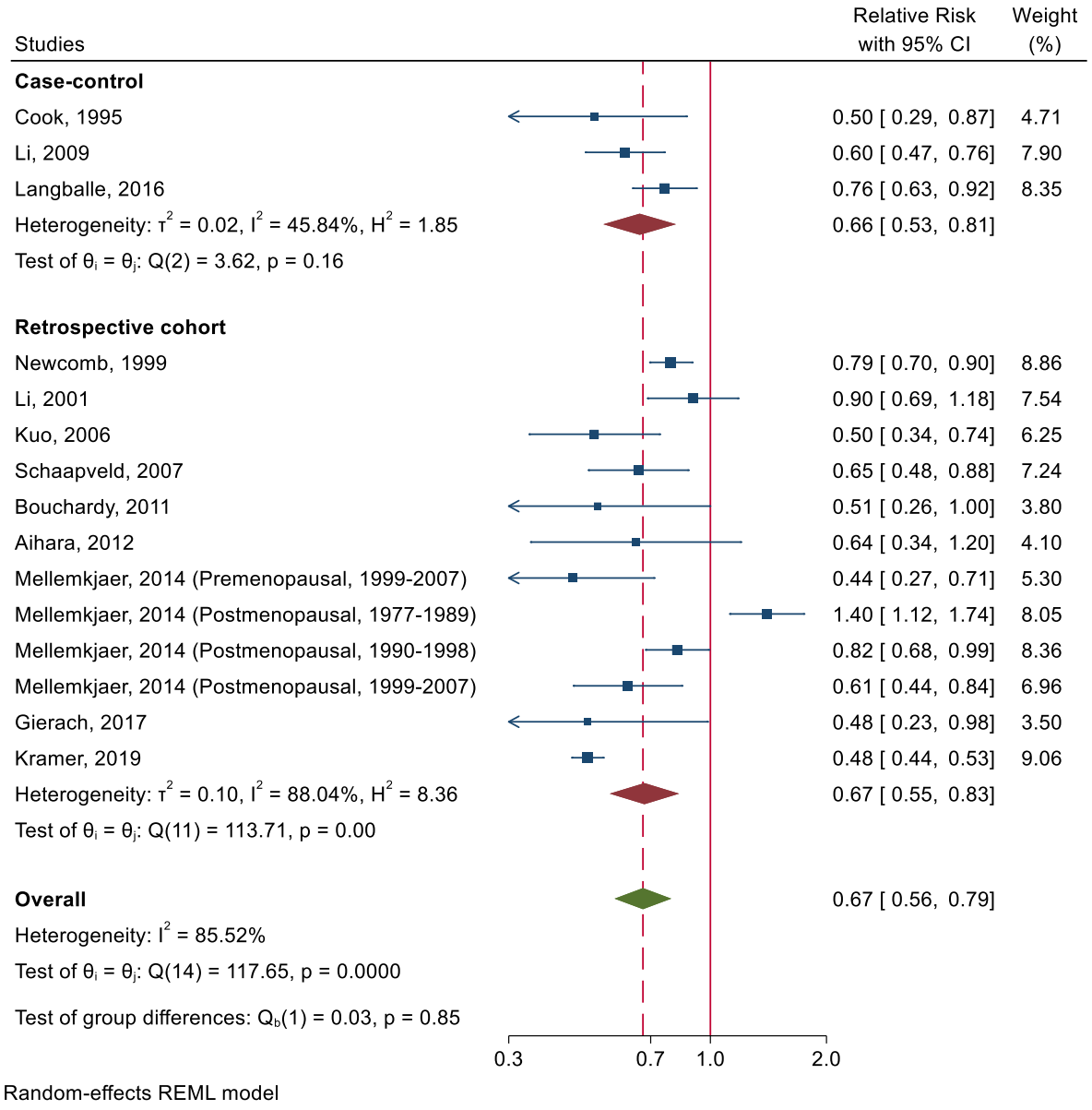

Figure S6.4: Forest plots of the observational studies assessing the association between ever use of endocrine therapy and the risk of contralateral breast cancer among primary breast cancer survivors, after excluding Pierce 2006, Metcalfe 2011, Phillips 2013 and Gronwald 2014 (BRCA mutation carriers) and Guirreri-Gonzaga 2016 (low dose tamoxifen use) (n=12)

This forest plot depicts the relative risk of the association between any endocrine therapy and the risk of contralateral breast cancer by case-control and retrospective cohort study design, after excluding studies conducted among BRCA mutation carriers and low dose tamoxifen use. The studies were conducted among all primary breast cancer (irrespective of ER-subtype), except Li 2009, which was conducted among ER+ breast cancer survivors. Mellemkjaer 2014 reported estimates by menopausal status and calendar period of primary breast cancer diagnosis. All effect estimates are based on ever use of tamoxifen, except for Newcomb 1999, Schaapveld 2007, Bouchardy 2011 and Aihara 2012, which reported any endocrine therapy use, and Gierach 2017, which reported ever use of AIs. The studies are ordered in the plot by respective publication dates.

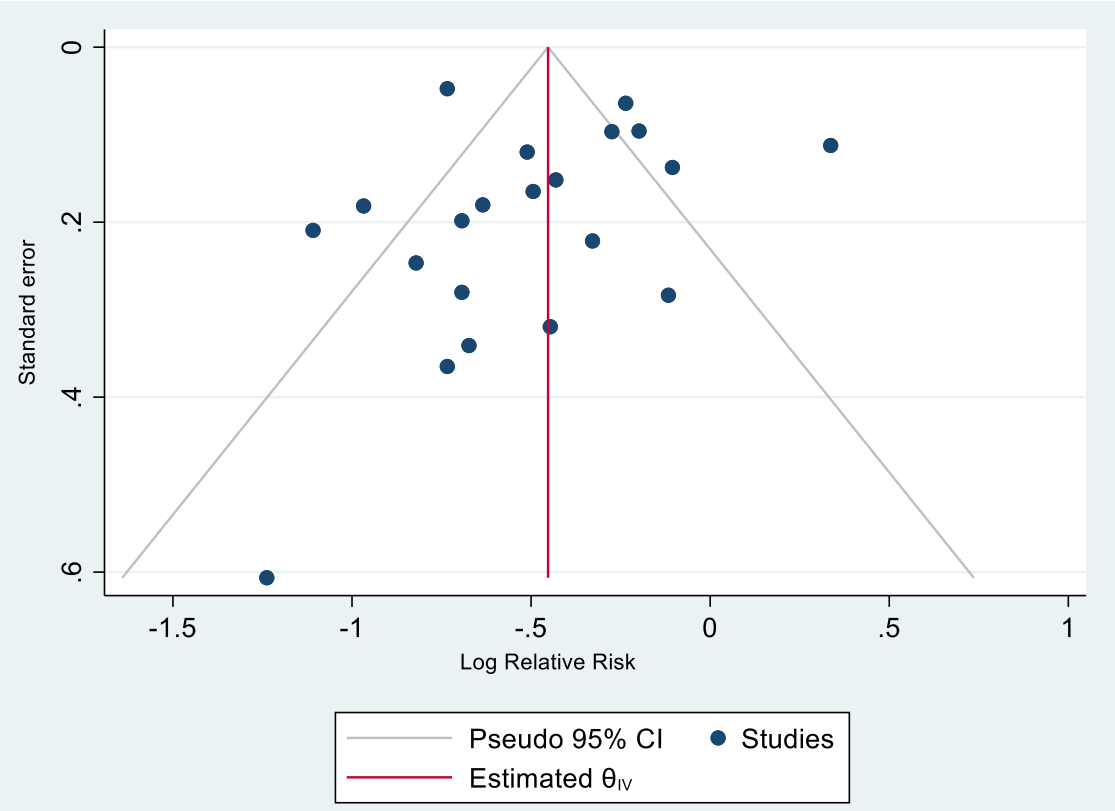

Figure S6.5: Funnel plot of risk estimates of the association between ever use of endocrine therapy among primary breast cancer survivors and the risk of contralateral breast cancer

Funnel plot of standard error by log relative risk of the association between endocrine therapy use and CBC risk for the studies included in the meta-analysis (n=17)

Chapter 7: Conclusions

Main findings and future directions

The findings of this dissertation suggest that one of the most widely used endocrine therapies, tamoxifen, modulates circulating hormones, estrogen and IGF-I, crucial determinants of breast carcinogenesis. The results suggested an approximately 13-27% decrease in select EMs among postmenopausal women, and a positive relationship between tamoxifen metabolites and EMs, among premenopausal women, indicating heterogeneity in this relationship by menopausal status. Evidence from prior literature suggests that tamoxifen reduces breast density, which is associated with subsequent reduction in breast cancer risk[24-26] and improved outcomes[28,29,291] among breast cancer patients. However, no association was observed between tamoxifen treatment and breast density in this study. Despite the observed decrease in IGF-I within one year of tamoxifen treatment, the results of this dissertation do not support a relationship between changes in IGFs and concomitant changes in breast density, among tamoxifen-treated women. However, it is important to note that the limited sample size may have affected the study power. Future longitudinal studies with a larger sample size are needed to understand the etiologic association between tamoxifen treatment, circulating hormones, and breast density in breast cancer risk and progression. Furthermore, this dissertation meta-analyzed findings from population-based studies and demonstrated that endocrine therapy, including tamoxifen use reduces the risk of CBC by approximately 40% in a real-world treatment scenario, in concert with the findings of the RCTs. The findings further suggested that this relationship varies by important determinants of breast cancer, including menopausal status and ER-subtype of CBC. The paucity of observational studies that have assessed AI use, and the duration and/or recency

of treatment highlight a gap in this research area. Since breast cancer is a heterogeneous disease characterized by genetic variations that influence tumor characteristics[319], the effectiveness of endocrine therapies on breast cancer prognosis and their maximum therapeutic benefit may vary based on the specifications of the primary tumor characteristics. However, limited studies have assessed this relationship by characteristics of the primary tumor (histology, stage, size) among breast cancer survivors. Hence, future research in this domain should be aimed at fully exploring the relationship between endocrine therapy use and CBC risk, by types of treatment, duration and/or recency of use, and tumor characteristics of the primary breast cancer using larger population-based observational studies to reflect the real-world treatment effects. Continued research in this domain may help to move in the direction of more personalized treatment, and ensure better care for breast cancer survivors.

Public health significance

Breast cancer, the most commonly diagnosed cancer in the United States as well as in the world, has an annual incidence of over 200,000[3,61]. Globally, breast cancer is also the leading cause of cancer mortality among women[61]. In the United States, early screening and the advent of new therapeutic approaches have drastically improved the survival rates of breast cancer[320], leading to an increased risk of CBC among the survivors[40,299,300]. Contralateral tumors are associated with worse disease prognosis and survival compared to primary breast cancer[321], and lead to considerable psychological and financial burden[91]. Given the high incidence of contralateral tumors among primary breast cancer survivors[40,299,300], it is important to improve the understanding of the effectiveness of preventative treatments in a real-world setting. Endocrine therapy has played a pivotal role in decreasing mortality and improving prognosis, among breast cancer patients[9,322]. The findings of this dissertation provided valuable evidence as to whether the

therapeutic benefits of endocrine therapy vary by select determinants of breast cancer, and improved upon existing meta-analyses by including these determinants in the analysis, within the context of a real-world treatment scenario.

Tamoxifen, one of the most widely used endocrine therapy, reduces the risk of breast cancer by more than 50%[197], and lowers breast cancer mortality by approximately 30%[41]. However, tamoxifen treatment is primarily effective after 5-10 years of use[323-325], and there is a limited way of ascertaining its treatment efficacy until the development or recurrence of breast cancer. This dissertation explored novel associations of the effect of tamoxifen on circulating hormones, and breast density, and the findings suggest that tamoxifen lowers EMs and IGF-I in circulation. This tamoxifen-mediated modulation of circulating hormones may be a surrogate marker of therapeutic response, and highlights the interplay between tamoxifen treatment and endogenous hormones in breast cancer etiology. Hence, with supporting evidence from future longitudinal studies, modulation in levels of estrogen metabolites and/or IGFs, may be repurposed as an objective measure of treatment response. Since an important issue associated with tamoxifen is poor adherence to treatment[315], positive findings from future research on the persistence of treatment effects after cessation of use and alteration in endogenous hormone levels as a surrogate marker therapeutic response may help improve adherence to the tamoxifen and improve prognosis among breast cancer patients. In conclusion, the broader significance of this dissertation lies in elucidating breast cancer etiology by improving our understanding of the interplay between breast cancer risk factors, circulating hormones and breast density, and protective factors, tamoxifen, and ascertaining the effectiveness of endocrine therapy, including, tamoxifen among subgroups of breast cancer patients.

Appendices

Appendix A: Observational studies assessing the association between endocrine therapy and risk of contralateral breast cancer risk among breast cancer patients

	Author, year	Study population	Study design	Exposure information	Outcome information	Confounders/ EMs	Result
Retrospective cohort studies							
1	I Kramer et al, 2019	-Invasive breast cancer patients, mostly HR+, taking ET/CT/combination, underwent surgery, diagnosed between 2003-2010. -Follow up till 2016 -Netherlands Cancer Registry - N = 83,144 -Median age at diagnosis: 58.5 years (BC); 63.9 years (CBC).	Retrospective Cohort Study	-Endocrine therapy, ET (Tamoxifen, AIs). -Chemotherapy, CT (taxane/anthracycline), trastuzumab, or combined -Data obtained from: Netherlands Cancer Registry	Metachronous CBC (>= 3 months after first BC diagnosis) -Netherlands Cancer Registry with linkage to PALGA: Dutch Pathology Registry	-Confounders: All other systemic therapies, age and Stage (III vs I–II) at first BC diagnosis. -Predictors of CBC in the Dutch population (age at diagnosis, TNM stage, treatment, and follow up interval) -Subgroup analysis by HR/HER2 status of primary BC and ER status of CBC	-Adjuvant CT (HR= 0.70, 95% CI = 0.62, 0.80), ET (HR= 0.46, 95% CI = 0.41, 0.52), ET + CT (HR= 0.35, 95% CI = 0.31, 0.39), TRA+CT (HR= 0.57, 95% CI = 0.45, 0.73) was associated with reduced CBC risk. -AIs associated with reduced CBC risk (HR= 0.32, 95% CI = 0.23, 0.44) compared to TAM use. ET use associated with decreases ER+CBC risk HR= 0.41, 95% CI = 0.36, 0.47) but not ER-CBC, compared to no ET

	Author, year	Study population	Study design	Exposure information	Outcome information	Confounders/ EMs	Result
2	G Gierach et al, 2017	-Patients diagnosed with invasive breast cancer at Kaiser Permanente (KP) Northwest or Colorado from January 1, 1990- Dec 31, 2008. -Survived at least 1 year following primary cancer diagnosis. -Exclusion criteria: CB removal at initial surgery, having metastatic disease, patients not treated with breast surgery, aged >= 85 years. -Median age= 60.6 years (24.9-84.9) N=7541	Retrospective Cohort Study	- TAM, AIs, other hormonal agents, chemotherapeutic agents, and radiotherapy. -Tamoxifen Use: Duration in years and time since last use (Current use, former use, <=3 years, 3-5 years, >= 5 years) in ref to nonusers of tamoxifen/used <90 days. - AI use was limited to "ever use". - Data obtained from electronic Kaiser Permanente(KP) prescription and medical records.	Incident CBC. Other second cancer diagnosis, death. Data: KP tumor registry	Confounders: AI use (with or without tamoxifen therapy), chemotherapy with an alkylating agent, and radiotherapy. -Subgroup analysis by ER status of CBC and age at diagnosis of primary BC (as a proxy for menopausal status)	-Use of TAM (former and current) is associated with decreased risk of CBC. -Yearly use was associated with reduced CBC risk (RR= 0.76 (95% CI, 0.64-0.89) compared with nonusers. -AI use without TAM was also associated with reduced CBC risk (RR: 0.48; 95% CI, 0.22-0.97) when compared to nonusers. -Among TAM users, risk of ER+ CBC was significantly reduced among current (RR: 0.68; 95% CI, 0.54-0.84) and former users whose BC was ER+. -Among ER- primary BC survivors, TAM use was not associated with ER+ CBC and ER-CBC.
3	Guirreri-Gonzonga et al, 2016	-Women diagnosed with ER+ primary DCIS between January 1997-December 2008, who underwent BCS -Median age (IQR) at primary BC: 53 (46-62) years	Retrospective Cohort Study	-Tamoxifen (low dose) - RT	Primary: Ipsilateral recurrence. Secondary: Contralateral Breast Cancer	Confounders: Age, radiotherapy, BMI, margins, DCIS grade.	-Low dose TAM: HR= 0.89, 95% CI: 0.51, 1.55)

	Author, year	Study population	Study design	Exposure information	Outcome information	Confounders/ EMs	Result
		-Median follow-up duration (IQR): 7.73 (5.81-9.67) years					
4	L Mellemkjaer et al, 2014	-Women aged >= 30 years at invasive BC diagnosis identified from Danish Breast Cancer Cooperative (DBCG) database during the period 1977-2007. - Inclusion criteria: undergone mastectomy, breast conserving surgery with axillary sampling, or clearance for their BC, and had been enrolled in a DBCG program -Initial n=37533 (Users: 15863, Non-users: 21670)	Retrospective Cohort Study -Followed up after 4 months of BC surgery. - Followed up: Jan 1, 1978 - Dec 31, 2009.	Tamoxifen -Analyzed as time dependent yes/no; current/past use, duration, time since start of tamoxifen (<1, 1-4, 5-9, >10+ years), and time since last use (<5, 5-9, 10+ years). - Users: Treatment dates reported in DBCG Non-users: Not been prescribed to TAM. -Data: Clinical DBCG database (Use of TAM was linked to Danish Cancer Registry).	Metachronous CBC (>= 4 months after primary BC) Data: Danish Cancer Registry	Confounders: Age at first BC, histology of first BC, and RT (yes/no), CT (for some user vs nonuser combinations). EM: Menopausal status and calendar periods of the DBCG program.	-TAM use was associated with a reduced risk of CBC (HR =0.44: 95% CI: 0.27 to 0.71) compared to no use. -This reduction in risk differed by menopausal status and period during the first 10 years after TAM cessation only
5	KA Phillips et al, 2013	-Female BC patients with BRCA1 or BRCA2 mutation enrolled between September 1,1993-December 2, 2009, in 3 cohort studies IBCCS, kConFab, BCFR from Europe, Australia, New Zealand, the United States, and Canada. - Recruited through BC family clinics and population-based recruitment.	Cohort Study (Both retrospective and prospective analyses were done)	Tamoxifen Use. -Data: Self-reported at cohort entry and follow up -Ever use (took it for any period of time) vs never use	Contralateral Breast cancer Data: Self-reported and/or collected by linkage to a cancer registry	Confounders: Age, country of residence. EM: ER status, Sensitivity analyses: Stratified by menopausal status, adjusting for chemotherapy use, histologic subtype, adjusting of first BC oral contraceptives or HRT after first BC diagnosis	Using combined prospective and retrospective data Tamoxifen use after first BC was associated with a reduction in CBC risk for -*BRCA1* (HR=0.38; 95% CI, 0.27 to 0.55) mutation carriers. and, -*BRCA2* (HR=0.33; 95% CI, 0.22 to 0.50) mutation carriers.

	Author, year	Study population	Study design	Exposure information	Outcome information	Confounders/ EMs	Result
		-Exclusion: History of other invasive cancers or tamoxifen use before first BC diagnosis -N=2464 (BRCA1=1583; BRCA2= 881)					
6	T Aihara et al, 2012	Japanese women with unilateral BC, underwent surgery, whose primary tumor was either PTis or PT1mic selected from 8 different hospitals in Japan till December 2010. -N= 2074 (pTis=1905 ; pT1mic = 169) - Mean age = 54.1 (SD=12.2)	Retrospective Cohort Study	-Endocrine therapy (Tamoxifen, AI) -Radiotherapy -Data collected using a standardized electronic data collection form	Incidence of CBC, -Recurrence of Ipsilateral BC, - Total incidence of BC (sum of IBC recurrence and incidence of CBC)	Confounders: pTis/pT1mic, type of surgery, age at surgery	-Incidence of CBC was 3.6/1000-person years (95% CI: 2.1-6.1) among those who received ET. -TAM users have 3.8/1000 (95% CI: 2.2, 6.5) person years incidence of CBC. -AI users have 2.3/1000-person years (95% CI: 0.3, 16.5) incidence of CBC.
7	M Schaapveld et al , 2007	-Women diagnosed with stage I-IIIA breast cancer between Jan 1989-Jan 2002 at regional the Cancer Centers of the Netherlands (Netherland Cancer Registries, NCR*). -N=45,229	Retrospective Cohort study -Median follow up: 5.8 years	-Adjuvant systemic therapies. ➢ Hormonal therapy ➢ Chemotherapy -Data collected from: Medical records from NCR using registration and coding manual	-Metachronous Contralateral Breast Cancer (Synchronous CBC, death as competing risks). All-cause mortality. -Cancer registries of the Netherlands. PALGA and national hospital databank.	Confounders: Age, morphology, stage, and treatment at index cancer diagnosis. EM: Age, radiotherapy	-The cum incidence of metachronous CBC increased at 0.4% per year, 5.9% at 15 years follow-up. -Adjuvant HT (HR 0.58; 95% CI 0.48–0.69) and CT (HR 0.73; 95% CI 0.60–0.90) were associated with a decreased CBC risk compared with no adjuvant therapy.

	Author, year	Study population	Study design	Exposure information	Outcome information	Confounders/ EMs	Result
					-Mortality Data: Medical records through linkage with municipal population registries or National Death registries of the Central Bureau for Genealogy.		-Metachronous CBC was associated with worse survival (HR 1.44; 95% CI 1.33–1.56), association observed for stage II and higher cancers.
8	C Li et al, 2001	- Women diagnosed with primary invasive breast cancer at age >= 50 years during the period January 1990-December 1998. N=8981 (TAM =4654; No TAM= 4327)	-Retrospective Cohort Study. -Followed up at least 6 months post diagnosis of primary cancer	Exposure: Tamoxifen. Data: Medical record abstraction.	Contralateral BC Data: Cancer Surveillance System (CSS), a cancer registry of 13 counties in the Washington state	Confounder: patient's age and year of diagnosis of the first breast cancer, tumor stage Stratified by: Hormone Receptor profiles of both primary BC and CBC.	-Use of TAM was associated with reduced risk of CBC (HR=0.90, 95% CI: 0.7 to 1.2). -This reduction was seen among ER+, PR+, and ER+/PR+ tumors only -TAM use was associated with decreased risk of ER+ CBC but increased risk of ER- CBC. The diff in risk was significant. -This result was observed even after restricting to women with ER+ primary tumors.
Case-control studies							
1	R Langballe et al 2016	-Women aged <= 54 diagnosed with invasive and in situ BC (WECARE I) or invasive BC (WECARE II). -Identified through 8 population registries throughout USA, Canada, and Denmark from Jan 1, 1985-Dec 31, 2008.	-Matched Case Control Study. -WECARE I & WECARE II.	-Chemotherapy and hormonal(tamoxifen) therapy -Data: Retrieved from medical records. If unavailable, self-reported was used.	Contralateral breast cancer -WECARE I: Both invasive and in-situ CBC. WECARE II: Invasive CBC	Confounders: Age at diagnosis of the first primary BC, first degree family history of BC among, lobular histology and stage of first BC, ER/PR status of the first BC and radiotherapy.	-TAM use was associated with reduced CBC risk (RR=0.76; 95% CI: 0.63-0.92) compared to no use. -The risk of CBC was reduced for longer duration of current TAM users compared to non-users (RR=0.73; 95% CI: 0.55-0.97).

	Author, year	Study population	Study design	Exposure information	Outcome information	Confounders/ EMs	Result
		-Cases were diagnosed with CBC (no later than Dec 31, 2001). -Controls were women with no CBC and had no prophylactic mastectomy during the at-risk period of the cases, and had no prior or intervening cancer diagnoses. Study: WECARE I and II -N= 1521 cases and 2212 controls (matched on age and year of diagnosis)	-Matched in a 1:2 ratio (case: controls) in WECARE I and 1:1 ratio in WECARE II by age at first BC, calendar-year of first diagnosis, cancer registry, race/ethnicity, radiotherapy (WECARE I). .	CT and TAM therapy included date of administration, type of drug, reason of treatment. Ever Use vs Never use; duration and time since last use	-Data: Medical records were used for tumor characteristics (cancer registry was also used) for first BC and CBC	>Analyses for the effect of CT included terms for RT and HT. >Analyses for the effect of TAM included RT and CT, and HT other than tamoxifen EM: Time since diagnosis, age at first diagnosis, year of BC diagnosis, first degree family history of BC, BMI, histology and ER/PR status of first BC. -For CT: CT regimens. -For TAM: Duration of use, time since last use at reference date Interaction was tested between TAM use and CT use	-Among TAM users, the risk of ER-positive CBC RR=0.75; 95% CI: 0.58-0.96) was decreased compared to no TAM. No association with ER-CBC. -Use of chemotherapy (ever versus never use) was associated with a significantly reduced RR of developing CBC (RR=0.71; 95% CI: 0.59-0.85).
2	Gronwald et al., 2014	-Cases were women with BRCA1 or BRCA2 mutation diagnosed with bilateral breast cancer (cases) and unilateral breast cancer (controls) on or after January 1st, 1970 -Controls were matched to cases on year of birth, age of diagnosis of primary breast cancer, BRCA mutation, and regional residence.	-Matched Case Control Study.	-TAM (ever/never) use • Ever use: Taken for a period equivalent to the period between first BC diagnosis and CBC diagnosis of the case -Data obtained from questionnaire administered in person and telephone interviews	Contralateral breast cancer -Data: Hereditary Breast Cancer Clinical Study	Confounders: Radiotherapy, chemotherapy	-All women: OR= 0.53, 95% CI: 0.37, 0.75) -BRCA1 mutation: OR= 0.58, 95% CI: 0.39, 0.85 -BRCA2 mutation: OR= 0.39, 95% CI: 0.19, 0.83

	Author, year	Study population	Study design	Exposure information	Outcome information	Confounders/ EMs	Result
		-Hereditary Breast Cancer Clinical Study -10 participating countries					
3	KW Reding et al, 2010	Participants: CBC patients (cases) identified from 5 different cancer registries (4 in US and 1 in Denmark) diagnosed between Jan 1, 1985, and Dec 31, 2000. -Controls with unilateral BC (UBC). -Carriers of BRCA1/BRCA2 mutations N : Cases= 708, Controls =1399 BRCA1/BRCA2 mutation carriers: 181	-Matched Case Control Study (The WECARE study. -Controls matched to cases in 2:1 ratio on birthyear, year of diagnosis, registry region, race. They were 1:2 counter matched on registry reported radiation treatment of the UBC.	-Tamoxifen (y/n) - Chemotherapy (y/n) -Regimens: cyclophosphamide, methotrexate, 5-fluorouracil (CMF) , and CAF/CEF. Other anthracycline based or multiple regimens Data: Medical records, hospital records	Contralateral Breast Cancer Data: 5 cancer registries (4 from USA) and 1 in Denmark (Danish Breast Cancer Cooperative Group)	Confounder: Age	-TAM was associated with a significant reduced CBC risk in non-carriers (RR = 0.7 [95% CI: 0.6-1.0) but not significant among the carriers (RR = 0.7 [95% CI: 0.3-1.8]. -CT was associated with lower CBC risk both in non-carriers (RR = 0.6 [95% CI: 0.5-0.7]) and carriers (RR = 0.5 [95% CI: 0.2-1.0]; P value = 0.04).
4	CL Li et al, 2009	Cohort: Patients with first primary invasive stage I-IIIB, ER+ breast cancer at age 40-79 in the four county Seattle Puget region from Jan 1990-September 30,2005. Cases: Diagnosed with ER+ invasive primary BC and CBC >=6 months after their first BC diagnosis from July 1, 1990, to March 31, 2007 -N= 367 Controls: diagnosed only with ER+ primary breast cancer -N= 728	-Population based nested case control study.	-Adjuvant Tamoxifen therapy, TAM -Interviewer administered telephone interviews and medical record reviews on use of all forms of adjuvant HT. -Information included drug names, doses, frequency of use, start and stop dates, side effects and complications, and any interruptions or changes in patterns of use were noted.	-Contralateral Breast Cancer (ER+/PR+, ER-, ER-/PR-) -Data: Identified through Cancer Surveillance system and medical record reviews.	Confounders: radiation therapy for first breast cancer EM: age, BMI, and the interval between first breast cancer diagnosis and reference date, on the relationships between adjuvant hormonal therapies and risk of different subtypes of contralateral breast cancer defined by hormone receptor status.	-Users of TAM had reduced risk of CBC (OR = 0.6; 95% CI: 0.5-0.8) compared to non-users. -Use of TAM for >5 years is associated reduced risk of ER+ CBC (OR = 0.4; 95% CI: 0.3-0.7) and ER+/PR+ CBC (OR = 0.5; 95% CI: 0.3-0.8).

	Author, year	Study population	Study design	Exposure information	Outcome information	Confounders/ EMs	Result
			-Controls were individually matched to cases in a 2:1 ratio on age, year of diagnosis, county at first diagnosis, race/ethnicity, SEER historic stage of first breast cancer (localized versus regional), and survival (at least through the time their matched case was diagnosed with contralateral breast cancer).	- Total durations of use of any adjuvant HT was estimated with the above information.			
5	L Berteisen et al , 2008	Women aged <= 54 years with invasive primary breast cancer without distant metastases. -Diagnosed between Jan 1, 1985-December 31, 1999. -Identified from 5 population registries of United States (4 centers) and Denmark (1 center). Eligibility: Cases : Second primary invasive or in situ BC (CBC) diagnosed >= 12 months after the initial diagnosis.	-Matched Case Control Study. -Women's Environment, Cancer and Radiation Epidemiology Study (WECARE). -Matched in 1:2 (case: control) ratio on year of birth (in 5-year strata), year of diagnosis (in 4-year strata), registry region, and race and were counter matched on radiotherapy (yes or no)	>Hormone therapy (tamoxifen), >Chemotherapy -Regimen 1: CMF (cyclophosphamide, methotrexate, and 5-fluorouracil) -Regimen 2: CEF/CAF (cyclophosphamide, epirubicin/Adriamycin, 5-fluorouracil) >Radiotherapy. Data: Retrieved from medical records. Date of administration, type of drug; reason of treatment.	Contralateral Breast Cancer Data: Hospital and physician medical records	Confounders: Age at diagnosis of the first primary BC, family history of BC among first-degree relatives, lobular histology of the first BC, and stage of first BC (local or regional). >Analyses for the effect of CT included terms for RT and HT. >Analyses for the effect of TAM included RT and CT, and HT other than tamoxifen EM:	-Using CT associated with reduced CBC compared to no CT use (RR=0.57, 95% CI = 0.42,0.75). -Using TAM associated with reduced CBC compared to no TAM use (RR=0.66, 95% CI = 0.50,0.88) -CT (RR=0.53, 95% CI = 0.34,0.83) and combination of CT and TAM was associated with reduced risk of CBC (RR=0.43, 95% CI = 0.23,0.80) compared to women only with surgery.

	Author, year	Study population	Study design	Exposure information	Outcome information	Confounders/ EMs	Result
		Controls : No CBC and had no prophylactic mastectomy during the at-risk period of the cases, and had no prior or intervening cancer diagnoses. N= 1792 (634 cases and 1158 controls) Median age at diagnosis of first BC = 46 years (range 23-54 years)		Telephone Interview conducted with structured questionnaire to gain info on BC risk factors		Time since diagnosis, age at first diagnosis, menopausal status, family history of BC, BMI, histology & ER status of first BC, CT regimens. Interaction was tested between TAM use and CT use	- Combination CT+TAM was associated with reduced CBC risk (RR=0.36, 95% CI = 0.23,0.56) compared to no TAM + no CT.
6	J Gronwald et al, 2005	-Cases: Women with bilateral breast cancer and BRCA1 or BRCA2 mutation. N= 285 -Controls: Women with unilateral breast cancer and BRCA1 or BRCA2 mutation. N= 751 -Cases and controls are carriers of mutation in the same gene BRCA1/BRCA2 -From 10 countries	Matched case control study -Matched on age of birth, age at diagnosis of first BC, BRCA1/BRCA2 mutation and oophorectomy status (y/n) -Average follow up: 7.4 years	Exposure: Tamoxifen -TAM use: Given as treatment for primary BC but not recurrent BC -Duration, dose, dates of treatment . - Questionnaire administered in person and telephone interviews	Contralateral Breast Cancer Questionnaire administered in person and telephone interviews	-Confounders: Other treatments received (RT and CT), smoking (ever/never), parity and oral contraceptive use (ever/never). -EM: BRCA1/BRCA2 mutation, Oophorectomy status, menopausal status, time since first BC diagnosis (1-5 years/5-10 years)	-TAM use was associated with reduced risk of CBC (OR=0.47, 95% CI: 0.30, 0.74). -This effect was also significant among BRCA1 OR=0.48, 95% CI: 0.29, 0.79) and BRCA2 OR=0.39, 95% CI: 0.16, 0.94) mutation carriers.
7	SA Narod et al, 2000	Cases: Bilateral breast cancer with BRCA1/BRCA2 mutation. -N= 209 Controls: Unilateral disease with BRCA1/BRCA2 mutation. -N=384 Average age at second BC= 45.5 years	-Matched Case Control study. -Matched on age and age at diagnosis, BRCA1, BRCA2 mutation, residence at time of diagnosis. -Follow up period: 9.7 years	Exposure: Tamoxifen -Data: Interview or by self-administered questionnaire.	CBC -Data centers from 8 different countries	Confounders: Other treatments received, ethnic group, parity, and smoking habits	-TAM is associated with reduced risk of CBC among BRCA1 (OR =0·38, 95% Cl 0·19–0·74) and for those with *BRCA2* mutations (OR=0·63, 95% CI: 0·20–1·50). -Risk of CBC reduced by 75% among women who used tamoxifen 2-4 years

	Author, year	Study population	Study design	Exposure information	Outcome information	Confounders/ EMs	Result
8	L S Cook et al, 1995	-Women with breast cancer. -Cases: Women with ovarian (n=34), endometrial (n=36), or CBC (188). -Controls: Did not develop a second primary malignancy. ovarian (n=89), endometrial (n=66), or CBC (328). -Matched age, year of BC diagnosis, and stage	-Nested case control study	Exposure: Tamoxifen, TAM -Abstracted from medical records validated against a computerized pharmacy database in a subset of 46 cases and 108 controls	CBC, Ovarian cancer, Endometrial cancer. -Hospital medical records	Confounders: Surgical treatment, radiation, and chemotherapy	-TAM is associated with decreased risk of CBC (mOR = 0.5; 95% CI = 0.3-0.9). -Duration of TAM use >1 year (mOR = 0.4; 95% CI = 0.2-0.9) and postmenopausal status at primary BC (mOR = 0.4; 95% CI = 0.2-0.8) is associated with decreased risk too.

BC: breast cancer, CBC: contralateral breast cancer, BCM: breast cancer mortality, ACM: all-cause mortality, CT: chemotherapy, ET: endocrine therapy, TAM: tamoxifen, AI: aromatase inhibitors, EM: effect modifier; mOR: multivariable odds ratio

Appendix B: Randomized clinical trials assessing the association between endocrine therapy and risk of contralateral breast cancer risk among breast cancer patients

	Author, year	Study population	Study design	Exposure information	Outcome information	Confounders/ EMs	Result
1	T Ruhstaller et al, 2019	-Postmenopausal women with ER+/PR+, or both early BC. -N=6416 (Enrolled=8010)	-Randomized Control Trial (Four arm, phase III, double blind). -BIG 1-98 long-term follow-up study (BIG 1-98 LTFU). -Median follow up: 12.6 years (Max follow up 17.7 years)	- Monotherapy with letrozole or tamoxifen for 5 years. -Sequential therapy with Letrozole for 2 years followed by TAM for 3 years. -Sequential therapy with Tamoxifen for 2 years followed by letrozole for 3 years. -Dosage Letrozole: 2.5 mg daily Tamoxifen: 20 mg daily. -Data: Clinic visit, telephone contact with patient, physician, or family member , or Danish Breast Cancer Collaborative Group(DBCG).	- Primary: Disease free survival (including invasive BC recurrence, invasive CBC, any second cancer, or death w/o a previous cancer event) -Secondary: Overall survival, distant recurrence-free interval, and invasive BCFI, breast cancer mortality. -Data: DBCG	-EM: Chemotherapy	-Letrozole use was associated with reduced risk of CBC (HR=0.62, 95% CI: 0.36, 1.09) for years 0-5 and (HR=0.47, 95% CI: 0.23, 0.97) for years 5-10, compared to TAM use. - This effect was reversed after >10 years (HR=1.35, 95% CI: 0.53, 3.41). -DFS was also reduced among letrozole users (HR=0.91, 95% CI: 0.81, 1.01) compared to TAM users.
2	DeCensi et al., 2019	-Women aged ≤75 years and diagnosed with HR-positive, or unknown breast cancer (ADH, DCIS, LCIS) -N= 500	-Randomized (multicenter, phase III) control trial. -Randomized in a 1:1 ratio to tamoxifen or placebo group.	-Tamoxifen (5 mg/day oral) vs placebo	Primary: Invasive breast cancer or DCIS Secondary: Incidence of ADH or LCIS, endometrial cancer, other	Confounders: Age and menopausal status.	-TAM was associated with reduced risk of CBC (HR=0.25, 95% CI: 0.07, 0.88)

	Author, year	Study population	Study design	Exposure information	Outcome information	Confounders/ EMs	Result
					second primary cancers, deep venous thromboembolic events, coronary heart disease, bone fractures, cataract, and menopausal symptoms		
3	John F Forbes et al 2016	-Postmenopausal women aged 40-70 years diagnosed with locally excised, hormone receptor positive ductal carcinoma in situ (DCIS) within 6 months before randomization. -Randomized between March 6, 2003-Feb 8th, 2012. -Final n = 2938 (A= 1434; T=1478) -Median age: 60.3 years (IQR 56.1-64.6)	-Randomized (Double Blinded multicenter, placebo) control trial. -Randomized in a 1:1 ratio to anastrozole or tamoxifen group. -Median follow up = 7.2 years (IQR 5.6 -8.9).	-Tamoxifen (20 mg/day oral) + ANA placebo. vs -Anastrozole (1 mg/day oral) + TAM placebo Duration: 5 years (Followed up at 6 months, 12 months, and then annually)	Primary: All DCIS (Invasive and new or recurrent DCIS). Including local recurrence, distant recurrence, or isolated CBC events. Secondary: BC mortality, other cancers, CVD, fractures, fractures, adverse events, non-BC deaths.	Confounders: Age, BMI, HRT use, grade, margins and radiotherapy subgroups. - Randomization was stratified by major center.	-No statistically significant difference was observed in overall deaths, cause-specific death, non-BC, overall recurrence, CVD events between the two groups. -Invasive (HR= 0.68, 95% CI: 0.36-1.29) CBC risk was reduced among ANA group compared to TAM, but it was not significant -Fractures, musculoskeletal adverse events, and hypercholesterolemia were significantly higher in the ANA group.

	Author, year	Study population	Study design	Exposure information	Outcome information	Confounders/ EMs	Result
4	RG Margolese et al 2016	-Postmenopausal women with noninvasive HR-positive DCIS or DCIS and lobular carcinoma in situ treated with lumpectomy and whole breast irradiation, from Jan 6, 2003 – June 15, 2006. -N=3104 (T=1552; A=1552). -Data collected up to Feb 28, 2015. -Median duration of follow up: 9 years (IQR 8.2-10.0).	-Randomized (double blind phase 3) control trial. -Randomly assigned in 1:1 ratio to receive anastrozole (+ tamoxifen placebo) or tamoxifen (+ anastrozole placebo) for 5 years	-Anastrozole (ANA) (oral) – 1 mg/day. -Tamoxifen (oral) – 20 mg/day. -Duration: 5 years	-Primary: BC free interval (any BC event including local, regional, or distant recurrence, or CBC, invasive disease or ductal carcinoma in situ). -Secondary: Disease free survival (time to recurrence, second primary cancers (excluding skin cancers, all-cause mortality. - Also, Ipsilateral BC, contralateral BC, non-breast second primary cancers, osteoporotic fractures, overall survival	Confounder: Age EM: Age (<60 vs >=60 years). Treatment, age group comedo necrosis, palpable mass at presentation.	-ANA was associated with reduced risk of BC free interval compared to TAM (HR 0·73 [95% CI 0·56–0·96], p=0·0234). This effect was significant in women younger than 60 years of age. -ANA was associated with significant reduction of both total (HR 0·64 [95% CI 0·43–0·96], p=0·03). and invasive CBC (HR 0·52 [95% CI 0·31–0·88], p=0·01). -No difference was observed for overall survival in both the groups.
5	MM Regan et al, 2016	-Premenopausal women with invasive HR+ HER2-ve early BC. -Randomly assigned between November 2003 and March 2011.	-Follow up study of 2 RCTs, TEXT (Tamoxifen and exemestane Trial) and SOFT (Suppression of Ovarian Function Trial).	-Tamoxifen, Exemestane. -Women were randomly assigned at a 1:1:1 ratio to 5 years of exemestane plus OFS (ovarian function suppression),	-Breast Cancer Free Survival (BCFI) (defined as time from random assignment to first appearance of	Confounders: Age, nodal status, tumor size and grade, ER, PR, and Ki-67 expression levels. EM: Cohort (by trial and CT use)	-Patients who received only TAM (SOFT-no CT) had a 5-year BCFI as 96.1%, where those who also used OFS (TEXT) had improved 5-year BCFI by 3.6%.

	Author, year	Study population	Study design	Exposure information	Outcome information	Confounders/ EMs	Result
		- Participants assigned between November 2003 and March 2011(TEXT) & December 2003 and January 2011 (SOFT) -N=4891 -Median follow up= 6 years (TEXT); 5.6 years (SOFT)	-Randomly assigned in 1:1 ratio to 5 years of exemestane +OFS or 5 years of TAM+OFS (TEXT). - Randomly assigned in 1:1:1 ratio to 5 years of exemestane +OFS or 5 years of TAM+OFS, or TAM alone (SOFT)	tamoxifen plus OFS, or tamoxifen alone. -Random assignment was stratified according to use of prior chemotherapy, lymph node status, and intended initial method of OFS (if randomly assigned to OFS).	invasive breast cancer recurrence (local, regional, or distant) or invasive contralateral breast cancer.	and treatment assignment	-Exemestane plus OFS improved 5-year BCFI by 5.4% and 7.4% versus tamoxifen plus OFS and tamoxifen alone. -There was 10% to 15% improvements in 5-year BCFI with exemestane + OFS versus tamoxifen alone, 5% or more in 5-year BCFI with exemestane + OFS versus tamoxifen + OFS.
6	J Rosell et al, 2017	-Postmenopausal women <75 years of age with early stage invasive breast cancer who did not have any recurrence/CBC after 2 years of TAM use. - Mean age: 62.7 years (2-year group); 62.6 years (5-year group) -N=4128	-Randomized Control Trial (Swedish BC Group; 5 BC study organization). - Randomized to receive 2-year vs 5 year of tamoxifen	Tamoxifen -Duration: Administered for 2 years and 5 years -Dose:20 mg (daily) doses in two centers and 40 mg (daily) doses at the other three centers.	Second primary cancers including CBC; Lung cancer; Endometrial cancer Data: Swedish Cancer Registry	EM: Trial center	-Compared to two years of TAM use, 5 years of TAM use was associated with decreased incidence of contralateral breast cancer (HR: 0.73; 95% CI 0.56–0.96). -A similar decreased incidence was seen among lung cancer, but the incidence of endometrial cancer increased among the 5-year users of TAM.

	Author, year	Study population	Study design	Exposure information	Outcome information	Confounders/ EMs	Result
7	N Zdenkowski et al, 2016	-Postmenopausal women with HR-positive unilateral/bilateral invasive BC who completed ≥ 4 years of ET (AI, TAM, ovarian suppression, or sequential). -Recruited more than 1 year prior from 29 sites in Australia and New Zealand. -N= mm(letrozole); 179 (observation) -Median follow up = 3.9 (IQR: 3.1-4.8) -Median age : L + obs group = 65 (60.0-70.7) obs group = 64.3 (59.2-68.7).	-Randomized, (multicenter open label phase III) trial. (LATER). -Women randomized to oral letrozole or placebo. Trial treatment was for 5 years. -Randomized between May 16, 2007 – March 14, 2012.	Letrozole vs placebo -Dose: 2.5 mg/daily -Duration: 5 years; from Feb 2010). -Follow-up at 6 and 12 months; annually thereafter	-Primary: Invasive breast cancer events (primary, local, regional, or distant recurrence, or CBC). -Secondary: Disease free survival (DFS), overall survival, and cause-specific mortality, and toxicity.	EM: Institution, prior ET, axillary node status	-Annual rate of invasive BC was 0.3% in the L group and 2.4% in the observation group. -The difference in the invasive BC event is 8.4% (95% CI: 3.8%, 13.0%, log rank P= 0.0004) between letrozole vs observation.
8	PE Goss et al, 2013	-Postmenopausal women with HR-positive primary BC identified between June 2, 2003 – July 31, 2008. -Median age: Exemestane grp = 63.9 years Anastrozole grp = 64.3 years. -N= 7576 (E= 3789, A= 3787)	-Randomized open-label, multicenter multinational phase III trial. -MA.27. -Randomly assigned to receive exemestane vs anastrozole -Median follow up: 4.1 years	Exemestane vs anastrozole (With or without celecoxib; discontinued later) Duration: 5 years	-Primary: Event-free survival (EFS), defined as time from random assignment to time of locoregional or distant disease recurrence, new primary breast cancer, or death from any cause.	Stratified by lymph node status, prior adjuvant chemotherapy, aspirin use, celecoxib group, trastuzumab use	There was no difference in hazard of EFS (HR= 1.02 (95% CI, 0.87 to 1.18; *P* = .85), distant disease-free survival, and disease specific survival among those who received exemestane vs anastrozole.

	Author, year	Study population	Study design	Exposure information	Outcome information	Confounders/ EMs	Result
					Secondary: Overall survival, distant disease–free survival, CBC; Disease-specific survival.		
9	Water et al., 2013	-Postmenopausal women diagnosed with estrogen (ER) and/or progesterone receptor (PR)-positive breast cancer -N= 9766	-Randomized (multicenter, phase III) control trial. -Randomized in a 1:1 ratio to tamoxifen or placebo group.	-Exemestane (25 mg/day for 5 years) or a sequential regimen consisting of tamoxifen (20 mg/day for 2.5–3 years, followed by exemestane 25 mg/ day for 2.5–2 years) vs placebo -CT, RT	Primary: Invasive breast cancer or DCIS Secondary: Incidence of ADH or LCIS, endometrial cancer, other second primary cancers, deep venous thromboembolic events, coronary heart disease, bone fractures, cataract, and menopausal symptoms	Confounders: Tumor and treatment characteristics (country of residence, histological grade, tumor stage, nodal stage, ER/PR status, type of surgery, RT, CT	-No significant association between TAM and CBC across age groups -65-74 years: HR= 1.03 (95% CI: 0.60, 1.77) - ≥ 75 years: HR=1.31, 95% CI: 0.64, 2.68)
10	J Cuzick et al., 2011	-BC patients with unilateral/bilateral DCIS randomized between May 1990 – August 1998. -UK DCIS trial -Median follow up: 12.7 years (range 0.2-9.9).	-Randomized Control Trial -UK/ANZ DCIS trial (updated results of the trial) -2X2 way randomization -Randomized to either RT or TAM, stratified by	-Radiotherapy -50 Gy in 25 fractions over 5 weeks -Tamoxifen, TAM -Dose: 20 mg/daily -Duration: 5 years	Primary -Invasive ipsilateral BC (for RT). Contralateral BC and DCIS (for TAM). -Recurrent DCIS, invasive IBC,	Stratified by whether patients received alternate treatment/not	-TAM reduced the risk of CBC (HR 0.44, 95% CI 0.25–0.77, p =0.005) compared to no TAM

	Author, year	Study population	Study design	Exposure information	Outcome information	Confounders/ EMs	Result
		-N=1694 - Followed up till: Oct 1, 2008	screening assessment center		contralateral DCIS or invasive tumor, BC mortality and cause-specific mortality (for both RT and TAM)		
11	A Hackshaw et al, 2011	-BC patients aged >50 years, had early breast cancer confined to one breast (T1-T3, N0 or N1, and M0), taking tamoxifen (20 mg) for 2 years. -Recruited between January 1987-February 1997 (71 centers across UK, Ireland, Greece, Turkey, Belgium, Poland, Hong Kong). N=3449 (2-year group = 1724 ; 5-year group= 1725)	-Multicenter International Randomized trial. -The Cancer Research UK "over 50s" trial. -Randomized to 2 years vs 5 years of TAM treatment - Median follow-up = 10.1 years	Tamoxifen -Dose: 20 mg/daily -Received either 2 years vs 5 years	-Event-free survival (EFS), -Risk of recurrence (local or distant or contralateral BC), -BC mortality, -Overall survival (OS), -CV events (i.e., nonfatal or fatal), and death as a result of a CV event.		-Among women who used 5 years of TAM, the risk of contralateral breast cancer was significantly reduced (HR: 0.70; 95% CI, 0.48 to 1.00) compared to 2 years. -Significant reduced risk of EFS, recurrence and BC mortality was also observed
12	M Regan et al, 2011	-Postmenopausal women with ER+/PR+- early BC who underwent surgery enrolled from 148 hospitals of 27 countries. -N= 8010	- Randomized, phase 3, double-blind trial (BIG 1-98). -Compares 5 years of TAM or letrozole monotherapy, or sequential treatment with 2	-Letrozole Dose: 2·5 mg/daily -TAM Dose: 20 mg daily -Duration: 5 years	Primary: Disease free survival (invasive BC relapse, second primaries [CBC and non-breast], or death without previous cancer event).	EM: -Monotherapy model: Randomization option (2-arm/4-arm) and CT use. -Sequential therapy: CT use -	-Letrozole monotherapy was significantly associated with reduced DFS (HR 0·82 [95% CI 0·74–0·92], overall survival HR 0·79 [0·69–0·90], DRFI HR 0·79 [0·68–0·92], BCFI HR

	Author, year	Study population	Study design	Exposure information	Outcome information	Confounders/ EMs	Result
		-Median follow up= 8.1 years (range 0-12.4 years)	years of one of these drugs followed by 3 years of the other.		Secondary: OS, distant recurrence-free interval (DRFI), and BC-free interval (BCFI)		0·80 [0·70–0·92] when compared to TAM.
13	J Cuzick et al, 2010	-Postmenopausal women with early stage operable invasive BC. -Median age = 72 years (IQR 65-91)	-Randomized Clinical Trial (Analysis of ATAC trial). -Assigned in 1:1:1 ratio, active ANA +TAM placebo; active TAM+ ANA placebo; active ANA + active TAM -Median follow up: 120 months (range 0-145)	-Anastrozole (ANA) Dose: 1 mg/daily -Tamoxifen Dose: 20 mg/daily -Duration: 5 years	Primary: Disease free survival, DFS. Secondary: time to recurrence, time to distant recurrence, censoring at deaths without recurrence; CBC; death after recurrence; and OS.	Stratified by HR-status	-Patients treated with A had improved DFS, lower time to recurrence, and lower time to distant recurrence compared to those treated with tamoxifen. -Incidence of CBC was lower in ANA group compared to the TAM group (HR= 0.68, 95% CI: 0.50-0.91) -Similar results were seen among HR+ patients with ANA and CBC risk (HR= 0.62, 95% CI: 0.45-0.85).
14	S Alkner et al 2009	-Premenopausal women with unifocal, stage II invasive breast cancer and received surgery.	-Randomized Clinical Trial. -Randomized to 2 years tamoxifen vs no adjuvant systemic therapy	Exposure: Tamoxifen	Metachronous CBC. -Second tumor developed >=3 months after first primary BC	Confounders: age, ER status, HER2 status, Nottingham histological grade, tumor size, lymph node status	-TAM was associate with reduced risk of CBC (HR=0.50, 95% CI: 0.28, 0.88). -TAM reduced risk of CBC in both ER+ and ER- patients, but significant

	Author, year	Study population	Study design	Exposure information	Outcome information	Confounders/ EMs	Result
		-Enrolled and randomized from two study centers in Sweden from 1986 to 1991. -N=564	-Median follow up: 14 years			EM: Age Interaction: ER status and TAM	reduction was seen among ER+ patients (HR=0.43, 95% CI: 0.20, 0.94).
15	ATAC Trial Group. JF Forbes et al, 2008	-Postmenopausal women with localized invasive BC who received 5 years of TAM -N= 6241 (TAM=3116; ANA=3125) -Mean age: 64 years (at study entry)	-Randomized clinical trial. -Median Follow up : 100 months (for 1 year) after 5 years of initial study. -Assessed in the total population and the HR+ sub-population	Anastrozole = 1 mg/daily Tamoxifen = 20 mg/daily -Mean duration of treatment : 4.11 years (SD: 1.65) for ANA and 3.97 (SD=1.71) for TAM	Primary: Disease free survival, DFS Secondary: time to recurrence, CBC, time to distant recurrence; death after recurrence; and overall survival	Not adjusted for covariates	-The study outcomes, DFS, CBC, TTR, and TTDR improved in both the total population and the HR+ sub population -For HR+ patients: DFS (HR= 0·85 ; 95% CI 0·76–0·94, p=0·003); CBC (HR 0·60 ; 95% Ci: 0·42–0·85), p=0·004; TR HR 0·76 (0·67–0·87), p=0·0001; TTDR HR 0·84 (0·72–0·97), p=0·022)
16	JN Ingle et al, 2008	-Postmenopausal women with HR-positive BC who had received ~5 years of adjuvant tamoxifen therapy. -N=5187	-Randomized Control Trial (MA.17). -Double blind placebo-controlled trial. -Median follow-up: 64 months (range 16-95 months)	Letrozole (2.5 mg/daily)	Primary: DFS (Any BC recurrence, and CBC events) Secondary: DDFS, overall survival, CBC	Confounder: Menopausal status (at the start of TAM); time on TAM before randomization. EM: receptor status, axillary node status, adjuvant CT	-Reduced risk of CBC (HR 0.61, 95% CI 0.39–0.97); DFS (HR 0.68, 95% CI 0.55–0.83) among those using letrozole vs those using placebo -Patients receiving letrozole within 3 months of TAM was associated with

	Author, year	Study population	Study design	Exposure information	Outcome information	Confounders/ EMs	Result
							reduced risk of DFS and CBC.
17	PE Goss et al, 2005	-Postmenopausal women who received 5 years of tamoxifen treatment ER+/PR+ or both BC. -N= 5170 (L=2583; Placebo: 2587). -Median Age: 62 years	-Randomized double-blind placebo-controlled trial (MA.17). -Randomized to receive letrozole vs placebo -Median follow up: 30 months	Letrozole -Dose: 2.5 mg/daily -Duration: 5 years	Primary: Disease free survival (time from randomization to the earliest recurrence of breast cancer or a CBC). Secondary: Overall survival, OS	Confounder: menopausal status at the start of tamoxifen treatment; duration of tamoxifen treatment. EM: HR-status, lymph node status, and prior CT.	-Letrozole use was associated with reduced risk of CBC or recurrence (adj HR: 0.59; 95% CI: 00.45-0.76). -No difference was seen in OS (HR: 0.82; 95% CI: 00.57-1.19).
18	R Jakesz et al, 2005	-Postmenopausal women aged <80 years (ABCSG trial 8) or 75 years or younger (ARNO 95) with hormone-sensitive invasive BC. -Completed 2 years' adjuvant oral tamoxifen -N= 3224 (A=1618; T=1606)	-Combined analysis of two multicenter randomized control trials, -ABCSG trial 8 and the ARNO 95 trial. -Randomized to receive or TAM or switch to ANA for 3 additional years. -Median follow-up: 28 months	Anastrozole Dose: 1 mg/day Tamoxifen Dose: 20 or 30 mg/day Duration: 5 years (TAM 2 years + 3 years TAM vs 3 years ANA)	Primary: Event free survival, (distant metastasis, contralateral BC)	EM: Country Stratified by age, tumor grade, tumor stage, nodal status, and participating centers.	-There was a 40% decrease in the risk for an event in the anastrozole group as compared with the tamoxifen group (HR= 0·60, 95% CI 0·44–0·81, p=0·0009).
19	RC Coombes et al, 2004	-Postmenopausal women with ER-positive/unknown invasive primary BC who remained free of disease after receiving adjuvant TAM for 2-3 years.	-Randomized, double-blind trial. -Randomized to receive exemestane, E or TAM to complete total 5 years of ET.	Exemestane Dose: 25 mg Tamoxifen Dose: 20 mg (some 30 mg)	-Primary: DF (includes recurrence of BC at any site, CBC, or death from any cause).	Confounders: Prognostic factors (ER receptor status, nodal status, chemotherapy, use of HRT)	-Exemestane significantly reduced the risk of time to CBC (HR, 0.44; 95% CI, 0.20 to 0.98; P=0.04). -There was no statistically

	Author, year	Study population	Study design	Exposure information	Outcome information	Confounders/ EMs	Result
		-N=4742 (recruited between February 1998-February 2003 from 37 countries)	-Median follow-up: 30.6 months		-Secondary end points included OS, incidence of CBC, and long-term tolerability		significant difference in overall survival (HR, 0.89; 95% CI, 0.67 to 1.17). -DFS was reduced among exemestane group as compared with the TAM group was 0.67 (95% CI, 0.56 to 0.82)
20	AU Buzdar et al, 2003	-Postmenopausal women with operable invasive BC following completion of primary therapy, who were candidates to receive adjuvant ET, July 1996-March 2000. -N=9366 (*ANA*=3125, TAM=3116 and COMB= 3125)	-Randomized clinical (double-blind) trial. -ATAC ('Arimidex', Tamoxifen Alone or in Combination) -Compared TAM with ANA alone, and the combination of ANA + TAM -Median follow up: 33.3 months	Tamoxifen Dose: 20 mg Anastrozole ('Arimidex') Dose: 1 mg -Combination of anastrozole plus tamoxifen	Primary: Disease free survival Secondary: Incidence of CBC, time to recurrence, TTR.		-ANA use reduced the risk of CBC by 58% (OR=0.42, 95% CI: 0.22-0.79) -ANA use improved DFS (HR=0.81, 95% CI 0.71–0.96) compared to TAM. ANA was superior to the combination therapy for both DFS and TTR.
21	PE Goss et al, 2003	-Postmenopausal women with BC who has completed approx. 4.5-6 years of TAM. -August 1998-September 2002 -N=5187	-Randomized (Double blinded placebo) controlled trial. -Randomized into treatment with letrozole and placebo group.	Letrozole Dose: Duration: Year 5-Year 10 Clinical evaluation, routine blood work, evaluation of toxic effects was performed semiannually in 1st	Disease free survival (time from randomization to the recurrence of the primary disease or the development of a new primary	Stratified by HR-status, lymph node status, and previous CT use.	-Letrozole was associated with reduced CBC risk (HR=0.57; 95% CI: 0.43, 0.75). Similar effect was seen among node +ve/-ve CBC.

	Author, year	Study population	Study design	Exposure information	Outcome information	Confounders/ EMs	Result
		2593 (letrozole group); 2594 (tamoxifen group)	-Median follow up: 2.4 years	year and then annually	breast cancer in the contralateral breast) Secondary end points: OS, quality of life and long-term safety. Mammography was performed annually		-Improvements in DFS, distant metastases, reduced BC deaths were observed in the L group compared to placebo.
22	Schmid et al, 2003	-Postmenopausal women with stage I/II, HR+ BC. -From December 1990-Dec 1995 - N= 1986 (TAM=996; Combination: 990)	-Randomized Clinical Trial (ABCSG Trial 6). -By the Austrian Breast and Colorectal Cancer Study Group (ABCSG). -Randomized to receive TAM for 5 years vs Aminoglutethimide for first 2 years + 5 years TAM. - Data collected at ABCSG trial center. -Median follow up: 5.3 years	-Tamoxifen monotherapy Dose : 20 mg Duration: 5 years -Aminoglutethimide Dose:125 mg; 250 mg Duration: First 2 years	Primary: DFS (loco regional, distant, or combined), and OS (death) - Mammography	Confounder: treatment, tumor grade, tumor stage, lymph node status, age, ER, PR, surgical procedures, and radiotherapy	-No significant difference in recurrence (P = 0.89) and survival (P=0.74) between combination and TAM group. -No significant difference in prevalence was seen for CBC among the two groups
23	Swedish Breast Cancer Cooperative Group, 1996	-Postmenopausal women aged <= 75 years with operable, axillary lymph node negative/positive	-Randomized Control Trial. -Randomized to 2 years or 5 years TAM therapy.	Tamoxifen -Treatment assigned by telephone call or letter to the office of	Primary: Event free survival (first of any of the local-regional	EM: Treatment center	-There was significant improved event-free survival with 5 years of TAM in

	Author, year	Study population	Study design	Exposure information	Outcome information	Confounders/ EMs	Result
		invasive breast cancer enrolled from 1983-1991 from Sweden. N= 3545	-By Swedish Breast Cancer Cooperative Group -5 participating centers in Sweden for randomization, treatment and follow up. -Median follow up: 5.5 years	each of the 5 organizations	recurrence, distant metastasis, contralateral breast cancer, or death, irrespective of the underlying cause) and Overall survival -Secondary: the local-regional recurrence, distant metastasis, contralateral breast cancer, or death. Data: Mortality was checked through record linkages to regional population registers.		relation to 2 years of treatment (HR= 0.82, 95% CI: 0.71, 0.96). -CBC risk was reduced (HR= 0.74, 95% CI: 0.43, 1.29). -All cause mortality all improved in the 5-year group (HR= 0.82, 95% CI: 0.69, 0.99)
24	B Xing et al, 1996	-Female primary breast cancer patients identified from 1st January 1985 –31st December 1990 from the Dept of Surgery at Beijing Institute for Cancer Research. -N=351.	-Randomized Control Trial	-Adjuvant Tamoxifen therapy. Dose: 20 mg daily Duration: 2-5 years. N=523 -Placebo group; w/o any tamoxifen therapy	CBC		-The incidence of CBC in the treated group was 1.5% (8/523), and that in the control group was 3.7%. -Patients assigned to receive 5 years of tamoxifen, compared with 2 years of tamoxifen, experienced statistically

	Author, year	Study population	Study design	Exposure information	Outcome information	Confounders/ EMs	Result
							significant improvements in event-free survival (HR = 0.82; 95% CI = 0.71- 0.96) and OS (HR = 0.82; 95% CI = 0.69- 0.99). (13/351, P < 0.05).
25	LE Rutqvist et al, 1995	-Postmenopausal BC patients aged <= 71 years who have undergone surgery were randomly allocated from November 1976 – May 1990. -N-=2729 (1372 allocated to TAM and 1357 to the placebo group)	-Randomized Control Trial. -Stockholm Trial. -Randomized to receive either 2 years of adjuvant tamoxifen therapy or no adjuvant therapy (Placebo).	Exposure: Tamoxifen -High risk patients were allocated 4 groups postoperative radiation therapy, postoperative radiation therapy plus tamoxifen therapy, chemotherapy, or chemotherapy plus tamoxifen therapy	Second primary malignancies (CBC, endometrial, gastrointestinal , colorectal, and stomach cancers) -From retrospective linkage to the Swedish Cancer registry		-Significant reduction of CBC (P=0.008) among patients treated with tamoxifen. -There was an increase of risk endometrial, gastrointestinal, stomach and colorectal cancers among tamoxifen users.
26	LE Rutqvist et al, 1991	-Postmenopausal BC patients aged <= 71 years who have undergone surgery were randomly allocated from November 1976 – September 1, 1986. -N=1846 (TAM =931; Placebo= 915)	RCT (Stockholm Trial). -Randomized to receive either 2 years of adjuvant tamoxifen therapy or no adjuvant therapy (Placebo).	Tamoxifen therapy -High risk patients were allocated 4 groups postoperative radiation therapy, postoperative radiation therapy plus tamoxifen therapy, chemotherapy, or chemotherapy plus tamoxifen therapy	Incidence of CBC		-There was a significant reduction of contralateral breast cancer in the TAM group versus that in the placebo (29 versus 47 cases, respectively; P = .03). -The cumulative incidence at 10 years in the TAM group and the control group was

	Author, year	Study population	Study design	Exposure information	Outcome information	Confounders/ EMs	Result
							5% and 8%, respectively.
27	B Fisher et al, 1989	Women aged <70 years with lymph node negative, ER+ BC -Randomized from Jan 4, 1982- Dec 31, 1987, from US and Canada. -N: 2818	-Randomized (double blinded) Clinical Trial (B-14 study). -Follow till Mar 14, 1988	-Tamoxifen Duration: 5 years Dose: 20 mg/ day -Placebo	-DFS (local recurrences, regional and distant metastases, recurrence of tumor in the ipsilateral breast, CBC, second primary tumor, and death due to causes other than cancer) -OS	-Stratified by age, tumor size at examination, type of operation, ER receptor concentration. Interactions: Treatment and stratification variables	-Significant difference in DFS between TAM users vs nonusers (P<0.00001) -There was reduction in CBC incidence too.

BC: breast cancer, CBC: contralateral breast cancer, BCM: breast cancer mortality, ACM: all-cause mortality, CT: chemotherapy, ET: endocrine therapy, TAM: tamoxifen, AI: aromatase inhibitors, EM: effect modifier

Appendix C: Association between circulating IGFs and odds of breast cancer, prior to tamoxifen initiation

IGFs (ng/ml)		Model 1*	Model 2**
	N	OR (95% CI)	OR (95% CI)
IGF-I			
T1	57	1.00	1.00
T2	73	2.16 (0.90, 5.22)	1.82 (0.73, 4.50)
T3	64	1.47 (0.59, 3.68)	1.35 (0.52, 3.50)
p trend (p-het)		0.37 (0.21)	0.62 (0.42)
IGF-I (continuous)[a]		1.61 (0.76, 3.43)	1.63 (0.73, 3.64)
IGFBP-3			
T1	54	1.00	1.00
T2	69	2.31 (0.92, 5.84)	2.32 (0.90, 5.97)
T3	71	2.57 (1.02, 6.46)	2.65 (1.03, 6.83)
p trend (p-het)		0.06 (0.11)	0.05 (0.11)
IGFBP-3 (continuous)[a]		1.03 (0.99, 1.06)	1.03 (0.99, 1.07)
IGF-I: IGFBP-3 Ratio[b]			
T1	64	1.00	1.00
T2	63	0.81 (0.35, 1.85)	0.77 (0.33, 1.80)
T3	67	1.06 (0.47, 2.39)	0.96 (0.40, 2.28)
p trend (p-het)		0.86 (0.79)	0.94 (0.80)
IGF-I: IGFBP-3 Ratio (continuous)		1.08 (0.75, 1.55)	1.05 (0.71, 1.54)

OR and 95% CI were estimated from logistic regression analyses. Model 1* adjusted for matching variables, age (ordinal), race (categories), menopausal status (categories)); Model 2**adjusted for matching variables (age (ordinal), race (categories), menopausal status (categories), BMI at baseline (ordinal), and baseline sound speed (ordinal).

[a]Concentrations per 100 ng/ml. b IGF-I: IGFBP-3 Ratio: IGF-I/IGFBP-3 * 100. Tertile values (ng/mL):- IGF-I: T1 <106.126, T2 = 106.126-<141.113, T3 ≥141.113; IGFBP-3: T1 <3408.10, T2 = 3408.10-<4141.89, T3: ≥4141.89; IGF-I: IGFBP-3 Ratio: T1 <0.029011, T2 = 0.029011-<0.035482, T3 ≥ 0.035482.IGFs= insulin-like growth factors, IGF-I= insulin-like growth factor-I, IGFBP-3= insulin like growth factor binding protein 3

Appendix D: Proportion of EMs in relation to total EMs among postmenopausal women

Estrogen and EMs (pmol/l)	Baseline visit
	Proportion (%)
Parent estrogens	41.5
Estrone	33.8
Estradiol	7.7
2-Hydroxylation pathway	26.7
2-Hydroxyestrone	16.1
2-Hydroxyestradiol	4.0
2-Methoxyestrone	3.7
2-Methoxyestradiol	2.7
2-Hydroxyestrone-3methyl ether	0.2
4-Hydroxylation pathway	1.6
4-Hydroxyestrone	1.3
4-Methoxyestrone	0.3
4-Methoxyestradiol	0.1
16-Hydroxylation pathway	30.2
16a-Hydroxyestrone	4.4
Estriol	12.1
16-Ketoestradiol	7.9
16-Epiestriol	3.3
17-Epiestriol	2.4

Each EMs are presented as a proportion of the total estrogen metabolite concentrations at baseline among postmenopausal women

Appendix E: Distribution of participant characteristics among tamoxifen treated breast cancer patients and untreated women in the comparison group, prior to tamoxifen initiation

Participant characteristics	**Tamoxifen-treated group (n=53)**	**Untreated group (n=141)**	**p-value***
	N (%)	N (%)	
Age (years), Mean (SD)	51.6 (8.8)	52.3 (8.1)	0.62
BMI (kg/m^2), Mean (SD)	30.4 (7.1)	30.8 (7.6)	0.75
Age (years)			
<45	11 (20.8)	29 (20.6)	0.86
45-<50	13 (24.5)	33 (23.4)	
50-<55	14 (26.4)	31 (22.0)	
≥55	15 (28.3)	48 (34.0)	
BMI (Kg/m^2)			
<25	13 (24.5)	33 (23.4)	0.96
25-30	14 (26.4)	40 (28.4)	
>30	26 (49.1)	68 (48.2)	
Race			0.50
White	17 (21.1)	46 (32.6)	
Black/African American	32 (60.4)	90 (63.8)	
Other	4 (7.6)	5 (3.6)	
Education			0.87
High School or Less	17 (32.1)	41 (29.1)	
Some College	15 (28.3)	45 (31.9)	
College or Higher	21 (39.6)	55 (39.0)	
First Degree Relative with breast cancer			0.19
No	37 (69.8)	111 (78.7)	
Yes	16 (30.2)	30 (21.3)	
Age at Menarche (years)			0.26
≤ 12	30 (56.6)	79 (56.0)	
13	8 (15.1)	34 (24.1)	
≥ 14	15 (28.3)	28 (19.9)	
No. of live births			0.24
0	7 (13.2)	34 (24.1)	
1-2	27 (50.9)	60 (42.6)	
≥3	19 (35.9)	47 (33.3)	
Age at First Birth (years)[a]			0.05
<20	10 (21.7)	40 (37.4)	
20-24	9 (19.6)	30 (28.0)	
25-29	15 (32.6)	21 (19.6)	
≥30	12 (26.1)	16 (15.0)	
Menopausal Status			0.07

Participant characteristics	Tamoxifen-treated group (n=53)	Untreated group (n=141)	p-value*
	N (%)	N (%)	
Premenopausal	37 (69.8)	78 (55.3)	
Postmenopausal	16 (30.2)	63 (44.7)	
Former Use of Menopausal Hormone Therapy[b]			0.45
No	11 (31.3)	49 (77.8)	
Yes	5 (68.8)	14 (22.2)	
Endocrine Symptoms in the past two weeks			
Hot Flushes			
No	33 (62.3)	78 (55.3)	0.38
Yes	20 (37.7)	63 (44.7)	
Joint pain			
No	27 (50.9)	60 (42.6)	0.29
Yes	26 (49.1)	81 (57.5)	
Tertiles of baseline sound speed (m/s)			
<1442.07	7 (13.2)	46 (32.6)	0.007
1442.07-<1449.52	17 (32.1)	48 (34.0)	
≥1449.52	29 (54.7)	47 (33.3)	

*p values for differences in participant characteristics by case and comparison group. *****P values generated from t-tests (continuous variables) and Chi-square tests (categorical variables). [a]Among parous women (Cases=46; Comparison group=107). [b]Among postmenopausal women (Cases=16; Comparison group=63)

Appendix F: Newcastle - Ottawa quality assessment scale and rationale of scoring eligible studies

Manuscript 3: Endocrine therapy use and the risk of contralateral breast cancer: A systematic review and meta-analysis

Exposure: Endocrine therapy use; Outcome: Contralateral breast cancer (CBC)

COHORT STUDIES

Note: A study can be awarded a maximum of one star for each numbered item within the Selection and Exposure categories. A maximum of two stars can be given for Comparability.

Selection

1) Representativeness of the exposed cohort

a) truly representative of the average in the community *****
b) somewhat representative of the average in the community *****
c) selected group of users e.g., nurses, volunteers
d) no description of the derivation of the cohort

Studies that selected participants from large population-based databases including cancer registries are representative of exposed women in the community, and hence will receive a star. Studies that have selected their population from hospitals and/or clinical institutes will not receive a star.

2) Selection of the non-exposed cohort

a) drawn from the same community as the exposed cohort *****
b) drawn from a different source
c) no description of the derivation of the non-exposed cohort

Studies that selected non-users of endocrine therapy from the same population as the exposed cohort, they will receive a star. Otherwise, they will not receive a star.

3) Ascertainment of exposure

a) secure record (e.g., surgical records) *****
b) structured interview *****
c) written self-report
d) no description

If the study uses medical records for ascertainment of endocrine therapy use, they will receive a star. Studies using self-reported and/or interviewer administered questionnaire for ascertainment

of endocrine therapy use will not receive a star.

4) Demonstration that outcome of interest was not present at start of study

a) yes *****
b) no

Cohort studies that state that the study population did not have a history of contralateral breast cancer will receive a star.

Comparability

1) Comparability of cohorts on the basis of the design or analysis
a) study controls for _____ (select the most important factor) *****
b) study controls for any additional factor ***** (This criterion could be modified to indicate specific control for a second important factor.)

The most important factors for the association between endocrine therapy use and the risk of CBC include age/menopausal status. Additional important adjustment factors include other BC treatments (Chemo, radiotherapy), stage, histology, HR-status etc. Studies that have adjusted for age and/or menopausal status will receive one star, and those that adjusted for age/menopausal status, and treatment factors/stage/histology will receive two stars.

If any studies, tests for these covariates using statistical tests, but does not include them in the final model, citing proper reason, for e.g.: covariates not associated with the exposure, endocrine therapy, and/or the outcome, CBC, hence, not meeting definition of a confounder, they will receive the star(s) depending on which covariates were tested.

Outcome

1) Assessment of outcome

a) independent blind assessment *****
b) record linkage *****
c) self-report
d) no description

Studies that assessed the outcome, CBC via medical records and/or cancer registry will receive a star. Studies that use self-reported data, or that do not provide any information will not receive a star.

2) Was follow-up long enough for outcomes to occur

a) yes (select an adequate follow up period for outcome of interest) *****
b) no

Based on the guidelines of the NOS scale, first, the minimum follow-up time to develop CBC was determined. If the studies reported the follow-up period with a mean/median and a range, and the mean/median is longer than the required minimum, the study will receive a star.

From previous literature using SEER data, the mean (SD) time to development of CBC was reported to be 8.7 (5.9) years, while the median (range) was 7.8 (0.1–25.0) years[318]. A different study using SEER data reported a median (range) follow-up of 7.9 (1.00-24.9) years to develop CBC[300]. Hence, eligible studies in this meta-analysis that has a mean/median follow-up of ≥1 year, will receive a star.

3) Adequacy of follow up of cohorts

a) complete follow up - all subjects accounted for *****
b) subjects lost to follow up unlikely to introduce bias - small number lost - > ____ % (select an adequate %) follow up, or description provided of those lost) *****
c) follow up rate < ____% (select an adequate %) and no description of those lost
d) no statement

Cohort studies included in this meta-analysis use time to event data, and hence, the study population are censored at loss to follow-up or death. Studies using population-based cancer registry linkage is expected to have complete/near complete follow-up data and will receive a star. No studies included using cancer registry linkage in this meta-analysis have provided information on the proportion of linkage

CASE CONTROL STUDIES

Note: A study can be awarded a maximum of one star for each numbered item within the Selection and Exposure categories. A maximum of two stars can be given for Comparability.

1) Is the case definition adequate?

a) yes, with independent validation *****
b) yes, e.g., record linkage or based on self-reports
c) no description

If the studies clearly define cases of contralateral breast cancer and mentions validation of case-status by cancer registry and/or medical database records, they will receive a star.

2) Representativeness of the cases

a) consecutive or obviously representative series of cases *****

b) potential for selection biases or not stated

Studies that selected cases from larger population-based database and/or registry which are representative of CBC cases in a population and/or community, will receive a star.

3) Selection of Controls

a) community controls *****
b) hospital controls
c) no description

Studies that have selected controls from the same base population as the cases, and who essentially would have been cases had the outcome been present, would fulfil this criterion and will receive a star.

4) Definition of Controls

a) no history of disease (endpoint) *****
b) no description of source

Studies that define controls as the study population who were diagnosed with first primary breast cancer without any history of CBC, will receive a star.

Comparability

1) Comparability of cases and controls on the basis of the design or analysis
a) study controls for _____ (Select the most important factor.) *****
b) study controls for any additional factor ***** (This criterion could be modified to indicate specific control for a second important factor.)

The most important factors for the association between endocrine therapy use and the risk of CBC include age/menopausal status. Additional important adjustment factors include other BC treatments (Chemo, radiotherapy), stage, histology, HR-status etc. Studies that have adjusted for age and/or menopausal status will receive one star, and those that adjusted for age/menopausal status, and treatment factors/stage/histology will receive two stars. Additionally, matching on the aforementioned factors and the adjustment of the matching factors in the analysis will be reviewed.

If any studies, tests for these covariates using statistical tests, but does not include them in the final model, citing proper reason, for e.g.: covariates not associated with the exposure, endocrine therapy, and/or the outcome, CBC, hence, not meeting definition of a confounder, they will receive the star(s) depending on which covariates were tested.

Exposure

1) Ascertainment of exposure

a) secure record (e.g., surgical records) *****
b) structured interview where blind to case/control status *****
c) interview not blinded to case/control status
d) written self-report or medical record only
e) no description

Studies that assessed the exposure, endocrine therapy using medical records and/or structured interview blinded to case-control status will receive a star. Studies that use self-report, structured interview not blinded to case-control status, or that do not provide any information will not receive a star.

2) Same method of ascertainment for cases and controls

a) yes *****
b) no

Studies that assessed the exposure, endocrine therapy similarly among cases and controls will receive a star.

3) Non-Response rate

a) same rate for both groups *****
b) non-respondents described
c) rate different and no designation

Studies that mention that data availability and/or missing data was similar among cases and controls will receive a star. Those that do not mention missing data, or data availability will not receive a star.

Appendix G: Association between changes in circulating IGFs and volume averaged sound speed (VASS) measures among tamoxifen-treated women, who continued tamoxifen use till 12-months follow-up (n=49)

Change in IGFs (ng/ml)		**Change in VASS (m/s)**		
	N	**Model 1***	**Model 2****	**Model 3****
		β (95% CI)	β (95% CI)	β (95% CI)
Change in IGF-I[a]				
< -51.7	18	Ref	Ref	Ref
-51.7 - <-17.6	18	3.19 (-2.78, 9.18)	3.91 (-1.63, 9.44)	3.56 (-2.46, 9.57)
≥ -17.6	17	-2.73 (-9.03, 3.57)	-1.34 (-7.24, 4.57)	-1.10 (-7.25, 5.04)
p trend (p-het)		0.47 (0.18)	0.79 (0.19)	0.82 (0.31)
Change in IGF-I (continuous)[b]		-2.61 (-11.14, 5.92)	-0.6 (-8.54, 7.34)	-0.94 (-9.10, 7.21)
Change in IGFBP-3[a]				
< -177.9	18	Ref	Ref	Ref
-177.9 - < 269.7	17	-4.47 (-10.65, 1.70)	-4.25 (-10.21, 1.71)	-3.79 (-9.96, 2.38)
≥ 269.7	18	-5.05 (-11.23, 1.12)	-4.23 (-10.11, 1.64)	-3.73 (-9.84, 2.38)
p trend (p-het)		0.10 (0.20)	0.13 (0.24)	0.20 (0.35)
Change in IGFBP-3 (continuous)[b]		-0.34 (-0.83, 0.16)	-0.33 (-0.81, 0.15)	-0.29 (-0.79, 0.20)
Change in IGF-I: IGFBP-3 Ratio[a]				
< -0.01	18	Ref	Ref	Ref
-0.01 - < -0.007	17	-0.77 (-6.96, 5.42)	-1.93 (-7.67, 3.81)	-2.34 (-8.06, 3.38)
≥ -0.007	18	0.84 (-5.68, 7.37)	2.94 (-3.03, 8.92)	4.04 (-2.14, 10.22)
p trend (p-het)		0.82 (0.88)	0.40 (0.30)	0.30 (0.16)
Change in IGF-I: IGFBP-3 Ratio (continuous)		-0.39 (-4.54, 3.76)	0.36 (-3.42, 4.15)	0.34 (-3.60, 4.28)

Beta estimates and 95% CI were estimated from linear regression analyses. Model 1* Unadjusted; Model 2**adjusted for age (ordinal), BMI change at 12 months (ordinal), menopausal status (categories), baseline VASS (ordinal); Model 3***Adjusted for age(ordinal), menopausal status (categories), BMI change at 12 months (ordinal), baseline VASS (ordinal), and race (categories). [a]The categories are based on tertile cut points. [b]Concentrations per 100 ng/ml. VASS= volume averaged sound speed, IGFs= insulin-like growth factors, IGF-I= insulin-like growth factor-I, IGFBP-3= insulin like growth factor binding protein 3

Appendix H: Association between circulating IGFs and volume averaged sound speed (VASS) measures, among premenopausal tamoxifen-treated and untreated group, prior to treatment initiation

IGFs (ng/ml)	Tamoxifen treated group (n=37)		Untreated group (n=78)	
	N	β (95% CI)		β (95% CI)
IGF-I				
T1	9	Ref	25	Ref
T2	15	-1.71 (-14.24, 10.81)	28	0.39 (-8.47, 9.25)
T3	13	-13.52 (-26.37, -0.67)	25	6.28 (-2.78, 15.34)
p trend (p-het)		0.03 (0.06)		0.17 (0.31)
IGF-I (continuous)[a]		-15.73 (-27.89, -3.56)		7.01 (-1.39, 15.41)
IGFBP-3				
T1	8	Ref	25	Ref
T2	11	-20.21 (-32.88, -7.53)	28	-2.15 (-10.84, 6.54)
T3	18	-18.66 (-30.19, -7.12)	25	-0.93 (-9.86, 7.99)
p trend (p-het)		0.01 (0.004)		0.85 (0.88)
IGFBP-3 (continuous)[a]		-0.71 (-1.28, -0.14)		0.02 (-0.38, 0.42)
IGF-I: IGFBP-3 Ratio				
T1	12	Ref	26	Ref
T2	8	-3.87 (-19.39, 11.65)	27	-0.20 (-8.61, 8.21)
T3	17	-0.95 (-14.29, 12.38)	25	8.39 (-0.27, 17.05)
p trend (p-het)		0.89 (0.87)		0.05 (0.07)
IGF-I: IGFBP-3 Ratio (continuous)		-1.50 (-8.33, 5.32)		4.35 (0.42, 8.28)

Beta estimates and 95% CI were estimated from linear regression analyses. Model 1* Unadjusted; Model 2**Adjusted for age (ordinal), BMI (ordinal), and race (categories). p-het: p for heterogeneity. p-int: p for interaction between cases and controls (Model 2). aConcentrations per 100 ng/ml. Premenopausal Women: Tertile values (ng/mL):- IGF-I: T1 <118.903, T2 = 118.903-<145.267, T3 ≥145.267; IGFBP-3: T1 <3499.29, T2 = 3499.29-<4156.53, T3: ≥4156.53; IGF-I: IGFBP-3 Ratio: T1 <0.030480, T2 = 0.030480-<0.036372, T3 ≥ 0.036372. Postmenopausal Women: Tertile values (ng/mL):- IGF-I: T1 <96.5602, T2 = 96.5602-<133.087, T3 ≥133.087; IGFBP-3: T1 <3298.69, T2 = 3298.69-<4141.89, T3: ≥4141.89; IGF-I: IGFBP-3 Ratio: T1 <0.027707, T2 = 0.027707-<0.033464, T3 ≥ 0.033464. VASS= volume averaged sound speed, IGFs= insulin-like growth factors, IGF-I= insulin-like growth factor-I, IGFBP-3= insulin like growth factor binding protein 3

Appendix I: Relation of participant characteristics with volume averaged sound speed (VASS) measures, among untreated women in the comparison group (n=141) in the Ultrasound Study of Tamoxifen, prior to tamoxifen initiation

Participant characteristics	**N (%)**	**VASS (m/s) Mean (SD)**	**p-value***
Age at Baseline (years)			0.02
<45	29 (20.6)	1452.3 (16.6)	
45-<50	33 (23.4)	1456.0 (17.7)	
50-<55	31 (22.0)	1449.2 (13.4)	
≥55	48 (34.0)	1446.9 (13.2)	
BMI at Baseline (Kg/m^2)			<0.0001
<25	33 (23.4)	1461.1 (21.1)	
25-30	40 (28.4)	1451.7 (15.1)	
>30	68 (48.2)	1444.9 (8.0)	
Race			0.94
White	46 (32.6)	1449.3 (11.7)	
Black/African American	90 (63.8)	1451.4 (17.0)	
Other	5 (3.6)	1449.4 (15.7)	
Education			0.34
High School or Less	41 (29.1)	1448.7 (13.2)	
Some College	45 (31.9)	1453.4 (19.4)	
College or Higher	55 (39.0)	1449.8 (12.9)	
First Degree Relative with Breast Cancer			0.54
No	111 (78.7)	1450.2 (13.5)	
Yes	30 (21.3)	1452.2 (21.1)	
Age at Menarche (years)			0.24
≤ 12	79 (56.0)	1448.9 (13.6)	
13	34 (24.1)	1452.4 (17.8)	
≥ 14	28 (19.9)	1453.5 (16.9)	
No. of Live Births			0.65
0	34 (24.1)	1453.8 (19.5)	
1-2	60 (42.6)	1450.9 (15.5)	
≥3	47 (33.3)	1448.0 (11.3)	
Age at First Birth (years)[a]			0.16
<20	40 (37.4)	1449.4 (14.5)	
20-24	30 (28.0)	1448.3 (14.4)	
25-29	21 (19.6)	1452.7 (11.9)	
≥30	16 (15.0)	1448.6 (13.9)	
Menopausal Status			0.01
Premenopausal	78 (55.3)	1453.5 (16.1)	

Participant characteristics		VASS (m/s)	
	N (%)	Mean (SD)	p-value*
Postmenopausal	63 (44.7)	1447.1 (13.8)	
Former Use of Menopausal Hormone Therapy[b]			0.79
No	49 (77.8)	1447.2 (15.2)	
Yes	14 (22.2)	1446.4 (7.9)	
Endocrine Symptoms in the past two weeks			
Hot Flushes			0.84
No	78 (55.3)	1450.4 (15.4)	
Yes	63 (44.7)	1450.9 (15.9)	
Joint pain			0.18
No	60 (42.6)	1452.7 (16.0)	
Yes	81 (57.5)	1449.1 (14.8)	

*P values for differences in breast density across categories of each covariate. t-tests for covariates with two categories; Kruskal-Wallis test for covariates with 3 or more categories. [a]Among parous women (n=107). [b]Among postmenopausal women (n=63). VASS= volume averaged sound speed

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