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Assessment of Her2-neu in Breast Cancer Lines Upon Differential Exposures to Xenoestrogens

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This is to certify that the doctoral dissertation by

Abha Aggarwal

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Walden University
2015

Abstract

Assessment of *Her2*-neu in Breast Cancer Lines Upon Differential Exposures to Xenoestrogens

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Abha Aggarwal

Dissertation Submitted in Partial Fulfillment

of the Requirements for the Degree of

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Abstract

Synthetic xenoestrogens have differential estrogenic properties. Research has shown that exposures to xenoestrogens could promote breast cancer by disrupting normal function of the human epidermal growth factor receptor 2 (*Her2*) gene. Although animal models demonstrated a connection between xenoestrogen exposure and *Her2* activity, no study using human cells has systematically examined their carcinogenic potential influencing the *Her2* gene expression. Furthermore, breast cancer cells are phenotypically disparate (ER+, *Her2*+), with some phenotypes *(Her2+),* leading to more aggressive disease. This study aimed to dosimetrically assess the carcinogenic potential of commonly used xenoestrogens influencing *Her2* gene expression, and delineate cellular phenotypes at greater risk of more aggressive disease. The study assessed whether the composition, concentrations, and exposure duration of BPA, EE, NPH, and DDT significantly altered *Her2* copy numbers in estrogen and *Her2* receptor positive or negative breast cancer lines. Each line was randomly assigned to cases (exposed) and control (unexposed) groups using a randomized block design. Fluorescent in-situ hybridization measured *Her2* gene copies. Mann Whitney, Kruskal Wallis, and Incidence Rate Ratios revealed H*er2* copy gains in all 4 xenoestrogens and receptor types with persistent exposures. A 44% increase in *Her2* was observed in the normal ER and *Her2* line, marking a shift in its *Her2* status, and a 30-times greater risk was noted in the *Her2*+ lines. These findings promote positive social change by revealing all 4 xenoestrogens as risk factors for breast cancer. This information can be used by breast cancer advocacy groups, health educators, and steering committees to educate women and formulating policies.

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Dedication

I dedicate my dissertation work to my parents who taught me to believe in myself, work hard, and persevere. This work is truly a testament to all their teachings.

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Chapter 1: Introduction to the Study

Introduction and Epidemiology

In 2011, the incidence and mortality for breast cancer in the United States were 220,097 and 40,931 women respectively (Centers for Disease Control and Prevention [CDC], 2014). For 2015, is the American Cancer Society [ACS], (2015) estimated that 231,840 cases of invasive and 60,290 cases of in situ breast cancer would be diagnosed. Regardless of race and ethnicity, breast cancer is the most common type of cancer found in American women (CDC, 2014). In the United States, breast cancer is the leading cause of death in Hispanic women and the second leading cause of death in women of all other ethnicities. Currently, almost 2.9 million women are living with a history of breast cancer in the United States (ACS, 2015; Surveillance, Epidemiology, and End Results Program [SEER], n.d.). Breast cancer cases are further estimated to increase by 50% in the next 15 years in America (American Association for Cancer Research [AACR], 2015).

The global incidence rate of breast cancer was 1.7 million cases in 2012 (Ferlay, Soerjometram, Dikshit, Eser, Mathers, ... Bray, 2014). Breast cancer is also the main cause of death for women globally, with its worldwide mortality rates reaching 522,000 women in 2012 (Ferlay et al., 2014; International Agency for Research on Cancer [IARC], 2013). In 2012, the global incidence and mortality for breast cancer rose dramatically by more than 20% and 14% respectively compared to 2008 (IARC, 2013). Additionally, 6.3 million women worldwide were found to be living with a history of breast cancer diagnosed in the past five years (Ferlay et al., 2014; IARC, 2013). A woman's lifetime risk of breast cancer today has increased, from 1 out of every 11 found

in the 1970s to 1 in every 8 women (ACS, 2013; Valeron, Pestano, Luzardo, Zumbado, & Boada, 2009).

Financially, breast cancer accounted for $18.1 billion of annual health care expenditures in the United States alone, ranking highest amongst all cancer care expenditure for 2014 (National Cancer Institute [NCI], 2015a). Projections on the overall cost of breast cancer care using current statistics, have suggested that they will rise from $16.5 billion in 2010 to $23.24 billion in 2020, with the largest increments (40.9%) found to be in breast cancer (NCI, 2015b). Furthermore, a trend analysis of indirect costs of breast cancer, such as loss in time and economic productivity due to illness and mortality are projected to rise from $52.4 billion in 2010 to $102.26 billion by 2023, a 95.2% increase (Milken Institute, n.d.). Together, these statistics indicated that the breast cancer incidence, mortality, risk, and financial burden worldwide are on the rise, making breast cancer a public health concern.

Major risk factors known for breast cancer (e.g., genetic predisposition of BRCA1 and 2, parity, reproductive history, lactation, age at menarche) and the increased usage of screening mammography only account for a third of all breast cancer cases (Aube, Larochelle, & Ayotte, 2011; Buteau-Lozano, Velasco, Cristofari, Balaguer, & Perrot-Applanat, 2008; Davis & Sieber, 1997). Lifestyle factors account for remainder (Aube, Larochelle, & Ayotte, 2011). Current researchers have considered exposure to environmental toxins as a lifestyle factor, and they have studied it as a potential risk factor for breast cancer (Aube, Larochelle, & Ayotte, 2011; Rudel, Attfield, Schifano, & Brody, 2007). Environmental toxins, such as xenoestrogens, have been partially blamed

for the increase in breast cancer incidence (Aube, Larochelle, & Ayotte, 2011; Buteau-Lozano, Velasco, Cristofari, Balaguer, & Perrot-Applanat, 2008; Davis et al., 1997).

Estrogen and progesterone are two hormones that are required for normal breast development and function, but their unregulated stimulation by extrinsic estrogen such as xenoestrogens can de-regulate the cell-cycle and result in breast cell proliferation, inducing carcinogenicity (Brown & Lamartinere, 1995; Murray, Maffini, Ucci, Sonnenschein, & Soto, 2007; Recchia et al., 2004). Estrogen receptors (ERs) are activated by ligands (e.g., estrogen, xenoestrogens), and with the help of many cofactors and growth factors can regulate estrogen responsive genes (Arpino, Wiechmann, Osborne, & Schiff, 2008; McKenna & O'Malley, 2002). Also, required for normal breast development is human epidermal growth factor receptor 2 (*Her2*), a proto-oncogene, which can mutate into its oncogenic state causing breast carcinogenesis. The *Her2* proto-oncogene is found in two copies in the normal breast tissue, but in its mutated form there is an increase in the gene copy numbers, also known as *Her2* gene amplification or over-activation. In its mutated (amplified/overactive) form, it becomes into an oncogene (i.e., cancer-causing gene) inducing carcinogenicity of the breast tissue. These tumors present an aggressive phenotype encompassing high tumor proliferation rates, metastasis, and mortality (Gutierrez & Schiff, 2011; Slamon, Eiermann, Robert, Pienkowski, Martin, Press, … Crown, 2011). Importantly, the estrogen receptor (ER) cross communicates with the *Her2* receptors at the cellular surface for normal function of the cell, these signaling processes further activate *Her2* gene within the nucleus of the cell (*Her2* gene expression) and the phosphorylation of the nuclear ER (Jung, Park, Jun, Kong, Kim,

Kim, … Im, 2010; Stoica, Franke, Wellstein, Czubayko, List, Reiter, ...Stoica, 2003; Yang, Barnes, & Kumar, 2004).

Biologically, estrogen signaling can occur by distinct pathways: genomic or nongenomic. In the genomic pathway, the ligand activated ER binds to the DNA, which further activates protein kinase (i.e., mitogen activated protein kinase [MAPK]) and modulates genes that regulate cellular functions. On the other hand, the nongenomic activity occurs within minutes after the formation of ligand (i.e., estrogen and xenoestrogens) receptor complex. In the nongenomic pathway, ligand-activated ER with the help of coactivators activates *Her2*, which then increases the phosphorylation of MAPK and modulates the nuclear ER. This *Her2*-dependant kinase activity of the nuclear ER is an important and essential component of normal regulation and function of nuclear ER. However, unregulated stimulation of ER causes an increase in *Her2* expression, which then increases expression of coactivators, the MAPK kinase activity, and phosphorylation of nuclear ER (Jung et al., 2010; Montemurro, Cosimo, & Arpino, 2013).

How the xenoestrogens act in biological systems was a conundrum for many years. Only in the past decade or so has the research on their mechanistic properties gained some momentum. It has been observed that upon xenoestrogenic exposures, cellular ER gets activated within minutes, suggesting that xenoestrogens activate the nongenomic response of the ER (Bulaveya & Watson, 2004). Research done on their cellular membrane activity indicated that some xenoestrogens are slow-activators while others react quickly and are fast-activators (Bulaveya & Watson, 2004; Payne, Rajapakse,

Wilkins, & Kortenkamp, 2000). Similarly, nuclear transcriptional assays performed to assess their potency showed that some xenoestrogens are very weak (e.g., DDE), while others are somewhat weak (e.g., Bisphenol-A [BPA]), yet others are quite strong (e.g., Diethylstilbestrol [DES]) in their estrogenic activity (Silva et al., 2007). A couple of studies indicated that exposures with two xenoestrogens or multiple derivatives of a single xenoestrogen for a short time period (24 hours to 1 week) produce an additive effect on cellular membrane activity (Aube, Larochelle, & Ayotte, 2008; Rajapakse, Ong, & Kortenkamp, 2001). Together, these studies provided the insights that although xenoestrogens are categorically grouped under one umbrella, their biochemical properties are disparate and that react and interact differentially in biological systems.

Reporter gene assays conducted to study carcinogenicity of organochlorines (OCs) indicated that 1-day exposure of normal mammary cells using nanomolar (nM) concentrations increases the expression of a number of protein kinase genes, including the Her3/ERRB3 kinase (Valeron, Pestano, Luzardo, Zumbado, & Boada, 2009). Interestingly, the structure of *Her3* shows that *Her3* itself does not have a protein kinase domain and it has to bond with other *Her* family members, especially *Her2* for its kinase activity (Ross et al., 2009). A recent study showed that for a breast cancer cell line (MCF7) that had only ERs, the proliferative effect of xenoestrogens (OC mixture) was purely because of the estrogenic potential of the cells; whereas in another cell line (CAMA-1) with equal numbers of estrogen and androgen receptors (ARs), the cellular proliferation occurred due to the inhibition of androgenic receptors (Aube, Larochelle, & Ayotte, 2011). This suggested that xenoestrogens respond and interact differentially to

estrogen and androgen receptors. Studies conducted on xenoestrogens to assess their carcinogenicity have been performed using short-term exposures (up to 1 week) mainly with OCs and their derivatives (Aube, Larochelle, & Ayotte, 2011; Boada et al., 2009). However, because the biochemical nature of xenoestrogens is disparate, it follows that the carcinogenicity of xenoestrogens other than OCs, such as those commonly found in household products (e.g., BPA, NPH, and EE), used individually as well as in combination may be quite different from that of OCs. Additionally, breast cancer has been observed to have a long latency period (Marsden, Wright, Carrier, Moroz, & Rowan, 2012; Nadler & Zurbenko, 2013; Olsson, Baldetorp, Ferno, & Perfekt, 2003; Paez, Labonte, Bohanes, Zhang, Benhanim, Ning, … Lenz, 2011), whereas the aforementioned studies have only studied short-term exposures (24 hours to 8 days).

Furthermore, breast cancer cells have been found to be phenotypically different (e.g., ER+, ER-, *Her2*+, and *Her2*-) making breast cancer a heterogeneous disease (Montemurro, Di Cosimo, & Arpino, 2013). It has also been observed that for ER-positive breast cancers, specifically those with increased *Her2* gene copies, the ERs activate *Her2* signaling and vice-versa (Montemurro, Di Cosimo, & Arpino, 2013; Osborne, Zhao, & Fuqua, 2005). In *Her2* and ER-positive (i.e., *Her2*+/ER+) breast cancer cells; either *Her2* or ER can function as the promoter of cellular proliferation and survival (Wang, Morrison, Gillihan, Guo, Ward, Fu, ... Schiff, 2011). In this case, it is biologically plausible that some breast cancer cell-types (e.g., ER+/*Her2*+) may have a greater risk of breast carcinogenesis than others (e.g., ER-/*Her2*- or normal expression of ER and *Her2*) when exposed to xenoestrogens.

Some epidemiologic studies conducted on xenoestrogenic exposures suggested that the risk of having a more aggressive type of breast cancer is to those women that are ER negative, indicating that xenoestrogens are not only a risk factor for women that have an ER-positive status, but also to those with an ER-negative status. In fact, women with an ER-negative status had worse survival outcomes, and were resistant to therapy (Gammon, Wolff, Neugut, Eng, Teitelbaum, Brinton, ... Santella, 1999; Rosenberg, Zhang, Coogan, Strom, & Palmer, 2008). Importantly, Gammon et al. (1999) assessed the *Her2* status in women that were using over-the-counter contraceptive pills and the researchers found that breast cancer aggressiveness and prognosis in these women were positively associated with the overexpression of *Her2* oncogene. These findings were further supported by animal studies connecting an increase in *Her2* and *3* expression levels with exposure to OCs (Jenkins, Raghuraman, Eltoum, Carpenter, Russo, & Lamartiniere, 2009). Another study found that increased *Her2* gene expression causes shorter breast cancer latency period and faster progression rates when the mice were exposed with derivatives of DDT (o' p' Dichlorodiphenyltrichloroethane [DDT], and p' p' DDT) (Johnson, Ho, Cline, Hughe, Foster, & Davis, 2012). Collectively, these data suggested that the mechanism underlying breast carcinogenesis with xenoestrogen exposure observed in animal models may also correspond to that found in humans and the critical assessment of *Her2* is warranted.

Because breast cancer is a heterogeneous disease with a long latency period, assessing the carcinogenic potential of the commonly used synthetic xenoestrogens in relation to the *Her2* gene, with multiple and prolonged exposures using different breast

cancer cellular phenotypes, is important. In this exploratory research project, I aimed to do exactly that. This study was intended to provide a model for gene-environment interaction (GEI) that will aid in predicting the carcinogenic potential of four xenoestrogens (BPA, Nonylphenol [NP or NPH], ethinyl estradiol [EE], and DDT) commonly used in household products (e.g., plastics, oral contraceptives, pesticides) in relation to the *Her2* gene, as well as discern cellular phenotypes (i.e., ER+/- and *Her2*+/-) that may be more susceptible to aggressive disease upon prolonged exposures (7 to 8 weeks), individually and in combination. The results from this research may impact breast cancer risk factor assessment with xenoestrogen exposure/s, useful in decision-making for policy-level changes as well as advocacy purposes for its primary prevention and discerning cellular phenotypes that may be at a greater risk of breast cancer progression, which could be monitored for early intervention using biologically targeted therapies for its secondary prevention.

Background

Historically, cancer has been known primarily as a genetic disease with a long latency period (Barrett, 1993, Knudson, 2001; Pitot & Dragon, 1991). Both nonhereditary (i.e., somatic cell) and hereditary (i.e., germ cell) cancers are caused by genetic accidents (e.g., mutations) that disturb the cellular proliferation systems. However, a vast majority of cancers (>70%) occur due to somatic cell mutations and are not inherited (Cornelisse & Devilee, 1997; Knudson, 2001, Lee & Muller, 2010). Models of breast carcinogenesis provide evidence that carcinogenesis is a multistage process, accompanied either by the mutation/amplification of a proto-oncogene into its oncogenic form, or inactivation of a

tumor suppressor gene [TSG] (Barrett, 1993; Lee & Muller, 2010; Leedham & Tomilinson, 2012; Pitot et al., 1993). *Her2* is a proto-oncogene that has been implicated in breast carcinogenesis (Dressman, Baras, Malinowski, Alvis, Kwon, Walz, & Polymeropoulas, 2003; Hynes & Stern, 1994; Jung et al., 2010; Slamon et al., 1989).

As a proto-oncogene, *Her2* plays a pivotal role in cell-signaling processes for the normal growth and development of the mammary epithelia (Akiyama, Ogawara, Toyoshima, & Yamamoto, 1986; Slamon et al., 2011; Yardin & Sliwkowski, 2001). When the *Her2* proto-oncogene mutates (i.e., oncogenic form), it deregulates the cell-cycle; which then initiates uncontrolled cellular proliferation of the mammary cells (Gutierrez & Schiff, 2011; Slamon et al., 1989; Slamon et al., 2011). *Her2* gene is a member of the epidermal growth factor receptor (EGFR), encoding for a transmembrane tyrosine kinase (TK), which is an enzyme that is important for cellular signal transduction. This enzyme is a key regulator of normal mammary cell growth, but also plays a critical role in the development and progression of cancer (Gutierrez & Schiff, 2011; Hynes et al., 1994). Mutation of the *Her2* gene increases its gene copy numbers with in the nucleus, also known as *Her2* gene amplification. The gene amplification (i.e., increase in gene copy numbers) results in an increased production of *Her2* receptors on the cellular surface for which the gene encodes. Because amplification of the *Her2* gene (nucleus) directly results in the overexpression of the *Her2* receptors (cell surface), the gene amplification observed at the genomic level can thus be used as a proxy for the overexpression of its protein product (*Her2* receptors) at the cellular surface (Gutierrez & Schiff, 2011; Slamon et al., 1989; Slamon et al., 2011). Additionally, the terms *gene*

amplification, or the increase in gene copy numbers, and *gene overexpression* can be used interchangeably in the case of breast carcinogenesis (Dressman et al., 2003; Gutierrez & Schiff, 2011; McCormick, Lillemoe, Beneke, Schrauth, & Reinartz, 2002; Meng, Tripathy, Shete, Ashfaq, Haley, Perkins, …Uhr, 2004; Slamon et al., 1989).

Empirical data revealed that *Her2* is overexpressed in up to a third (30%) of incident breast cancer patients (Bertucci, Borie, Ginestier, Groulet, Charafe-Jauffret, Adelaide, …Birnbaum, 2004; Korkaya, Paulson, Iovino, & Wicha, 2008 Slamon et al., 1989;; Slamon et al., 2011). The percentage of patients with *Her2* overexpression has been found to increase (by 40%) with higher disease stage and progression (Meng et al., 2004). It has been widely demonstrated that when there is amplification or overexpression of the *Her2* oncogene, then the patient prognosis relates to a more aggressive type of breast cancer with disease progression, tumor invasion, fewer disease-free days, and worse survival outcomes lending to its poor prognostic value (Baselga & Swain, 2009; Gutierrez & Schiff, 2011; Johnson et al., 2012; Lindemann, Resau, Nahrig, Kort, Leeser, Annecke, …Harbeck. 2007; Slamon et al., 2011).

In vitro and vivo studies clearly showed that these exposures to xenoestrogens promote (a) mitosis and changes in breast tissue morphology (Brown & Lamartinere, 1995), (b) nuclear activity (Murray, Maffini, Ucci, Sonnenschein, & Soto, 2007; Recchia et al., 2004), and (c) cellular proliferation (Bulaveya et al., 2004; Mercado-Feliciano & Bigsby, 2008; Recchia et al., 2004). Additionally, the kinases used in bringing about these changes are estrogen receptor kinases 1 and 2 [ERK1/2] (Bulaveya & Watson, 2004). Interestingly, these cellular end-points and kinases overlap those when *Her2* is

amplified, inducing breast carcinogenicity (Ellsworth, Ellsworth, Patney, Deyarmin, Love, Hooke, ... Shriver, 2008; Zhang, Wolf-Yadlin, Ross, Pappin, Rush, Laufenburger, & White, 2005; Yang et al., 2004). Cross-talk between ER and *Her2* receptors exists, especially during breast carcinogenesis, and the cellular end-points with xenoestrogenic exposures overlap those when *Her2* is amplified. Hence, it is plausible that perturbing the normal levels of estrogens with xenoestrogenic exposures further heightens this chemical cross-communication between ER and *Her2* thereby leading to *Her2* mutation and its oncogenic activation, inducing breast carcinogenicity.

Population-based studies assessing the risk of breast cancer with xenoestrogenic exposures have employed methods susceptible to recall and other systemic biases. The exposure assessment was conducted using interviews or self-reports and women may not be able to identify these xenoestrogens correctly, thus leading to misclassification (Van Hoften, Burger, Peeters, Grobbee, Van Noord, & Leufkens, 2000). Some studies have used controlled that were suffering from benign breast disease or mammomegaly (Stellman, Djordevic, Britton, Muscat, Citron, Kemney, …Gong, 2000; Zheng, Holford, Mayne, Ward, Carter, Owens, … Tessari, 1999; Zheng, Holford, Mayne, Tessari, Ward, Carter, … Hoarzham, 2000). Whereas some other studies used small (≤20) sample sizes (Djorveck, Hoffmann, Fan, Prokopczyk, Citron, & Stellman, 1994; Falck, Ricci, Wolff, Godbold, & Deckers, 1992). Furthermore, the unknown and the variables cannot be controlled in epidemiological studies, thus making it hard to establish direct correlation or causality between various xenoestrogenic exposures and mammary tumor outcomes. Keeping these in mind, a more sensitive approach to assessing the carcinogenic potential

of the xenoestrogens is necessary. Molecular genetics technologies, such as fluorescent in-situ hybridization (FISH), provide a sensitive tool to observe and assess gene level changes after xenoestrogenic exposures (Johnson et al., 2012; Press, Slamon, Flom, Park, Zhou, & Bernstein, 2002).

Statement of the Problem

Synthetic xenoestrogens and their repeated exposure could chemically modulate the promotion and progression of breast cancer (Aube, Larochelle, & Ayotte, 2013; Brody & Rudel, 2003; Valeron, Pestano, Luzardo, Zumbado, & Boada, 2009). Breast carcinogenesis is known to occur by the activation of oncogenes, or inactivation of TSGs (Barrett, 1993; Lee & Muller, 2010; Pitot & Dragon, 1993). Patient data showed that the *Her2* oncogene is amplified (increased gene copy numbers) and overexpressed in almost a third (20% to 30%) of incident breast cancer patients (Korkaya, Paulson, Iovino, & Wicha,, 2008, Lee & Muller, 2010; Slamon et al., 1989; Slamon et al., 2011).

Additionally, a few population-based studies indicated that some xenoestrogenic exposures leading to aggressive breast cancer were found in women with ER-negative and *Her2*-positive status (Gammon et al., 1999; Rosenberg, Zhang, Coogan, Strom, & Palmer, 2008). Because breast cancer cells possess differential cellular receptor phenotypes, such as ER and *Her2* (positive or negative) (Gutierrez & Schiff, 2011; Slamon et al., 2011), it is possible that the carcinogenic potential influencing the *Her2* oncogene may differ for various xenoestrogens within these receptor types, rendering some phenotypes more susceptible to aggressive disease over others, and this also has not yet been assessed.

Furthermore, studies showed that xenoestrogens had an additive effect, but these studies only used binary exposures at a single time-point for a few hours (48 hours) or exposures to mixtures of OCs for a short time period (9 days) (Aube, Larochelle, & Ayotte, 2008; Rajapakse, Ong, & Kortenkamp, 2001). However, cancer is a disease that has a long latency period (Barrett et al., 1993; Marlow, Honeth, Lombardt, Cariatti, Hessey, Piplli, … Dontu, 2013; Nadler & Zurbenko, 2013; Pitot & Dragon, 1993). Also, most women are exposed daily to various xenoestrogens commonly used in household products. However, no study to date has critically examined how the concentration, duration, and type of xenoestrogen exposure influence the *Her2* gene and, in turn, cancer cell growth and proliferation in human cells. Additionally, prolonged, continuous (7 to 8 weeks) and multiple (3 to 4 xenoestrogens) exposures of commonly used household xenoestrogens, such as BPA, NPH, estrogen and DDT, have not yet been studied using human cells or cell lines.

Purpose and Objectives of the Study

Using an experimental case-control study design nested with in a randomized block design (RBD), this research study quantitatively assessed *Her2* copy numbers with FISH technology on four phenotypically disparate human breast cancer cell-lines (ER and *Her2* positive or negative lines) after exposing them to differential exposures with four commonly used xenoestrogens (i.e., BPA, NPH, DDT, and EE). Controls remained unexposed to any xenoestrogen. *Her2* gene copy numbers for the cases and controls were counted and differences evaluated for statistical significance. The study determined the relationship between *Her2* copy numbers with increasing exposure concentrations and

durations to various xenoestrogens applied individually or in combination to the cell-lines. This research produced molecular data that provide mechanistic insights on the workings of this oncogene with differential xenoestrogenic exposures further assisting in the evaluation of their carcinogenic potential and breast cancer risk assessment with the various breast cell phenotypes.

The main objectives of this case-control study were to

- assess the carcinogenic potential of commonly used xenoestrogens influencing the *Her2* oncogene and
- to discern cellular phenotypes that maybe more susceptible to more aggressive disease with xenoestrogenic exposures.

Theoretical Construct

Breast carcinogenesis occurs with the activation of oncogenes or the inactivation of TSGs (Lee & Muller, 2010; Pitot & Dragon, 1993). Exposures to chemicals and hormones, including xenoestrogens, can trigger the activation of oncogenes (Brody, Tickner, & Rudel, 2005; Davis, Bradlow, Wolf, Woodruff, Hoet, & Anton-Culver, 1997; Montemurro, DiCasimo, & Arpino, 2013). *Her2* is a proto-oncogene, needed for normal mammary cell development and function, but it can mutate and become oncogenic. *Her2* oncogenic overexpression is noted in up to 30% of incident breast cancer patients (Korkaya, Paulson, Iovino, & Wicha, 2008; Slamon et al., 1989; Slamon et al., 2011), with the number of patients increasing by 40% with disease progression (Meng et al., 2004). Mammary carcinogenesis is controlled by cross-talk that occurs between ERs and *Her2*, forming a positive feedback loop for cellular proliferation, survival tactics used by

tumor cells, and their invasion and migratory activities (Montemurro, DiCasimo, & Arpino 2013; Osborne & Schiff, 2005; Wang, Morrison, Gillihan, Guo, Ward, Fu, …Schiff, 2011). *Her2* can interact with ER once the estrogen receptor ligand complex is formed activating the Phosphoinositide 3-kinase (PI-3K) pathways (Jung et al., 2010; Montemurro, DiCosimo, & Arpino, 2013; Stoica et al., 2003). Its increased expression initializes the mitogen activated protein kinase (MAPK) pathway, which then relocalizes more ER from the nucleus to the cellular cytoplasm, thus forming a positive feedback loop for the *Her2* amplification (Jung et al., 2010; Montemurro, DiCosimo, & Arpino, 2013; Yang, Barnes, & Kumar, 2004). Once activated, *Her2* can take over this pathway by homo-dimers or hetero-dimers with its other family members thereby activating an autocrine loop, in which case *Her2* becomes self-sufficient for its renewal (Fiszman & Jasnis, 2011; Pinkas-Kramarski et al., 1998; Witsch, 2010).

The MAPK, also known as estrogen receptor kinase (ERK), is a protein found in a cell that communicates a signal from the cell surface receptor to the DNA found within the cell's nucleus. The cell's signaling is initiated when a ligand (e.g., growth factor, hormone, or xenoestrogen) binds to the receptor and ends when the DNA in the nucleus initiates transcription of a protein and produces a change in the cell, such as cellular growth. Thus, it is a signaling pathway governing some of the key cellular processes, such as proliferation, differentiation, and cell-survival (Duronio & Xiong, 2013; Fiszman & Jasnis, 2011; Witsch & Yarden, 2010). The MAPK/ERK pathway includes many proteins that communicate. When the proteins involved in the pathway have a mutation, the signals sent to the nucleus go awry, which is a necessary step for carcinogenesis.

MAPK/ERK pathway is found to be de-regulated in various diseases, including breast cancer (Duronio & Xiong, 2013; Orton, Sturm, Vyshemirsky, Calder, Gilbert, & Kolch, 2005).

Several pathways leading to breast cancer (e.g., radiation, estrogens, alcohol, and diet) were initially hypothesized by Davis, Bradlow, Wolf, Woodruff, Hoe, Anton-Culver (1993), one of these pathways also showed that xenoestrogens increase the estrogenicity of a cell above normal levels and this leads to the mutation of genes found in the 17q loci. Interestingly, the *Her2* gene maps to this area (17q11.2-17q12) of the human genome, and this pathway provided the theoretical construct for this exploratory research project. The nongenomic and genomic action of ER and its cross-talk with *Her2* buttress this construct (Jung et al., 2010; Montemurro et al., 2013).

Xenoestrogens for the Study

Xenoestrogen selection criteria were geared towards products found in almost every household across the globe or those that bio-accumulate.

Using the aforementioned criteria, the following xenoestrogens were selected:

- DDT is an insecticide that was produced in large quantities (approx. 22 million pounds) in the United States in the mid-1900s. One of its important properties is that it bio-accumulates. Due to this, even though DDT was banned in the United States in 1972, it still persists in the environment. More so, DDT is still being used as malarial vector control by many countries (e.g., India, Africa), and it can transported to other parts of the world from these countries (Agency for Toxic Substances & Disease Registry [ATSDR], n.d.).

- EE is an estrogen used in almost all formulations of present-day combined contraceptive pills. In the past few decades, the EE part of the pill has been reduced from 100ug to 20ug. Small amounts (6ug) of EE are also converted from 1 mg of norethindron acetate (NETA), which is a formulation used in hormone therapy (Chu, Zhang, Gentzschein, Stanczyk, & Lobo, 2007).
- BPA is a chemical used in making plastics and resins. Some of its mainstream products that are used every day are plastic containers for storage, baby formula bottles, soda bottles, plastic tubing used for various purposes, and dental sealants. The chemical bonds that form BPA are highly unstable and can degrade with normal use. Factors such as increase in temperature, pH, and even time can break these bonds. When these bonds break, BPA can easily enter the human body (Jenkins et al., 2009).
- NPH or NP is a subset of alkyl phenols. It is widely used in industrial detergents and surfactants, and is added to many consumer products like pesticides, paper manufacturing, dry-cleaning, paints, household cleaners, and cosmetics (Calafat, Kuklenyik, Reidy, Caudill, Ekong, & Needham, 2005).

Research Questions, Variables, and Hypotheses

Research Question 1: Do increasing concentrations of synthetic xenoestrogens significantly increase the *Her2* copy numbers?

Outcome/dependent variable: *Her2* gene copy numbers

Predictor/independent variable: Concentrations of xenoestrogens (0.000nM or unexposed control, .1nM, .01nM, .001nM) and Receptor types (ER and *Her2* positive and

negative).

Null ($H_0$1): There will be no significant increase in *Her2* copy numbers with application with increasing concentrations of xenoestrogens.

Alternate ($H_1$1): There will be a significant increase in *Her2* copy observed with the application of xenoestrogens with increasing concentrations.

Hypothesis 1: It is hypothesized that increasing the concentrations of the xenoestrogens will increase *Her2* copy numbers. It will also do so for each cell line or receptor type.

Research Question 2: Do the concentration at which the increments in *Her2* copy numbers become significant vary between the four xenoestrogens?

Outcome/dependent variable: *Her2* gene copy numbers

Predictor/independent variable: Xenoestrogenic exposures of BPA, NPH, DDT, and EE using .1nM, .01nM, .001nM concentrations.

Null ($H_0$2): A significant increase in *Her2* copy numbers occurred at similar concentration levels for all four xenoestrogens (BPA, NPH, DDT, and Estrogen).

Alternate ($H_1$2): A significant increase in *Her2* copy numbers occurred at different concentration levels for all xenoestrogens.

Hypothesis 2: It is hypothesized that *Her2* expressions will significantly increase for the four different xenoestrogens at different concentrations.

Research Question 3: Overall, is there a significant increase in the *Her2* gene copies between short-term (5 days) and persistent/long-term (50 days) exposures to the xenoestrogens?

Outcome/dependent variable: *Her2* gene copy numbers

Predictor/independent variable: Exposure duration (short-term: single, short-term vs. multiple, persistent), and Xenoestrogen (BPA, DDT, EE, NPH).

Null ($H_0$3): No significant increase will be found in *Her2* copy numbers between the short and long term applications of xenoestrogens.

Alternate ($H_1$3): A significant difference in *Her2* copy numbers will be found between short and long term xenoestrogenic exposures.

Hypothesis 3: It is hypothesized that increasing the duration of xenoestrogenic exposures will significantly increase *Her2* copy numbers overall and for each categorical xenoestrogen.

Research Question 4: Overall, does *Her2* expression vary significantly with each specific receptor type (i.e., cell line) upon exposure to xenoestrogens?

Outcome/dependent variable: *Her2* gene copy numbers

Predictor/independent variable: Receptor types/Cell lines (ER+/*Her2*+, ER-/*Her2*-, ER+/*Her2*-, and ER-/*Her2*+) and Exposure durations (single, short-term vs. multiple, long-term).

Null ($H_0$4): *Her2* copy numbers will not vary significantly between the different receptor types/cell lines upon exposure to xenoestrogens.

Alternate ($H_1$4): A significant difference will be found in the *Her2* copy numbers between the different receptor types/cell lines upon exposure to xenoestrogens.

Hypothesis 4: It is hypothesized that differential *Her2* copy number increase will be noted between the different receptor types/cell lines when they are exposed to

xenoestrogens. However, each cell line would show significant *Her2* copy number gains with multiple, persistent exposures compared to single, short-term exposures.

Operational Definition

Measuring the Dependent Variable

The dependent variable in this study was *Her2* gene copy numbers. They were measured after conducting FISH experiments by counting the number of orange signals of the *Her2* gene probe because each orange signal denotes a copy of the *Her2* gene. The *Her2* gene copy number was quantified in its absolute value, that is, total *Her2* copies observed per nuclei (McCormick, Lillemoe, Beneke, Schrauth, & Reinartz, 2002). As humans are diploid (i.e., have two homologous chromosomes in normal individuals; Bilous, Morey, Armes, Cummings, & Francis, 2006), the increase of *Her2* in its absolute value will be greater than 2 copies of the gene.

Measuring the Independent Variables

- Concentrations of the xenoestrogen/s were measured by their molar concentration, diluted to nanomolar (nM) concentrations. The specific concentrations used were .001nM, .01nM, and .1nM (Payne, Rajapakse, Wilkins, & Kortenkamp, 2000; Rajapakse, Ong, & Kortenkamp, 2001).
- Duration of application (one time application cultured for 5 days vs. daily application for 50 days (Jenkins et al., 2009).
- Number of xenoestrogens that were applied (exposed to1 xenoestrogen vs. exposed to all 4 xenoestrogens) (Payne, Rajapakse, Wilkins, & Kortenkamp, 2000; Rajapakse, Ong, & Kortenkamp, 2001).

Strengths

FISH technology was used to assess *Her2* gene copy numbers, which provided high-test sensitivity (95% to 97%) and specificity (97% to 100%) (Press et al., 2002), thereby yielding a high predictive value (Mass et al., 2005; Olsson, Jansson, Holmund, & Gunnarson, 2013; Sauter, Lee, Bartlett, Slamon, & Press, 2007). Specifically, in the case of breast cancer, researchers have observed that the *Her2* gene amplification or increased copy numbers found at the level of the gene directly corresponded to its transcribed mRNA and its protein overexpression (Dressman et al., 2003; Slamon et al., 1989; Slamon et al., 2011). Thus, these study data cut across two biologic processes (i.e., *Her2* oncogenic amplification and hence its protein overexpression). Because *Her2* measurements are not hindered by the number of xenoestrogens used, it did not overestimate or underestimate true values of xenoestrogenic exposures (Rajapakse, Ong, & Kortenkamp, 2001).

This work was conducted in a laboratory, where the exposure types, amounts, and durations were measured precisely, controlled, and monitored. In the realm of the laboratory, this study was performed ethically using human cell-lines, whereas it would be unethical to do so in human populations (Brody, Tickner, & Rudel, 2005). Additionally, the *Her2* probe-set is FDA-approved, which helped in the IRB approval. Furthermore, the experimental design (Trochim & Donnelly, 2007, p. 158) increased the validity of this study, because random assignment of the flasks was performed for the test and control groups for each line.

Lastly, this exploratory research was conducted in a laboratory using breast cancer cell-lines. However, FISH experiments can be performed with fresh or archived tissue samples (Garimberti & Tosi, 2010; Schruter, LeBrun, & Harrison, 2002) and FISH reproducibility is high (Garimberti & Tosi, 2010; Press et al., 2002), making further research possible using either prospective or retrospective study design to gather data at the population level.

Limitations

Conducting experiments with cell-lines required extreme caution with respect to how long the cell-lines had already been cultured and passaged in the bio-repository before their receipt in the laboratory, as cell-lines with high passage numbers (> 40) can easily change their genetic conformation in response to stress produced by the culturing environment (American Type Culture Collection [ATCC], 2007). Due to this reason, extra precaution was used when ordering the cell lines to make sure that the ordered lines had a low passage number (< 40).

The cell repository had limited data on the lines, thus matching of data was not an option for other breast cancer risk factors (e.g., age, parity, breast-feeding, diet, smoking, and alcohol history). Another potential weakness could have been low yield of cells to work with after treatment with various xenoestrogens, which would be technically challenging. To combat this challenge FISH experiments were conducted using interphase nuclei. Interphase FISH is performed without a high yield of actively dividing cells (Ohlschlegel, Zahel, Kradolfer, Hell, & Jochum, 2012; Ohlschlegel, Zahel,

Kradolfer, Hell, & Jochum, 2013; Olsson, Jansson, Holmund, & Gunnarson, 2013; Press et al., 2002; Schruter, LeBrun, & Harrison, 2002).

Generalizability

Each normal healthy breast cell does have two copies of the *Her2* proto-oncogene (Akiyama et al., 1986; Slamon et al., 2011). However, because this research was conducted using breast cancer cell-lines, the data were limited to cell-lines. Even so, the lines themselves were derived from humans. Breast cancer cell lines selected were ER- and *Her2*-positive or -negative because of the following reasons: (a) ER and *Her2* are the main drivers of breast carcinogenesis (Gutierrez & Stoica, 2011), (b) xenoestrogens mediate their effects via ERs (Mercado-Feliciano & Bigsby, 2008; Stoica et al., 2003), and (c) ER cross-communicates with *Her2* receptors in breast carcinogenesis (Jung et al., 2010; Stoica et al., 2003). Different combinations of these two receptor types (e.g., *Her2*+/ER- and *Her2*-/ER+) were taken into consideration in the selection of these breast cancer cell-lines.

The four cell-lines used were MCF7, BT474, MDA-MB-231, and SKBR3, and they had already been categorized as ER and *Her2* positive or negative. The specific classification for each line was as follows: MCF7 (ER+/*Her2*-) BT474 (ER+/*Her2*+), SKBR3 (ER-/*Her2*+), and MDA-MB-231 (ER-/*Her2*-) (Chang, Chiu, Tseng, Chang, Chien, Wu, & Lui, 2006; Johnson et al., 2010; Wang, LiU, Wu, Hong, Yang, Liu, ... Gu, 2010). The generalizability was limited beyond those receptors (e.g., insulin-growth factor receptors, progesterone receptors, androgenic receptors) that are also found on the cellular surface of a mammary cell.

Significance and Social Change

Breast cancer still remains a public health concern (Aube, Larochelle, & Ayotte, 2011; Valeron, Pestano, Luzardo, Zumbado, & Boada, 2009). Synthetic xenoestrogens are found in varying quantities in commercial products that are available quite easily (e.g., herbicides, plastics, pesticides, contraceptives) to which women in all societies and all over the world are constantly exposed (Cohn, 2011; Darbre & Charles, 2010; Inifo-Nunez, Herreros, Eucinas, & Gonzalez-Bulnes, 2010; Kuch, Metzer, & von der Trenck, 2010; Qui, Suri, Bi, Sheng, & Fu, 2010; Vogel, 2009). If commonly available synthetic xenoestrogens do increase the risk of breast cancer, their ease of availability needs to be curtailed. Studying the risk associated with these compounds in relation to breast cancer can provide clues that could lead to its primary prevention (Aube, Larochelle, & Ayotte, 2011; Boada, Zumbado, Henriquez-Hernandez, Almeida-Gonzalez, Alvarez-Leon**,** Serra-Majem, & Luzardo 2012; Buteau-Lozano, Velasco, Cristofari, Balaguer, & Perrot-Applanat, 2008; Davis et al., 1997). Thus, reducing the risk factors of breast cancer would not only affect women, but also many families whose support structures they are. This would not only translate in financial terms, but also emotional terms for many children and husbands, who will not lose their mothers or spouses to breast cancer mortality. Furthermore, as synthetic xenoestrogens are found all over the world, this research would be far reaching, helping not only the immediate community in the fight against breast cancer, but also the global community.

As cancer is primarily a multistage genetic disease (Croce, 2008; Hanahan & Weinberg, 2011; Knudson, 2001; Vogelstein & Kinzler, 2004), integrating molecular

technologies into risk assessment methodologies can provide a powerful tool for gaining insights into oncogenic alterations that occur in response to xenoestrogenic exposures (Bishop, 2010). Such oncogenic alterations offer the potential to understand the nature of the deregulated oncogene leading to carcinogenesis, thereby improving understanding of the molecular mechanisms underlying breast cancer pathogenesis and progression.

This research provides a model for GEI that will aid in predicting the carcinogenic potential of four commonly used synthetic xenoestrogens influencing the *Her2* oncogene, a biomarker of breast carcinogenesis. Additionally, the study discerned cellular phenotypes that are more susceptible to aggressive disease with these exposures. Taken together, these results impact breast cancer risk assessment with xenoestrogenic exposures and provide mechanistic insights useful in decision-making for policy-level changes for its primary prevention and advocacy against the usage of xenoestrogens, especially for those women that are at an increased risk of disease progression.

Definitions of Terms Used

Allele is one member of a pair of genes. It is located on a specific position of a specific chromosome (Ellsworth et al., 2008).

Androgen receptors are responsible for the male phenotype. This nuclear receptor is activated by the binding of testosterone and Dihydrotestoterone (i.e., androgenic hormones) (Walters, Simanainen, & Handelsmann, 2010).

Bioaccumulate is defined as the increase in concentration of contaminated air, water, or food in living things due to their slow metabolism or excretion (Environmental Protection Agency [EPA], 2012).

Carcinogenesis is the process of malignant transformation leading to the creation of cancer (Barrett, 1993).

Catenin is a class of proteins that play an important role in cellular adhesion (Zhang et al., 2005).

Chromosome carries hereditary information, is formed of condensed chromatin, and is located in the nucleus of a cell (Akiyama, Sudo, Ogawara, Toyoshima, & Yamamoto,1986).

Dimerization is the process when two molecules link via covalent bonding (Tzahar & Yarden, 1997).

Endocytosis is the dissociation of dimers within the cell (Lenferink, Pinkas-Kramarski, Van de Poll, Van Vugt, Klapper, Tzahar, …Yarden, 1998).

Estrogen (E2) is a female sex-steroid hormone (Tora, White, Brou, Tasset, Webster, Scheer, & Chambon 1989).

Estrogen receptor (ER) is the receptor found in the cell through which estrogen (ligand) mediates its effect (Tora et al., 1989).

Enzyme-linked immunosorbent assay (ELISA) is a colorimetric assay that uses antibodies to identify the presence or absence of a protein (Engvall & Pearlmann, 1971; Konecny, Meng, Untch, Wang, Bauerfeind, Epstein, …Pegram, 2004)

Fluorochromes are specific DNA sequences that are labeled with fluorescent probe (Nitta, Hauss-Wegrzyniak, Lehrkamp, Murillo, Gaire, Farrell, …Grogen, 2008).

Immunohistochemistry is an assay that localizes antigens in cells of tissue-sections using antibodies that specifically bind to the antigens (Press et al., 2002).

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Isoform is when a protein exists in different configurations. They can be quite similar to each other, but are not exactly alike, and can perform different functions (Stoica et al., 2003).

Ligand is a molecule (e.g., hormone or growth factor) that binds to a specific receptor forming a ligand-receptor complex? These ligand-receptor complexes are important as they can modulate signal transduction and gene transcriptional activities of a cell (Akiyama, Sudo, Ogawara, Toyoshima, & Yamamoto, 1986).

Homodimer is when the dimerization occurs with like molecules (*Her2-Her2*) (Tzahar & Yarden, 1997).

Homologs are DNA sequences that have similarity, and share a common ancestry (Vennstrom & Bishop, 1982).

Heterodimer is when the dimerization occurs with two disparate molecules (*Her2-Her3*) (Tzahar & Yarden, 1997).

Molarity (M) is defined as the concentration of a solution expressed as moles of solute per liter of solution (Brown, Le May, & Burstein, 2002, p. G-9).

Oncogene is a gene that causes cancer when it is mutated or over-expressed (Barrett, 1993).

p-arm is the short arm of the chromosome (Akiyama, Sudo, Ogawara, Toyoshima, & Yamamoto, 1986).

Phosphorylation activates an effector molecule from its inactive state in order to convert one form of signal (i.e., stimulus) into another (e.g., cellular growth) (Tzahar & Yarden, 1989).

Proto-oncogenes are genes that are required for the normal growth and development of cells and tissues (Barett, 1993).

Sensitivity measures the proportion of true positives that are correctly identified by a test (Press et al., 2002).

q-arm is the long arm of the chromosome (Akiyama et al., 1986).

Specificity measures the proportion of true negatives correctly identified by a test (Press et al., 2002).

Southern blot is an electrophoretic technique used in genetic testing. It is used to separate sequences of DNA that have been digested with enzymes which breaks the DNA into fragments. These fragments are then blotted onto a membrane and hybridized with labeled probe to detect the fragment containing the gene of interest (NCBI, 04).

Tumor Suppressor Genes (TSGs) are required for the cell-death (apoptotic) processes of a normal cell (Barrett, 1993).

Ubiquitinization is a process that modifies and degrades proteins (Zhang et al., 2005).

Summary

Research data from in vivo, in vitro and some population-based studies have established that xenoestrogens are a risk factor for breast cancer (Bulaveya & Watson, 2004; Charlier, Albert, Herman, Hamoir, Gaspard, Mevrisse, & Plomterix, 2003; Gammon et al., 1999; Johnson et al., 2012; Maras et al. 2005; Recchia, Vivacqua, Gabriele, Carpino, Fasanella, Rago, ...Maggiolini, 2004; Warner, Eskenazi, Mocarelli, Gerthoux, Samuels, Needham, ...Brambila, 2002).

The designs of population-based studies pose technical challenges for exposure measurements as they are riddled with recall bias and misclassification, the variables cannot be controlled to establish causality or direct correlation between exposure and tumorigenesis, and conducting randomized trials to gather population-level information with xenoestrogen exposures is not an ethical option, but a costly one to detect low-level risk (i.e., less than twice) incurred by xenoestrogens. However, because most women around the globe are exposed to xenoestrogens, studying the risk that they pose and modifying these risk factors will have a great public health impact even though they only account for low levels of relative risk (Aube, Larochelle, & Ayotte, 2011; Brody, Tickner, & Rudel, 2005; Johnson et al., 2012).

The carcinogenic process occurs primarily due to activation of an oncogene or turning off a tumor suppressor gene. In the case of breast cancer, empirical patient data provide evidence that the *Her2* oncogene is activated in up to 30% of breast cancer patients (Gutierrez & Schiff, 2011; Slamon et al., 1999; Slamon et al., 2011). Additionally, the percentage of patients with *Her2* oncogenic activation grows by an

additional 40% as the cancer progresses to the next stage (Meng et al., 2002). Current reviews for xenoestrogenic exposures-related breast cancer have now marked the epidermal growth family and its receptors (HER) as one of the risk factors for breast carcinogenesis (Fucic, Gamulin, Ferencic, Katic, & von Krauss, 2012).

Further, a population-based study strongly suggested that the *Her2* oncogene is activated with xenoestrogenic exposures and these patients mostly are ER negative, but their breast cancer was more aggressive with a shorter latency period (Gammon et al., 1999). Studies using mouse models showed that *Her2* gene is activated upon xenoestrogenic exposures (Aube, Larochelle, & Ayotte, 2011; Jenkins et al., 2009), and the activation of the *Her2* gene causes accelerated tumor progression (Jenkins et al., 2009; Johnson et al., 2011). These studies further buttressed the hypothesis that similar processes of carcinogenesis maybe are occurring in both humans and mice, making it imperative to study the carcinogenetic potential of xenoestrogens for breast carcinogenesis in relation to the *Her2* gene using human cell with different phenotypes.

This research project determined that significant (p = .000) increase in the *Her2* copy numbers did occur with persistent xenoestrogenic exposures, occur in all receptor types (ER and *Her2* positive or negative), and with all four categorical xenoestrogens (BPA, DDT, EE, and NPH) in individual and combined exposures. These gains in *Her2* copies occurred at nanomolar concentrations (.001nM) for all four xenoestrogens. Chapter 2 reviewed existing literature and discussed how some research has suggested an association between *Her2* overexpression with xenoestrogen exposure. The chapter began with a description of models and theory of carcinogenesis that provided the

foundation and the theoretical framework for this study. A brief overview of estrogen, its receptors, and *Her2* family of receptors is provided. A detailed discussion of the *Her2* oncogene itself and its oncogenic potential specifically for breast carcinogenesis, as well as the chemical connections found between ER and *Her2*, which play a pivotal role in breast carcinogenesis followed. The chapter also discussed the sensitivity and specificity of different technologies used in the assessment of *Her2* gene. Finally, the chapter ended with an in-depth discussion of the research performed (laboratory and population-based) on xenoestrogens and breast cancer along with their outcomes, and discussed implications of past research for future work.

Chapter 3 described the methodology used in this project to answer the research questions. Further, it discussed the use of nonparametric analysis as a means to analyze the relationship between the dependent variable (*Her2* oncogene) and the independent variables (differential concentration and durations of exposures with individual xenoestrogen as well as a combined exposure of all four xenoestrogens). The chapter also included a description of the cell-lines, experimental protocols, ethical considerations, measures, and analysis of the data.

Chapter 2: Literature Review

Introduction

This literature review established the need for research in the area of xenoestrogenic exposures, especially with respect to how persistent and combinatorial exposure influence oncogenic expression of *Her2* in relation to breast cancer. The review encompassed the current knowledge of the carcinogenesis process and *Her2* as an oncogene for breast cancer, which provided the theoretical construct of this proposal. Because xenoestrogens have estrogenic properties, the review provided a broad overview of the ER and the cross-communication between *Her2* and ER. I discuss the properties of xenoestrogens, especially the research done using animal models that has shown *Her2* gene activity upon xenoestrogenic exposures. The review then ends with the main focus on the epidemiologic studies conducted using xenoestrogens, mainly organochlorines and pharmaceutical estrogens, and a summary providing the existent gaps that need to be filled by continued research such as this one.

The papers and some books used for this review were either accessed electronically through databases such as Pubmed Central, Medline, Google Scholar, Partners Healthcare Library, and Academic Search Premier (Walden University), or they were obtained from various books and journals that the Cytogenetics Core Laboratory at Brigham and Women's Hospital (BWH) subscribes to. The terms used in the various databases were *xenoestrogens, breast cancer, estrogen receptor, EGFR2, Her2-neu or Her2/neu, Her2*, and *Her2 oncogene*. The search was conducted in English. Primary articles were also obtained from review articles found using the above terms.

Carcinogenesis

The Process

Cell-division is a process by which normal cells reproduce in tissues. Under normal circumstances, this process is tightly controlled by genes and chemical messengers, such as growth factors and hormones that relay messages to specific genes (Duronio & Xiong, 2013; Park & Lee, 2003; Sherr, 1996). When genes guarding the cell-division processes undergo genetic changes either by mutation, amplification/increase in gene copy numbers, or chromosomal translocations, genetically aberrant cells are formed and the cell-division process is perturbed, which leads to uncontrolled cellular proliferation and differentiation resulting in carcinogenesis (Collins, Jacks, & Pavletich, 1997; Knudson, 2001; Sher, 1996; Valente, Gray, Michalak, Pinon-Hofbauer, & Scott, 2013; Vogelstein & Kinzler., 2004). Cancer is principally a genetic disease of somatic mutations with a latent phase of up to 30 years (Anderson et al., 1992; Barrett, 1993; Knudson, 2001; Nadler & Zurbenko, 2012; Vogelstein & Kinzler., 2004).

Models and Mechanisms

Carcinogenesis occurs in multiple steps (Barcellos-Hoff, Lyden, & Wang, 2013). Cancer initiation occurs when genes controlling either normal cellular growth and/or death (i.e., apoptosis) undergo genetic changes (i.e., mutation) forming genetically aberrant cells and its progression occurs when the genetically aberrant cell multiplies and further undergoes a series of genetic changes (Armitage & Doll, 1954; Fearon & Vogelstein, 1990; Lee & Muller, 2010; Moolgavaskar & Knudson, 1981). Carcinogenesis occurs due to an accumulation of genetically altered clones arising from a single

transformed cell that undergoes secondary and/or tertiary changes (Croce, 2008; Hanahan & Weinberg, 2011).

Mechanisms for carcinogenesis are characterized by three stages. First, *initiation* is an irreversible change in a cell, usually genetic. Genetic changes can be amplification, mutations, chromosomal rearrangement, or aneuploidy. Second, *promotion* is the process by which the initiated neoplastic cell divides resulting in its clonal expansion. Third, *progression* marks the irreversible onset from benign to malignant form (Barrett, 1993; Hilton, Graham, & Clarke, 2013; Pitot & Dragon, 1991). Figure 1 depicts the steps of the carcinogenic process.

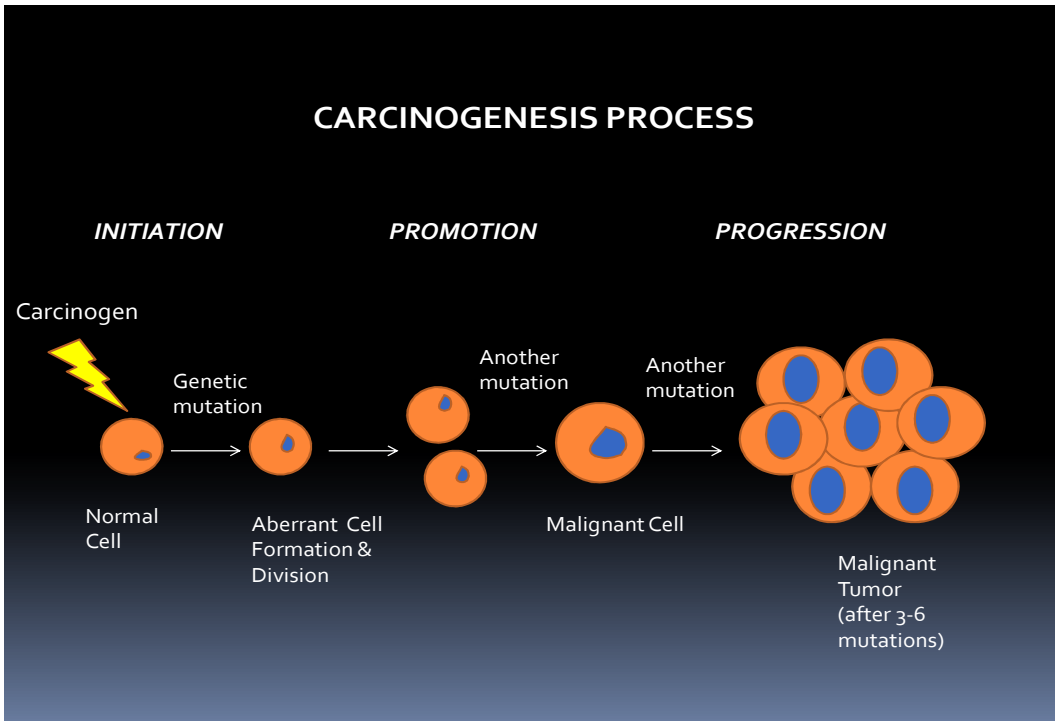

Figure 1. The process of carcinogenesis. The illustration shows the genetic events occurring with exposures to carcinogen/s leading to the conversion of a normal cell into a malignant one.

Histopathology on breast tumor sections bears evidence to the multistep processes of carcinogenesis (Xu et al., 2002). For example, dysplasia occurs during the initial stages of breast cancer in which only the cellular morphology changes without metastasis, and carcinoma marks its later stages; here the cancer has metastasized to other organs (Barett, 1993; Hartmann et al., 2014; Pitot et al., 1993; Virnig, Tuttle, Shamliyan, & Kane, 2010; Xu et al., 2002). Ductal hyperplasia is when the cells of the mammary duct are proliferating at a faster rate than normal, but the cellular structure and form (i.e., cellular morphology) remains normal (Wagoner, Laronga, & Acs, 2009; Xu et al., 2002). Atypical hyperplasia occurs when the cellular proliferation and morphology become deviant from the norm and this is a precursor of carcinoma in situ (Hartmann et al., 2014; Virnig, Tuttle, Shamliyan, & Kane, 2010). Carcinoma in-situ occurs when there is uncontrolled cellular proliferation and the cellular morphology is abnormal, but these cells are still within the tissue itself. Invasive carcinoma occurs when the cells from the carcinoma in-situ have starting invading other tissues or have now metastasized to the surrounding tissue/s. In this specific case the abnormal/cancerous cells are now not only in the mammary duct but have also metastasized to other parts of the breast and lymph nodes (Virnig, Tuttle, Shamliyan, & Kane, 2010; Xu et al, 2002).

Two classes of genes are involved in the process of carcinogenesis: oncogenes and tumor-suppressor genes (TSGs). Both these types of genes provide different cellular responses (Gutierrez & Schiff, 2011; Lee & Muller, 2010). Proto-oncogenes are the normal counterparts of an oncogene and are required for normal growth and development (e.g., the *Her2* proto-oncogene is necessary for normal breast growth and development;

Slamon et al., 2011). Proto-oncogenes promote normal cellular growth in numerous ways. Some proto-oncogenes produce hormones or mitogens effecting signal transduction, whereas others produce cellular receptors and are sensitive to hormones (Anderson, Reynolds, You, & Maronpot, 1992; Croce, 2008; Gutierrez & Schiff, 2011). The proto-oncogene can mutate into its oncogenic form with excessive or continued exposure to chemicals or ligands, such as hormones and xenoestrogens (Barrett, 1993; Davis et al., 1999; Montemurro, Di Cosimo, & Arpino, 2013). The transformation of a proto-oncogene into its oncogenic (e.g., *Her2*) form confers a growth and survival advantage to the cells that carry the mutated gene. Due to this growth advantage, the mutated cells accumulate over time leading to tumor formation (Bishop, 1991; Croce, 2008; Gutierrez & Schiff, 2011; Lee & Muller, 2010).

Contrastingly, TSGs control the cell-death processes of an abnormal or mutated cell, and when TSGs become dysfunctional they cannot block the cancerous cells from growing, thus the cancerous cell loses its capacity to senesce and keeps on proliferating (Lee & Muller, 2010; Valente et al., 2013). Usually, both oncogenes and TSGs are required in tumorigenesis (Barrett, 1993; Croce, 2008, Lee & Muller, 2010; Vogelstein et al., 2004). This has been observed in the case of breast cancer where p53, a TSG is deleted, and *Her2* an oncogene is overexpressed simultaneously in patients suffering with the disease (Ismail, Aly, Khaled, & Mohamed, 2009). TSGs are also known as anticancer genes, because inserting TSGs suppresses uncontrolled cellular growth and induces normal morphological characteristics in neoplastic (i.e., cancerous) cells (Huang et al., 1988; Valente & Strasser, 2013).

Another contrasting feature of TSGs when compared to oncogenes is that while oncogenes are dominant, meaning a single mutational event can activate them; the TSGs are functionally recessive requiring a "two-hit" inactivation process, meaning that genes found in both of the chromosome homologs must undergo a mutational event in order for the TSGs to become inactivated. This process is known as the Loss of Heterozygosity (LOH). In the case of oncogenes, the mutation is a somatic event; whereas in TSGs this event can either be somatic or inherited or both (Knudson, 1971; Knudson, 1973; Lee & Muller, 2010). The proteins coded by the TSGs suppress the cell cycle and/or promote cell senescence or both by deregulating the signaling pathways. The diverse functions of the TSG proteins can be categorized as follows:

1. Repress gene expression required for cell division when a cell's DNA is damaged and cannot be repaired (Lee and Muller, 2010).
2. When DNA damage occurs and cannot be repaired, TSGs then initiate processes of programmed cell death (apoptosis and autophagy, a type of cell death were cytoplasmic processes engulf a cell is found to be controlled by p53, a TSG) (Sherr, 2004; Maiuri, Malik, Morselli, Kepp, Criollo, Mouchel, ... Kroemer, 2009; White & DiPaola, 2009; Hotchkiss, Strasser, McDunn, & Swanson, 2009).
3. Some TSG proteins maintain contact inhibition and thereby suppress metastasis. Contact inhibition is a process by which normal cell arrest their cellular growth and proliferation when they come in contact with other cells. These processes are found to be lost in cancerous cells (Partanen, Nieminen,

& Klefstrom, 2009; Hirohashi & Kanai, 2005; Beltrami, Kim, & Gordon, 2013).

4. Mutations in the DNA repair proteins are also categorized as tumor suppressors because mutations in the DNA repair genes increases cancer risk (Brady, Jiang, Johnson, Jarvis, Kozak, … Attardi, 2011; Valente & Strasser, 2013). It has also been observed that mutation rates increase with decrease in DNA repair genes, this further leads to inactivation of additional TSGs and the activation of oncogenes (Markowitz, 2000; Saal, Gruvberger-Saal, Pearson, Lovgren, Jumppanen, Staaf, … Borg, 2008; Valente & Strasser, 2013).

Presented below are the main features of oncogenes and tumor suppressor genes in a tabular format.

Table 1

Main Attributes of Oncogenes and TSGs

Oncogenes	**Tumor Suppressor Genes (TSGs)**
Dominant	Recessive, Loss Of Heterozygosity (LOH)
Gain of cellular function (e.g., proliferation)	Loss of function (e.g., programmed cell death)
Somatic origin	Somatic or inherited or both in origin

Note: Self-made on Microsoft Word, 2007, using cited information on Oncogenes and TSGs.

Loss of TSGs increases chromosomal instability and the life of a cell in normal and transformed human cells (Dalton, Yu, &Yang, 2010). Some oncogenic mutations may also disrupt the normal apoptotic processes of a cell, thereby leading to initiation,

progression, and metastasis (Angelini, Fluck, Pedersen, Parra-Palau, Guiu, … Arribas, 2013; Shortt & Johnstone, 2012). Contrastingly, other oncogenic changes promote apoptotic processes hence promoting selective proliferation and survival of certain cells by blocking programmed cell senescence of these cells thus immortalizing specific clones of cells that have the growth advantage (Lowe, 2000; McDonnell, Deanne, Platt, Nunez, Jaeger, McKearn, … Korsmeyer, 1989; Vaux, Cory, & Adams, 1988). Comparative genomic technologies have also revealed genes are duplicated or deleted in cancers corresponding to the amplifications found in oncogenes and deletions of TSGs in the human genome (Bell, 2010)

Cancer is also a latent disease. Cancer latency has been studied in mouse and human models. Marsden, Wright, Carrier, Moroz, & Rowan (2012) injected tumor cells from bone marrows of mice with breast cancer into mammary fat pads of normal mice; which resulted in tumor formation in the disease-free mice in two months after injection. These tumors were found to be highly metastatic as tumors were also observed in kidneys, lungs, and livers of the normal mice. Olsson, Jansson, Holmund, & Gunnarson (2013) had performed DNA analysis on 17 women suffering with breast cancer, who had undergone radiation therapy. Studying the S-phase (Synthesis phase of the DNA replication cycle), they found that the median S-phase index for radiation induced tumors was 14%, corresponding to a median latency period of approximately 22 years. The researchers found that a high S-phase index correlated to a shorter latency period and vice-versa for a low S-phase index.

Nadler & Zurbenko (2012) developed a model called the Weibull Model Extension to study cancer latency. This model uses survival analysis curves to estimate cancer latency. This model is assumption free and relies only on the hazard distribution of cancer development, thus making it a flexible model to study cancer latency. Using this model for various cancers, they found that pancreatic, lung and liver cancers have short latency period (range: 8.5-13.5 years), whereas other cancers such as myeloid leukemia, stomach, melanoma, and breast have the longest latency period (range: 22.8-30 years).

Human Epidermal Growth Factor Receptor 2 (*Her2*)

Attributes, Role in Development, & Interactions with Her Family of Receptors

Human epidermal growth factor 2, also known as *Her2*, *Her2-neu*, or *ErbB2* is a proto-oncogene, and that plays a critical role in signal transduction for the normal growth and development of the breast tissue. The *Her2* gene encodes for the *Her2* receptor protein. It belongs to human epidermal receptor (Her) family. The *Her2* protein is one member of a family of closely related proteins, composed of Her1, 2, 3, and 4 (Lupu, R., Cardillo, Harris, Hijazi, & Rosenberg, 1995; Slamon et al., 2011; Yardin & Sliwkowski, 2001). After a ligand binds to the *Her* receptor, the receptor binds to another receptor closely related or similar in structure, a process called dimerization initializing phosphorylation, activating signal transduction processes, resulting in various cellular processes such as cellular growth and proliferation. When the receptor binds to a similar receptor, it is called homodimerization; and when it binds with a closely related receptor, it is heterodimerization (Ghosh, Narasanna, Wang, Liu, Chakrabarty, Balko, … Arteaga,

2011; Tzahar, Waterman, Chen, Levkowitz, Karunagaran, Lavi, … Yarden, 1996). Biochemical research showed that these receptors interact with a wide-range of growth factor ligands. The ligand and the receptor form ligand-receptor complexes and modulate signal transduction and gene transcriptional activities of a cell (Akiyama, Sudo, Ogawara, Toyoshima, & Yamamoto, 1986; Gutierrez & Schiff, 2011).

Ligands of the EGF family of growth factors binding to the various *Her* family of receptors fall into the following 3 categories: a) EGF and heparin-binding (EGF-HB) bind only to *Her1*/EGFR (Aceto, Duss, MacDonald, Meyer, Roloff, Hynes, & Alj, 2012; Higashiyami, Abraham, Miller, & Klagsburn, 1991), b) Betacellulin (BTC) binds to *Her1* and 4 (Riese et al., 1996; Emede, Kostler, & Yarden, 2010), and c) Neu differentiation factors (NDFs), like Heregulin bind to *Her3* and *4* (Aceto et al., 2012; Plowman et al., 1993; Witsch, Sela, & Yarden, 2011). Although *Her2* itself does not have a ligand-binding site, but it alone can partner with all of the other receptors of the *Her* family (i.e., *Her1, 3,* or *4*) forming dimers and inducing receptor tyrosine phosphorylation (DeFazio-Eli, Strommen, Dao-Pick, Parry, Goodman, & Winslow, 2011; Emede, Kostler, & Yarden, 2012). Further, *Her3* does not have a kinase site, and requires *Her2* to activate the phosphorylation process in order to achieve its cellular end-point (Emede, Kostler, & Yarden, 2012; Fisman & Jasnis, 2011; Tzahar & Yarden, 1998). In fact, *Her2* is the most preferred co-receptor for dimerization found in *Her* receptor family (Graus-Porta, Beerli, Daly, & Hynes, 1997; Fiszman & Jasnis, 2011; Emede, Kostler, & Yarden, 2012). *Her1, 3,* and *4*; especially *Her1* and *3* compete to dimerize with *Her2* (Aceto et al., 2012; Pinkas-Kramarski et al., 1998). By way of its association with different *Her* family

receptors and dimerizing with them, the *Her2* molecule has achieved a wide array of signals transmitted into the cell. *Her2*'s fluid dynamics have achieved many more cellular processes (e.g., proliferation, invasion, migration) compared to any of the other *Her* molecules (Emede, Kostler, and Yarden, 2012; Fiszman & Jasnis, 2011; Tzahar et al., 1997; Witsch et al., 2010). In fact, heterodimers formed with *Her2* are more stable and its signaling is more potent compared to any other homodimers or heterodimers formed within the Her family of receptors (Emede, Kostler, & Yarden, 2012; Fiszman & Jasnis, 2011; Karunagaran, Tzahar, Beerli, Graus-Porta, Ratzkin, …Yarden, 1996).

Another important characteristic of the *Her2* molecule is its slow rate of endocytosis, and furthermore they are recycled to the cellular surface where they become active all over again, resulting in increased activity sustained over a much longer time-period and is proposed to play a role in breast tumorigenesis (Lenferink et al., 1998). *Her* family receptors' mitogenic index examined by its proliferative capacity has shown that *Her2-Her3* heterodimers have the highest mitogenic potential followed closely by *Her1-Her2* (proliferative index of 10.5 and 9.6 respectively), when compared to all of the other *Her* family homo and heterodimers (proliferative index ranging from 0-5). Further, these hetero-dimers (i.e., *Her2*-with 1 and 3) have been found mostly in breast carcinomas. Interestingly, it was also observed that *Her2-Her2* homodimers do possess some mitogenic potential (proliferative index of 3.5) even though they do not have any ligand-binding domain (Ghosh et al., 2011; Pinkas-Kramarski et al., 1998). This property of *Her2* may have important implications, especially in the case of breast cancer where these ligandless receptors could induce a positive feedback loop for phosphorylation

resulting in cellular proliferation without any outside stimuli also known as the autocrine loop (Aceto et al., 2012; Witsch, Sela, & Yarden, 2010). Proximity Ligation Assays (PLAs) detects the formation of protein-protein complexes in a single molecule. PLAs performed on 321 patient tumors detected *Her2-Her2* and *Her2-Her3* complexes allowing for the in vivo detection of these molecules, a significant association (p = <.00001) was noted between homodimerization (*Her2-Her2* complex) and gene amplification of *Her2* (Spears, Taylor, Munro, Cunningham, Mallon, Twelves, … Bartlett, 2012).

Aceto et al. (2012) studied the role of *Her2/Her3* activation as a unit in breast cancer. They induced normal mammary cells with *Her2* and Her3 vectors, *Her2*, and Her3 vectors. Normal mammary cells with empty vehicle were used as the control. After culturing the cells for two weeks they performed a3D morphological analysis of the culture revealed that the normal cells formed small round structures and so did the cells that only had Her3. But, most of the cells (~70%) with *Her2* alone became larger structures, and almost all of the cells (~90%) that were co-expressing *Her2* and *Her3* showed complete lack of polarity; which characterizes highly invasive cellular structures.

Because the *Neu* gene was initially found in rat neuroblastoma, and later, its normal counterpart was discovered in rats and humans, thus, work done in rat models could bear important implications for the human (Hung, Schechter, Chevray, Stern, & Weinberg, 1986; Witsch, Sela, & Yarden, 2010; Yamamoto et al., 1986). *Her2* homologs are also involved in the origin and development of erythroblastoma in chickens (*ErbB*) (Vennstrom & Bishop, 1982; Witsch, Sela, & Yarden, 2010).

Her2 gene and protein

The *Her2* proto-oncogene spans a 190 Kilobase (Kb) region mapped to chromosome 17q11.2 - q12, encoding a 185 kilo Dalton (kDa) trans-membrane glycoprotein (Emede, Kostler, & Yarden, 2012). A healthy breast cell has two copies of this gene (Akiyama, Sudo, Ogawara, Toyoshima, & Yamamoto, 1986; Witsch, Sela, & Yarden, 2010). When activated, *Her2* initiates the tyrosine kinase activity which results in a signal that is sent from the membrane of the cell where these receptors (i.e., *Her2* protein product) are located to its nucleus. This signal transduction ultimately leads to gene activation and various other cellular processes depending on the dimerization molecule involved with *Her2* (Fiszman & Jasnis, 2011). Basically, *Her2* sends control signals to the nucleus from the membrane, thereby instructing them to grow, divide, and make repairs. It has great networking capabilities and kinase capacity, which makes it a potent activator of cellular functions (Fiszman & Jasnis, 2011).

An initial correlation study on the *Her2* gene and its protein product was performed by Slamon et al. (1989) on 51 samples breast cancer that over-expressed *Her2* using Southern, Western, Northern blots and IHC to assess the gene amplification, RNA, and the protein status respectively. The correlation between gene amplification and its over expression was found to be significant ($p = <.0001$). In all 51samples (100%), two of the three measures (i.e., Western blot, Northern blot, and IHC) used to assess the protein product showed concordance with its gene amplification. Complete concordance was observed in 46 samples (90%) by all three measures. The discordance in the remaining four samples occurred due to a dilution factor in Western blot; where the

tumor sample is mixed with normal stroma. Another study by Dressman et al. (2003) also performed expression profile analysis of *Her2* amplification in primary breast tumors which showed a significant correlation between gene amplification and its protein expression ($r = 0.76$, $p = .005$), further validating that the high expression levels is occurring due the amplification of the *Her2* gene in breast cancer.

Her2 Oncogene and Breast Carcinogenesis

Amplification or copy number increase of the *Her2* oncogene in breast cancer leads to genomic instability (Ellsworth et al., 2008; Szasz, Li, Eklund, Sztupinzki, Rowan, Tokos … Kulka, 2013). Genomic instability leads to increased cellular proliferation, and motility/migration (Asrani, Keri, Galisto, Brown, Morgan, Ghosh, … Winkles, 2013). These factors further translate in to tumor invasiveness and metastases (Laurin, Huber, Pelletier, Houalla, Park, Fukui, … Cote, 2013; Johnson, Seachrist, DeLeon-Roderiguez, Lozada, Miedler, Abdul-Karim, & Keri, 2010), as well as increased angiogenesis and decreased cell death (Konecny et al., 2004; Ye & Lu (2010).

Her2 Amplification and Genomic Instability

Amplification of the *Her2* can be used as a proxy for the lack of stability in the entire genome. Ellsworth et al. (2008) investigated the changes found in the entire genome in relationship to the *Her2* copy number status in patients suffering from invasive breast cancer.

Her2 copy number changes were assessed by FISH in 181 patients ($n = 181$). The FISH uses the centromeric as well as the Her2 gene probe. The number of signals of the centromeric (CEP), and the *Her2* probe were analyzed and compared. An amplification

was defined when the ratio of the *Her2* vs. CEP was >2 signals. For allelic imbalances spanning the entire genome micro satellite markers (two markers per chromosome) were used. Allelic imbalance (AI) was determined using the following criteria: when a given marker showed less than or equal to 0.35 allelic ratios. Co-relation between *Her2* status and AI was done non-parametrically. Non-parametric assessment does not confer to any assumptions (Cubash, Hanish, Schuz, Neugut, Karsdaedt, … Jacobson, 2013; Paxton, Chang, Courneya, & Pierce, 2012; Siegel & Castellan, 1988, p.34) thus making them less stringent, however, since they digress from tight associations or assumptions they are more flexible to accommodate complex data-sets as they can grow along with its complexity. Mean allelic levels for *Her2* positive patients were significantly more when compared to patients that were *Her2* negative (27% vs. 19% respectively, $p = <.005$). Also, stratifying by chromosome regions, *Her2* positive tumors had more AIs. Additionally, AI patterns downstream of the *Her2* gene (i.e., 17q12 to q21) using markers D17S250 and D17S579 found in this area showed that half of all *Her2-positive* tumors had allelic imbalances for both markers. This area has other genes of importance in breast cancer (e.g., TPO2A, BRAC1, and BRCA2). This indicates that many genes from the 17q area of the genome are altered in *Her2* positive tumors.

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Szasz, Qiyuan, Sztupinszki, Rowan, Tokes, Szekely, ... Kulka, J. (2013) conducted another correlation study using ER+, PR+, and *Her2*+ tumors to evaluate the correlation between genomic instability with respect to receptor status. The researchers assessed chromosome instability in 4 genes (FOXM1, TOP2A, TPX2, AURKA) also

known as CIN4, using gene expression arrays in 186 tumor samples. Receptor positive cells were defined using IHC for ER and PR positive status, and *Her2* was measured using FISH. The level of CIN was defined by the patient's clinical outcomes (size, vascular invasion, necrosis, disease-free survival). It was observed that ER+ and PR+ tumors had an inverse relationship with CIN4 expression (p=.001 and .017 respectively); whereas the *Her2* expression and amplification correlated inversely with the CIN4 expression (p =.001 and .013 respectively). These results show that ER-negative and *Her2*-positive expressions are associated with increase in CIN4 expression and worse clinical outcomes.

Cellular Proliferation, Migration, Invasion, & Directional Persistence

Her2 amplification can induce cellular growth and migratory activities. Dimerization activated via ligands recruits different partnering molecules in a signaling cascade which relay different types of messages to produce various cellular responses, and each message is specific for a particular response.

In a large study, Zhang et al. (2005) ingeniously provided for the cause and effect relationship in biological systems using mass spectrometry (MS) together with wound-healing assays plus fluorescent imaging to decipher phosphorylation of effector molecules, cellular proliferation and migratory response. First, to determine the activation or deactivation of effector molecules (clusters of peptides) a Human Mammary Epithelial Cell (HMEC) line with normal *Her2* expression, a *Her2* over expressed line, and sera-free control were used. All were stimulated with EGF (100ng/ml) or HRG (80ng/ml) (i.e., ligands), and their phosphorylation sites compared with MS. The MS data-set projected

different molecules with increased or decreased phosphorylation levels. When *Her2* is amplified it activates and deactivates various effector molecules providing regulatory functions for the internalization, degradation, and recycling of the receptor. Decreased phosphorylation leads to decreased ubiquitinization of the receptor. Ubiquitinization is a process that modifies and degrades proteins; its decreased activity marks an increase in time before the receptor is degraded.

Second, cellular proliferation was quantified in all three lines using tritiated thymidine uptake after the stimulation with ligands. Only the *Her2* over-expressed was noted with significant increase in cellular growth compared to the control (30,000 & 40,000 CPM with HRG and EGF treatments respectively vs. 15,000 CPM in control, $p = <.05$). Third, quantification of cellular migration models by wound healing projected that cellular migration is highest with EGF stimulus when *Her2* is over-expressed. Interestingly, the *Her2* over-expressed line always showed increased migratory activity (0.3 inches in 6 hours) when compared to the parent line (0.1 inch in 6 hours). The results of this study provide evidence that increased expression of *Her2* is the driving force for cellular proliferation and migratory response. Hence, *Her2* may not only be involved in the cancer initiation, but also its progression.

Aceto et al. (2012) performed migration assays using normal mammary cell line (MCF-10A) induced with *Her2*, Her3, or *Her2* and Her3 vectors. Control had empty vehicle as vector. These assays showed that a significant number of cells migrated with *Her2* alone (three and a half times more than the control, $p = <.003$), but a much greater number of cells migrated when *Her2* and 3 co-expressed (5 times more than the control, *p*

= <.002). Another recent study by Asrani et al. (2013) found that the fibroblast growth factor-inducible 14 (Fn14) , a member of the Tumor Necrosis Factor (TNF) receptor super family is also over expressed in breast cancers that over express *Her2*. This growth factor increases the migratory and invasive capacity of *Her2* over expressed tumors. In transgenic mice, *Her2* directly induces the expression of Fn14. Carrying this forward on human breast cancer cell line (MCF7) transfected with *Her2*; the researchers found that the human line also has increased Fn14 and MMP9 expression. Ablation of Fn14 expression with siRNA (RNA sequences that silence the expression of specific genes) decreased the migratory and invasion response even when *Her2* was being over expressed and suggested that Fn14 is an important downstream effector molecule for *Her2* in its migratory and invasive cellular response.

Tumor metastasis is a two-way process requiring not only cell movement, but also cellular invasion. *Her2* has shown to play a pivotal role in the invasion processes of breast carcinogenesis. Kumar et al. (2000) investigated *Her2* mediated cellular migration and invasion using cell-lines with or without *Her2* receptor. Using time-lapse photography the researchers examined cellular processes produced by cell-lines in the presence and absence of *Her2* receptors when exposed to EGF family peptides (i.e., NDF, BTC, and EGF). It was observed that in the absence of *Her2*, the cellular migration was comparable to the control. Exposing the cells with *Her2* not only increased the tyrosine kinase activity compared to the control, but the increment was much more prolonged (>2 hours) when compared to a transient peak (30-40 minutes) found in the cells without *Her2*. Also, the migratory response started almost immediately (~5 minutes)

in lines with *Her2* receptors. A reduction in cell-to-cell contact was noted within a half-hour, leading to tissue breakdown and hap-hazard cellular movement; whereas cells without *Her2* receptors failed to participate in migratory processes, maintaining their original cell-to-cell contact. The cells without *Her2* did not invade the basement membrane at all with any of the treatments.

Johnson et al. (2010) highlighted one such mechanism is the activation p120 Catenin, which further induces activation of Rac1. The expression of p120 mRNA was found to be four times more in *Her2/Neu-positive* mice mammary tumors compared to the wild type tumors ($p = <.001$). Performing migration and invasion assays using *Her2* over expressing human breast cancer lines and the same lines were silenced for p120 Catenin using shRNA (silences target genes by RNA interference) showed that both cellular migration and invasion was significantly reduced (80% reduction, $p = <.05$) in the silenced lines even though these lines over expressed *Her2*. The Rac1 expression was also reduced by half ($p = <.05$) in the p120 Catenin silenced lines. These experiments indicated that Rac1 activated metastatic response of *Her2* positive breast cancer required for the activation of p120 Catenin, and that p120 Catenin is the mediated the Rac1 metastatic response when *Her2* was over expressed.

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activation of p120 Catenin, and that p120 Catenin is the mediator of the Rac1 metastatic response when *Her2* is over expressed.

Aceto et al. (2012) studied the effects of *Her2* and *Her3* co-expression and cellular invasion in breast cancer. Their research revealed that when *Her2* and *Her3* co-express, this increases the expression of IL-8; a critical factor that is involved in the invasion, migration, and proliferative processes of *Her2* over expression. They performed invasion assays using normal mammary cell line (MCF-10A) and induced it with *Her2*, *Her3*, or *Her2* and *Her3* vectors. Control was induced with empty vehicle. Although *Her2* alone showed an upward trend for the invasion assays, but it did not reach significance. The invasion assays only reached significance when *Her2* and 3 co-expressed (three fold more than control, $p = <.002$). Furthermore, gene expression profiling of *Her2* and *Her3* co-expression using an Affymetrix array platform identified 80 genes that were up-regulated forming the *Her2*/*Her3* unit gene signature. Of special mention here is the increased expression of IL-8, which was up-regulated the most (11 fold). Gene ontology analysis using Ingenuity® software showed that *Her2*/*Her3* gene signatures are enriched with pathways involved in cellular motility, invasion, migration, proliferation, apoptosis, and signaling. Interestingly, IL-8 was found to be involved in all of the aforementioned processes. To further confirm this finding whether IL-8 induces cellular invasion, they treated normal mammary cells (MCF-10A) with increasing concentrations (10ng/ml to 50ng/ml) of IL-8. Control was unexposed. Results revealed a positive linear relationship between increasing concentrations of IL-8 and induced invasiveness in the normal mammary cells. Twenty percent cells were found to be

invasive at 10ng/ml of exposure of IL-8, reaching 50% cells that were invasive with 50ng/ml of IL-8 exposure compared to the control ($p = <.05$). However, these invasive structures were not greater in numbers than those found with the co-expression of *Her2* and 3 (90% invasive structures, $p = <.05$). Also, analysis of 1,881 primary breast cancers (public dataset) showed that the *Her2* over expressed tumors always had increased IL-8 expression.

***Her2* Over-expression and Vascular Endothelial Growth Factor (VEGF):**

VEGF plays an important role in disease progression by aiding in the infusion of blood vessels to other tissues (angiogenesis) in the metastatic process of breast cancer cells. In a clinical cohort ($N = 603$) of primary breast cancer patients, Konecny et al. (2004) evaluated the association between *Her2* and VEGF expression, and the clinical outcomes with their expression levels. ELSA was performed using antibodies for VEGF isoforms and *Her2*, and the patients were divided according to their *Her2* and VEGF status into: a) normal *Her2* expression with no VEGF in low-risk group, b) *Her2* over expressed with no VEGF; and c) normal *Her2* expression with VEGF were both in the intermediate risk, and d) *Her2* over expressed plus VEGF constituted high risk group. A significant association ($p = <.001$) was noted between *Her2* over-expression and VEGF expression, with almost 80% (463/603) of the patients that over expressed *Her2* also had detectable VEGF expression profiles. Survival analysis showed significant differences between the four groups, with increased mortality for those in the high risk group and the most favorable survival indices found in the low risk group (log rank test $p = <.0092$).

Thus, *Her2* over expression is not only associated with VEGF expression, but in conjunction with VEGF results in high mortality rates.

Ye & Lu (2010) also found similar results on their assessment of the expression of *Her2* and VEGF on 117 post-operative breast cancer patients using IHC. Fifty patients with mammary gland hyperplasia were used as controls. Positive expression of *Her2* and VEGF was noted in the sample compared to the controls ($p = <.05$). A positive correlation was found between *Her2* and VEGF expressions ($p = <.05$, $r = 373$). Pathologically, both *Her2* and VEGF correlated to lymph node metastasis ($p = <.05$), however, no correlation was found with age, histological type, grade, and stage ($p = >.05$).

Schoppmann, Tamandl, Roberts, Jomrich, Schoppmann, Zwrtek, ... Birner (2010) further validated that *Her2* over expression is associated with an increase in VEGF. Using IHC, they studied the expression of VEGF (factor C), lymphatic microvessel density, lymphovascular invasion and *Her2* over expression on 150 randomly selected, node-positive breast cancer patients. Mann Whitney U test results showed that the cases that over expressed *Her2* (3+ IHC score) also expressed significantly greater ($p = .0006$) amounts of VEGF compared to those patients that did not over express *Her2*. Additionally, the lymphatic microvessel density showed a significant ($p=.012$) correlation with VEGF expression. This data suggests that *Her2* protein over expression influences tumor metastasis by increasing the production of VEGF factor C.

The aforementioned studies (Aceto et al., 2012; Asrani et al., 2013; Johnson, Seachrist, DeLeon-Roderiguez, Lozada, Miedler, Abdul-Karim, & Keri, 2010; Konecny

et al., 2004; Lauren et al., 2013; Schoppmann et al., 2010; Ye & Lu, 2010) performed on the mechanistic insights of how *Her2* mediated its carcinogenic potential have now elucidated how *Her2* effectively used many effectors (downstream elements) and mediated diverse singular effects—metastasis via migration, invasion, proliferation, and angiogenesis.

Accruing Her2 Amplification with Disease Progression

Historically, clinical data has shown that *Her2* gene amplification occurs in approximately a third (30%) of patients suffering from breast cancer (Slamon et al., 1989; Emede, Kostler, & Yarden, 2012). However, this diagnosis of *Her2* over-expression has been done only on primary tumors, whereas the remainders of the patients who do not show *Her2* gene amplification initially have not re-assessed for *Her2* amplification during their later stages. However, as the disease progresses it there could be more patients with *Her2* amplification.

Indeed, this is the case as many studies have shown that *Her2* over expression is a dynamic process, and it can be acquired over a period of time with disease progression. Meng et al. (2004) followed 24 breast cancer patients who were *Her2* negative for it's over expression. Their *Her2* amplification status was assessed prospectively before, during, and after treatment, or when the patient became chemo- refractory (i.e., the chemotherapy stops working on the tumor cells and the disease progressed to the next stage) using FISH probes (*Her2* and CEP17) on their circulating tumor cells (CTCs) in their blood. A total of 9 patients of the 24 (37.5%) did end-up with a *Her2* over-expression in their CTCs during disease progression. Another study by Hayes et al.

(2002), also using FISH techniques to quantify *Her2* levels in the CTCs evaluated 19 patients for their Her2 over expression levels with disease progression that were initially *Her2* amplification negative. It was observed that in 7 of the 19 patients (i.e., 40% of the patients) a rapid rise in *Her2* gene copies did precede disease progression.

Genomic instability and acquisition of *Her2* amplification with disease progression has been corroborated by Ismail, Aly, Khaled, & Mohammed (2009) where they evaluated the correlation p53, a tumor suppressor gene (TSG), and *Her2* and myc oncogene expression levels on breast tumor samples (n = 34) using FISH technology. Increase in copies or amplification of *Her2* and myc oncogenes, and deletions in the copies of p53 gene (TSG positivity rates) were assessed by scoring signals for each with respect to the centromeric signals. All three of these genes showed a significant correlation with each other, more so with regards to *Her2* over-expression (*Her2* and myc r = .511, p = .002; *Her2* and p53 r = .432, p = .01; myc and p53 r = .356, p = .03). Additionally, the frequency of the number of patients with *Her2* oncogene amplification or increase in its copy numbers did increase with advancement of the disease. Using tumor size, disease stage, and lymph node status as parameters of disease advancement, it was observed that 70%, 40%, and 50% of the patients showed positivity for *Her2*, myc, and p53 genes respectively when the size of the tumor was small (<3 cm) which ballooned-up to 92% (*Her2*, p = .005), 87% (myc, p = .0006), and 71% (p53, p = .01) with a larger tumor size (>3 cm). Similarly, for disease stage, the *Her2*, myc, and p53 expressions jumped from 75%, 56% (for both myc and p53) of the patients in early stages (I & II) to 95%, 89%, and 72% of the patients respectively for later stages of the disease

(III & IV). Noteworthy here is that only *Her2* over expression levels increased significantly between the early and the late stages (p = .008). For lymph node status from negative to positive, once again statistically significant increase only in *Her2* over-expression were noted (node negative mean = 1.69 ±0.25, node positive mean=2.49 ±0.22, p = .038), although the number of patients with a p53 deletion did increase tremendously from 42% in the node negative to 77% for the node positive category, but it still not statistically significant (p = .05).

Measuring *Her2* Using FISH Technology

This molecular technique is DNA based and detects targeted gene sequences. (Garimberti & Tosi, 2010). It is a DNA based technology which makes use of the fact that a DNA molecule consisting of two homologous strands, and can be denatured to single strands. The denatured DNA strand can only be re-natured with its homolog, thus remaking an exact replica of the initial double strand (Liehr, 2009, p.26-28; Garimberti & Tosi, 2010; Gasparini & Malazzi, 2006). A FISH probe is made of specific DNA sequences that renature to the gene in question. The target DNA is fixed on a glass-slide, and the probe DNA is tagged with a fluorescent reporter molecule. Then, both these single-strands are unified in a hybridization reaction and visualized under a fluorescent microscope (Liehr, 2009, p.26-28; Varga, Noske, Ramach, Padberg, & Moch 2013). Detection of the exact in-situ chromosomal location of a gene and its copy number changes can be delineated, quantified, and assessed (Liehr, 2009, p.26-28; Varga, Noske, Ramach, Padberg, & Moch, 2013). FISH can be studied in metaphase spreads or

interphase nuclei (Bishop, Garimberti & Tosi 2010; Gozetti & Le Beau, 2000; Pinhel et al., 2012).

Press et al. (2002) compared the accuracy between FISH and IHC tests employed for testing *Her2* levels. Gene amplification was first assessed using Southern blots (NCBI, 04) in 117 (n) breast cancer samples. FISH (*Her2*/CEP17 probe set, Vysis) and IHC (DAKO Hercep), sensitivity and specificity was evaluated compared to Southern blotting. Three or more signals marked amplification. Forty-two samples (36%) were amplified. FISH sensitivity was 95.4% (42/43 samples) and specificity 98.6% (72/73 samples). One hundred and fourteen samples were correctly identified by FISH for accuracy of 97.4%. Concordance (κ) with Southern blotting was 0.945 (CI=0.88-1.0). With IHC, only 30 of the 43 samples were identified for over expression of the protein, making its sensitivity 69.8%, however, all of the cancers that showed low expression were accurately categorized with this test yielding a specificity of 100%, and accuracy of 88.9%. Concordance (κ) values between IHC and Southern blots was only at 0.745 (95% CI=0.618-0.871). Other studies (Mass et al., 2005; Olsson, Jansson, Holmlund, & Gunnarson, 2013; Sauter et al., 2007) have also yielded very high predictive values (sensitivity: 95% to 97%, and specificity: 97% to100%) for the assessment of *Her2* with FISH.

FISH is the preferred technology over IHC is partly due to the fact that while IHC is subjective, FISH is quantitative (Bartlett et al., 2001; Jacobs, Gown, Yaziji, Barnes, & Schnitt, 1999; Thomson et al., 2001). A proficiency test conducted in 146 clinical laboratories, using *Her2* amplified / over expressed and low *Her2* amplification / low

expression showed that all laboratories using FISH were in 100% agreement; whereas those using IHC only 72% agreed (Pinkel et al., 1986). Due to these discrepancies between the two tests, American Society of Clinical Pathologists (ASCO) reviewed and changed its guidelines in 2011 for IHC scoring of *Her2* from 2+ and 3+ staining to be observed in 30% of the cells instead of 10% cells that was done previously (2005-2010). These changes in IHC scoring criteria has lowered its false-positive rates considerably, and increased the positive concordance rates between IHC and FISH from 72% to 95% (1083/1118 cases retrospectively analyzed using IHC and FISH). However, the cases where discrepancy still exists between the IHC and FISH results, the confirmatory analysis is still done using FISH technology, and the FISH results for *Her2* are considered definitive. Importantly, the guidelines for FISH analysis of *Her2* have remained constant over the past 12 years, and so has its specificity, sensitivity, and accuracy (Varga, Noske, Ramach, Padberg, & Moch, 2013). Another advantage with FISH technology is that besides interphase nuclei, it can be performed on metaphase also which allows a researcher to pin-point the exact chromosomal location of the aberration (Bishop, 2010; Gozetti & Le Beau, 2000).

The probe-set used for *Her2* testing is FDA approved (Park, Park, Koo, Yang, Kim, & Park, 2010; Wulfkuhle, Berg, Wolff, Langer, Tran, Illi, … Petrcoin, 2013), and is currently being used for clinical diagnosis, prognosis, and management of breast cancer patients (Burris, Rugo, Vukelja, Vogel, Borson, Limentani, … O'Shaughnessy, 2011; Fleming, Sill, Darcy, McMeekin, Thigpen, Adler, … Fiorica, 2010; Ohlschlegel, Zahel, Kradolfer, Hell, & Jochum, 2011; Slamon et al., 2011). Specifically, in this kit the probe

sequences are tagged by fluorescently labeled probes which recognize both the genetic sequences for the *Her2* gene and chromosome 17 centromeric regions. The *Her2* gene and the centromeric regions are labeled with different colored fluorochromes for an easy scoring of disparate signals (e.g., orange and green respectively) (Olsson, Jansson, Holmlund, & Gunnarson, 2013). The *Her2* amplification can be quantified using a fluorescent microscope by counting the *Her2* gene copies, and the CEP17 is used as an internal control to check for aneusomy (i.e., increase of the entire chromosome) of chromosome 17 (Nitta et al., 2008; Gutierrez & Schiff, 2011).

Estrogen and Its Receptor: An Overview

Human estrogen (E2) is synthesized in 3 forms by the ovary: a) estrone (E1), produced during menopause, b) estradiol (E2) predominantly found in non-pregnant women, and c) estriol (E3) produced during pregnancy. E2 mediates its effect via its receptors, estrogen receptor alpha (ERα) and beta (ERβ). ERs are nuclear receptors (NRs), meaning that it is found in the nucleus of a cell (Tora, White, Brou, Tasset, Webster, Scheer, & Chambon, 1998). Biologically, the function of ERα has been extensively studied in the case of breast cancer (Jung, Park, Jun, Kong, Kim, Kim, ... Im, 2010; Palmieri, Cheng, Saji, Zelda-Hedman, Srri, Weihua, ... Gustafsson, 2002). Both ERα and β are found in the normal mammary epithelium, but an increased level of ERα is noted in breast cancer. Almost 70% of breast cancers with ER-positive status expressed ERα (Renoir, Marsand, & Lazennec; 2013). Basically, E2 forms a complex with its receptor, known as a ligand-receptor complex. When ER is unbound to a ligand, it is found as a monomer bound to a protein called the heat-shock protein. Upon binding with

a ligand, hsp gets disrupted producing conformational changes of the receptor molecule, which induces receptor activation (Gutierrez & Schiff; 2011; Le Goff, Montano, Schodin, & Katzenellenbogen, 1994; Montemurro, Cosimo, & Arpino, 2013).

Classically, when activated, they form a homodimer with other co-regulatory agents. Together, they bind to the estrogen response element (ERE) contained in the promoter region of specific genes, and have the capacity to modulate the transcriptional activity of those genes (Kumar & Chambon, 1988; Montemurro, Cosimo, & Arpino, 2013). Besides the classical pathway of the ER functioning as transcriptional regulator, it is also proposed that E2 exerts its effect non-genomically (non-classical pathway) by interacting with growth factor receptors [e.g., epidermal growth factor (EGF), and insulin growth factor (IGF)], and cell signaling molecules (Kahlert et al., 2000). Furthermore, different ligands can change the conformation of the ER in differential ways (Montemurro, Cosimo, & Arpino, 2013; Osborne et al., 2003). Thus, ER transcriptional activity is controlled by specific ligand, co-regulatory molecules, their phosphorylation (i.e., activation), and promoter sequences present in distinct set of genes.

A study by Grober, Mutarelli, Giurato, Ravo, Cicatiello, DeFillppo, …Weisz (2011) was conducted using ERα positive cell line (MCF7). The researchers found that this line co-expressed ERβ. Analysis of their transcriptomes with CHIP-Sequencing (CHIP-Seq) technology for the entire genome surprisingly showed that there are 9702 ERβ sites vs. only 6402 ERα binding sites in the MCF7 line when it is stimulated with estrogen (E2). Further analysis of the binding sites by sequencing showed the presence of estrogen receptor elements (EREs) in ERα and ERβ. Additionally, ERα and β share

similar genomic targets, and in co-existence they compete for these genomic targets. However, the cellular response is opposite for ERβ vs. ERα. Cell proliferation assays for the MCF7 using miRNA line show that ERβ down regulates cell growth, unlike ERα which promotes cell growth. Thus, the ERβ receptors were able to modulate the effects of ERα receptors on gene transcription and cellular growth as was noted in the MCF7 cell line.

Signaling Pathways: Cross-Talk between ER and *Her2*

Phosphotidyl Inositol 3-Kinase/Akt Pathway (PI3-K/AKT Pathway)

Cellular signaling for the activation and control of gene expression by estrogen was found to be complex as well as multifaceted to say the least. Two major pathways of ER signal transduction were: a) phosphotidylinositol 3-kinase [PI 3-K/Akt], and b) MAPK pathways. Estradiol was able to bind to ERα directly. The estradiol bound to ERα interacted with *Her2* followed by the activation of the PI 3-K/Akt was indicative of the cross-communication that occurred between ERα and *Her2*.

An extensive research by Stoica et al. (2003) examined the activation of various signaling molecules, and their pathways upon stimulation by exogenous estradiol in ERα positive (MCF-7), and ER-negative (MCF-7/ADR) breast cancer lines. They determined the requirement of ERα itself, by exposing MCF7/ADR cells to17β estradiol, and immunoblots probed them with anti-phospho-Akt. A nine-fold increase in Akt activity was noted in the ER-positive line. No Akt activity was observed in the ER-negative line, and transfection with ERα restored the Akt activity. Further, the MCF-7 cells treated with the two isoforms of estradiol (α and β), only the β isoform showed a nine-fold in Akt

activity.

The researchers elucidated the mechanism and the kinase responsible for Akt activity, by treating MCF-7 cells with 17β estradiol that had been exposed to AG825 which selectively blocks *Her2*, and AG30 an EGFR inhibitor. The cells treated with *Her2* inhibitor did not induce any Akt activity; however, this response was not inhibited by the EGFR inhibitor, suggesting that *Her2* is a critical element for Akt pathway activation. As Akt also exists in three isoforms (1, 2, and 3), treatment with antibodies specific to each showed that ERα positive line (MCF-7) Akt 1 is expressed, and ER negative line (MDA-MB-231) produced activity with Akt 3, this indicated that different isoforms of Akt can be selectively activated depending on the ER availability, thus involving two distinct mechanisms for protein and gene expression. It can also be inferred from this data that in cells where *Her2* co-exist with ER (e.g., breast cells), exposure of the cells to estrogen itself or estrogen-like compounds (xenoestrogens) can activate their cellular growth and survival via these pathways.

Mitogen Activated Protein Kinase Pathway (MAPK Pathway/ERK)

Jung et al. (2010) found that co-activators, such as Matrix metalloproteinase-1 (MMP-1) expression increases when crosstalk occurs between the ER and *Her2* receptors via the MAPK pathway. These co-activators act as molecules that relay the message from the ER to the *Her2* receptors.

ER is mainly found in the nucleus of an ER-positive cell. However, when stimulated with estrogen, the ER interacts with *Her2* and initializes the MAPK pathway by inducing extra cellular signal regulated kinase 1 and 2 (ERK 1/2) activity (Lemmon &

Schlessinger, 2010). This further prompts re-localization of the ER from the cell's nucleus into its cytoplasm, increases *Her2* expression, aids in the progression of breast cancer, and makes the cancer resistant to therapy. Yang, Barnes, & Kumar (2004) had investigated the communications that occurred between *Her2* and ER, the signaling cascade in breast cancer cells, and the indispensable role that *Her2* plays in breast cancer pathogenesis. Breast cancer cells that did not express any *Her2*, as well as those that over-expressed *Her2* were used to perform confocal microscopy using immunofluorescence after their exposure to 17β estradiol. It was observed that upon *Her2* amplification there was physical movement of the ER from the nucleus into the cytoplasm. Vice-versa effects occurred when *Her2* expression was de-regulated with anti-*Her2* antibody, which is that ER, moved back into the nucleus from the cell's cytoplasm. Western blots plus confocal immunofluorescence with anti-ERK 1/2 showed that ERK 1/2 increased (8 fold) with the increase in *Her2*. This data shows that ER relocation from its primary position; the nucleus to the cellular cytoplasm is a downstream effect of increased *Her2* expression, and *Her2* interacts with the ER to produce this effect. Also, ERK 1/2 activity provides the fuel for the relocation of ER. Jung et al. (2010) found that co-activators, such as Matrix metalloproteinase-1 (MMP-1) expression increased when crosstalk occurred between the ER and *Her2* receptors via the MAPK pathway. These co-activators acted as molecules that relayed messages from the ER to the *Her2* receptors.

PI-3K, MAPK, and *Her2* Over Expression

How the PI-3K and MAPK pathways interacted with*Her2* over expression was

further detailed by Serra, Scaltriti, Prudkin, Eichhorn, Ibrahim, Chandarlapaty, … Baselga (2011). Using PI-3K inhibitors (BEZ235) on *Her2* over expressing cell lines (BT474 and SKBR3) resulted in a dose-dependent inhibition of PI-3K catalytic activity (i.e., phosphorylation) within 24 hours ($p = <.05$). In addition, a simultaneous increase was noted of a downstream effector (P90RSK) of the MAPK/ERK pathway. When similar tests were performed using *Her2*-negative lines (MCF7 and MDA-MB-468), activation of ERK/MAPK was not pronounced. To confirm the involvement of *Her2*, both BT474 and SKBR3 (*Her2*+ lines) were treated with anti-*Her2* agents; this prevented the phosphorylation of the ERK/MAPK pathway to occur.

Unique Properties of Xenoestrogens: Insights from Animal Models, Lines and Assays

Activate Protein Kinase Genes

Valeron, Pestano, Luzardo, Zumbado, & Boada (2009) used RNA arrays and studied the expression of protein kinase genes after they exposed human mammary cell line with DDT analogs, alderin and dieldrin at 0.18, 90, and 180 nM concentrations for a period of 96 hours (4 days). Their arrays results showed a sharp increase in the expression of protein kinase genes; such as KIT, ALK-1, and ERRB3/*Her3*. Noteworthy, is the finding of an increase in ERRB3/*Her3* kinase, but an inherent physical property of the ERRB3/*Her3* receptor is that the receptor itself does not have a kinase site, so it dimerizes with *Her2* for kinase activity in order to reach the cellular proliferative end point (Aceto et al., 2013; Fiszman & Jasnis, 2011; Ross et al., 2005; Tzahar et al., 1998). Thus, it may be that here also *Her2* mediated the kinase activity for *Her3* since *Her2* is its

preferential dimerization partner, and *Her3* cannot possibly activate the kinase on its own since it is physiologically incapable to do so on its own.

Estrogen Receptor Agonist that Activates Cellular Signaling

Xenoestrogens are estrogen receptor (ER) agonist. Exposure to small amounts of xenoestrogens leads to receptor (estrogen receptor) binding with the ligand (xenoestrogenic compound) forming a receptor-ligand complex. This receptor-ligand binding rapidly initiated activation of cell signaling molecules (ERKs). Bulaveya & Watson (2004) demonstrated the rapid changes that occur in cell signaling pathways specifically when xenoestrogens, such as DDE, bisphenol A (BPA), endosulfan, nonylphenol (NPH), coumestral, and dieldrin bind to estrogen receptors (ERs). Changes that had occurred during intracellular signaling were measured by performing ELISA for dose-dependent phosphorylation using prolactinoma lines. Time-periods used were between three to thirty minutes after exposure with concentrations from 10^{-8} to 10^{-10} M. Administration of an agonist at different concentrations in a dose-response experiment exhibited an uphill curve as one proceeds from the left to the right of the graph. Any activity of ≥120% compared to the control (ethanol) was considered statistically significant ($p = <.05$). Each xenoestrogen, except BPA produced rapid phosphorylation of the estrogen receptor kinases (ERKs) within 30 minutes after application reached statistically significant levels of response. Each xenoestrogen activated the ERKs in a unique fashion. Some (e.g., NPH and coumestral) produced dual activity peaks; whereas others (e.g., endosulfan) produced activity at all times with all concentrations tested, but none of these compounds was able to exactly copy the phosphorylation patterns of

estrogen (E2) itself. Nevertheless, they all initiated ERK activity in low dosage concentrations (i.e., nanomolar and picomolar amounts).

Differential ERK activity patterns were observed. The time of activation for the various xenoestrogens were divided into two groups: a) fast-phase responders with one activity peak in the first half or 6-10 minutes (e.g., DDE), and b) slow-phase responders that produced a single delayed peak in 30 minutes (e.g., endosulfan and nonylphenol). However, E2 was different, as it produced a bimodal ERK phosphorylation response with distinct periods of phosphorylation and deactivation; whereas all xenoestrogens produced only a monophonic response. Importantly, the study found that although xenoestrogens activated the ERK, but they had different dose-dependent patterns. Two basic patterns had emerged:

1) Some compounds were active in nano-molar as well as sub-pico molar concentrations (e.g., coumestral, E2, endosulfan, and nonylphenol), and
2) Others were active only at nano-molar concentration (e.g., DDE and dieldrin).

Since the ERK pathway is also activated by *Her2*overexpression (Fiszman & Jasnis, 2011), it is biologically plausible that in the above experiments *Her2* is also being over expressed with xenoestrogenic exposures.

Cellular Proliferation & Nuclear Compartmentalization

Xenoestrogens initiated cell growth. A study conducted by Mercado & Bigsby (2008) examined the role of PBDEs found widely in the environment, and act as endocrine disruptors. The study was conducted in vivo, and measured estrogenic activity with response to different dosage with various time intervals in two strains of mice. They

were either wild type (naturally occurring), or those in which the ERα gene had been removed (knockout) mice. All mice were injected with 75, 150, and 300 mg/Kg of DE-71 for 3 and 34 (persistent exposure) days respectively, and then their reproductive tracts were weighed as estrogen bioassays. An in-vitro assessment of the effect of these treatments was also done using MCF-7 breast cancer cell line and conducting cell proliferation assays. Cell proliferation assays found a significant increase in cell counts. A three-fold increase of DE-71 treated cells was obtained at 10uM concentration compared to DMSO control ($p = <.01$). Albeit, the cellular growth observed in the E2 treated cells was much higher (five-fold increase) with a much lower concentration (0.01nM). A noteworthy observation was that treatment with $>2.5 \times 10^{-5}$M concentration of DE-71 results in a sudden drop in the cell growth, indicative of its noxious effect to the cells beyond this strength. The estrogen assays revealed that only persistent treatment of 34 days increased the uterine weight substantially with estradiol treatment (8-12 times compared to the control, $p = <.001$), and with DE-71 (23% more than control, $p = <.05$). The knocked out mice had not produced any effect, which suggested that ERα is the receptor that got actively recruited.

Recchia et al. (2004) also examined the estrogenic nature of xenoestrogens, specifically BPA and 4-Nonylphenol (NPH). To assess this, the investigators created an estrogen response element (ERE) in MCF7 and T47D breast cancer cell-lines. An ERE is the promoter that initiated gene transcription when an estrogen agonist (ligand) binds to the ER (Klinge, 2000). Both lines were treated with 10 μM of BPA and NPH for 5 consecutive days, and then cell proliferative and transcriptional assays were performed.

Both BPA and NPH transactivated ERα. Nuclear compartmentalization had occurred when BPA and NPH were added to MCF7 cells. Furthermore, proliferative activity induced by BPA and NPH was observed for both MCF7 and T47D cell-lines were assessed by cell-proliferative assays. It was noted that similar amounts (i.e., concentration) of xenoestrogens induced transcriptional and proliferative response in the lines. However, when compared to the natural estrogen hormone, the proliferative activity of both xenoestrogens was reduced. It is noteworthy here that cellular proliferation were also induced and promoted with the over expression of *Her2* (Ellsworth et al., 2008; Szasz et al., 2013). Hence, the overlapping cellular growth and proliferation further provided biological plausibility that the *Her2* gene is also involved in the circuit of molecules when the xenoestrogens are applied to reach the specific cellular endpoints.

Induction of Cellular Growth at Low Concentrations

Cellular growth was characteristic of estrogenic effect. Xenoestrogens can induce cellular growth at very low concentrations. Maras et al. (2005) investigated the estrogenic properties of five perfluorinated compounds (xenoestrogens) by using a combination of in vitro assays. The capacity of these compounds to induce cellular growth in growth arrested MCF-7 breast cancer line was measured by E-screen assay, and the cell cycle analysis was done by flow cytometry. The E-screen is based on the ability of MCF-7 growth arrested cells to initiate growth in the presence of estradiol, and this is compared to the compound under scrutiny (Soto et al., 1995). De-regulated cellular cycle can lead to tumorigenesis due to increased cellular growth or decreased cell-death. Normally,

apoptosis occurs after a cell goes into its resting phase (Elledge, 1995). When the resting MCF-7 cells were exposed to fluorotelomer alcohols as they re-entered the synthesis phase (S-phase) within a day. A 35% increase was noted with exposure to estrogen, and 4-Nonylphenol (4-NP). There was a 31% and 29% increase in cells with 8:2 and 6:2 fluorotelomer alcohols respectively. Additionally, low concentrations (e.g., 10 μM) of the fluorotelomer alcohols induced cellular growth.

Changes Mammary Tissue Morphology

Increased cellular activity due to chemical exposure increased cancer susceptibility. It has now been known for more than a decade that some xenoestrogens can change the morphology of the mammary tissue, and initiate the proliferation of its cells in animal models. Brown & Lamatinere (1995) investigated these properties by conducting a case-control study on Sprague-Dawley rats. The experimental group of rats was subjected to acute exposures of 50ug/gm body weight of DES, DDT, genestin, and 25ug/gm body weight of TCDD, Arcolor 1221 and 1254. Controls were given sesame oil. Each group was assigned equal number (6) of rats, and exposed to the xenoestrogen for a week. Morphological changes were assessed on whole mount preparations of breast tissue. Cell proliferation was quantified using proliferating cell nuclear antigen (PCNA) an indicator of mitotic activity, and cell differentiation was analyzed using IHC.

Genestin and DES did significantly increased mammary cell growth as well as its differentiation when compared to controls (cell growth observed: 149 ± 7 mm^2 Genestin vs. 122 ± 10 mm^2 control, $p = <.05$, and gland differentiation observed: 43 ± 8 lobules with genestin; 43 ± 6 lobules with DES; 10 ± 1 lobules in control, $p = <.01$ and $<.001$

respectively). Although, not statistically significant, but, Arcolor 1221 and 1254 showed cellular proliferation. This could be due to insufficient dosage of these chemicals due to their weaker estrogenic properties. Interestingly, it was noted that TCDD inhibited the cellular proliferation of the mammary cells (81 ± 9 TCDD vs. 132 ± 6 mm^2 control, p = <.01). This could have occurred because TCDD's toxicity was killing the cells.

Latent Effects in Mammary Tumor Development

Exposure to xenoestrogen (e.g., BPA) during gestational age in minute amounts resulted in carcinogenesis of the breast tissue during adulthood. Murray, Maffini, Ucci, Sonnenschein, & Soto (2007) investigated the effects of prenatal exposure to BPA, and whether BPA exposure independently resulted in breast carcinogenesis during adult life. Sprague-Dawley rats were exposed to 2.5, 25, 250, or 1,000 micrograms (ug) of BPA per body weight per day from embryonic day 6 until day the first day after delivery via implanted pumps. Control rats were given a dose of 50% dimethyl sulfoxide. Female mice were specifically sacrificed on the 50th day or the 95th day after delivery, because mammary gland ductal growth and extension of the ducts into the fat pads is noticed on these days respectively. IHC on whole sections showed ductal hyperplasia in all of the animals. Three to four-fold increment was found in the formation of hyperplasic ducts in the experimental animals compared to the controls. The ductal size had also increased due to the active proliferation specifically of the luminal epithelial cells. H & E staining suggested differences in chromatin pattern, presence of nucleoli, and secondary lumina.

A much larger study conducted by Jenkins et al. (2009) utilized rats and investigated whether oral BPA exposure as a neonate could cause breast cancer with a

single carcinogenic exposure in the adult life. They exposed 32, 34, and 24 female rats to sesame (control), 25 ug/Kg body wt/day (25), and 250 ug/Kg body wt/day (250) of BPA respectively. Totally, 15 treatments were given. Eight rats from each group were taken on 21st and 50th days, and their mammary gland was excised to examine tumor progression in live tissue. On the 50th day, a rat from each litter was also exposed to a dose (30mg/Kg body weight) of DMBA, a known cancer causing agent, and sacrificed for tumorigenesis study. Mammary gland proteins were observed by immunoblots. Cell-death and growth was assessed by IHC on terminal end buds (TEDs), and confirmed by Ki-67 analysis.

Tumorigenesis was measured in two ways: a) tumor latency, and b) tumor burden. An increase in the tumor formation was noted with increasing doses of BPA (2.84, 3.82, and 5 respectively). Rats given doses of BPA 250 had a tumor burden that was statistically significant compared to control (p = .004). Additionally, tumor growth was observed in 65, 53, and 36.5 days for BPA 25, BPA 250, and controls respectively (p = .025) showcasing an inverse relationship that existed between tumor latency and BPA dosage. Noteworthy here is that although BPA 25 did show an increased tumor burden and decreased latency when compared to controls, it did not reach statistical significance for either (p = .131 and .058 respectively). However, this does not mean that the tumors were not forming at all or that they were forming earlier in rats that were not subjected to this xenoestrogen at all. Hence, it is plausible that if these exposures were carried out for a longer period of time (persistent exposures) the tumor burden and latency may have reached levels of significance.

Further, mammary terminal end buds (TEBs) were significantly more in the 50 day old rats when compared to the control (22% increase in cellular proliferation of TEBs, $p = <.001$), and a decrease in apoptosis was observed for the 50 day old rats compared to control (40% less apoptosis, $p = .001$). Apoptotic and proliferation proteins, Akt and phosphorylated Akt (pAkt) for 50 day old rats showed significantly higher expression levels (2- fold, $p = .001$; and ~ 2 fold, $p = .050$ increase in expression respectively) compared to control. Analysis of Progesterone receptor A and B (PR-A and PR-B), their co-activators (steroid receptor co-activator; SRC-1, 2, and 3), and *Her* family of tyrosine kinases using Western blots showed a 54% increment in PR-A protein expression in TEBs of 50 day old rats compared to controls. SRC-1, 2, and 3 were all found to be significantly over-expressed compared to control (3.5 fold, $p = .001$; 1.5 fold, $p = .003$; and 3.5 fold, $p = <.001$ increased expression respectively). Although there was an increase in *Her2-neu*, PR-B (a third more for both), and *Her3* (~2 fold increase), but only *Her3* reached significance (p=0.01). Once again, it is possible that *Her2* over expression could reach levels of significance once the exposures are persistent. Interestingly, since the *Her3* receptor itself does not have a kinase terminal required for phosphorylation to induce cellular changes, and to bring about these cellular responses *Her3* partners with *Her2* (Akiyama et al., 1986). Thus, the increase in *Her3* and *Her2* should be similar, but strangely enough only *Her3* reached levels of significance and not *Her2*. Further, the down regulation (a third) of ERα was observed which also occurs when *Her2* amplification reaches its autocrine potential.

Although this study was done in a rat model, it was the first to prove how early exposures to a xenoestrogen like BPA by lactation can have harmful effects during adulthood for the offspring with only a single dose of carcinogenic exposure. This study elucidates the manner in which BPA acts for breast tissue carcinogenesis. Further, the increased cellular proliferation and decreased cellular death plays a crucial role in tumorigenesis. Importantly, studies done in humans on breast cancer patients also showed that an increased expression of the steroid receptor co-activator-3 (SRC-3/A1B1) is linked to an increased expression of the *Her2* oncogene. Thus, it is likely that this study maybe carried over to the human model.

In a recent study, Johnson et al. (2012) had directly linked *Her2* gene expression with exposures to two metabolites of DDT (o'p' DDE and p'p' DDE) in a mouse model with the *Her2-Neu* proto-oncogene. Of these two metabolites, one (o'p' DDE) is an estrogenic isomer; whereas the other (p'p' DDE) is antiandrogenic. They locally injected 5 μg pellets of the DDT derivatives, individually and in combination for two months into the mammary fat pads (total: 4) of prepubertal mice. These exposures were at concentrations that have been found in the human mammary tissue. It was observed that the control mice also developed mammary tumors, suggesting that the breast cancer incidence was similar in all groups, but the p'p' DDE antiandrogenic isomer significantly increased the breast cancer progression rate (shorter latency period) compared to the control mice (90 vs. 147 days respectively, $p = <.02$). Although the rate of tumor progression was greater with o'p' DDE as compared to the control, but it not found to be significantly higher (126 days vs. 147 days respectively, $p = >.05$). These results

indicated that p'p' DDE accelerated breast cancer progression via hormonal and other actions, and the DDT isomers aided in breast cancer progression not initiation. Interestingly, the combinatorial exposures of both isomers had no affect on latency when compared to the control, suggesting that the actions of these two isomers are not synergistic.

Differential Estrogenic Strength of Different Xenoestrogens

Different xenoestrogens exhibited a diverse range of estrogenic activity. Some were mildly estrogenic whereas others were far more effective at a similar dosage of exposure. Due to this attribute, it is deemed important that each xenoestrogen be assessed individually. Silva, Scholze, & Kortenkamp (2007) studied the low dose (nanomolar dosage) responses in 24 known xenoestrogens using the E-screen assay. They found that estriol (E2) produced the highest level of proliferative response with the lowest dose (4.0 X 10^{-4} nM). Coumestral, a phytoestrogen was a hundred times less effective compared to estriol, and produced an effect at 0.55 nM. Surprisingly, in the case of β-endosulphan, it was noted that the concentration at which it produced a 1% effect was lower (140 nM) than its concentration required for no effect (150 nM). More so, many of the steroidal estrogens (e.g., estrone, estriol, hexestrol, and dienestrol) produced shallow dose-response curves, whereas many of the synthetic xenoestrogens did not.

Additive Effects in Combination

Xenoestrogens produced an additive effect when they were present in a combination as mixtures of different estrogenic compounds. However, this effect occurred only when each of the individual compounds formulating that mixture had equal

strength. Payne, Rajapakse, Wilkins, & Kortenkamp (2000) investigated whether additive effects of different estrogenic compounds could be calculated by individual dose-response effects of each compound found in the mixture using YES assay.

Dose-response assays were performed using equal strength of various xenoestrogens (e.g., o, p'DDT, genestin, 4-Nonylphenol (4-NPH), and n-4-octylphenol), individually and in combinations, using E2 as a positive control. Best-fit model predicted by absorbance readings for an individual chemical showed that 4-NPH produced a maximum response, similar to E2 (control). Individually, the chemicals showed a wide-array of effects. Even when the estrogenic compound showed the lowest maximal effect individually, it produced a large additive effect when it was combined with other estrogenic compound/s. A major drawback noted was that the best fit of the model and the readings on combined effects were quite similar only for binary mixtures, but with three compound mixtures the effects were a little under estimated; whereas it was vice-versa for four compound mixtures. Nonetheless, additive effects were observed in all the mixtures. This data could prove to be extremely important, especially in the case of estrogenic compounds that barely produced any detectable effects individually, albeit they could produce significant effects when applied in combination.

Following suite to this study, Rajapakse, Ong, & Kortenkamp (2001) used the YES bioassay and tested whether BPA and o, p'DDT produced an additive impact when each of these xenoestrogen is combined with the naturally occurring hormone 17 β-estradiol (E2). The researchers hypothesized that even weakly estrogenic compounds; such as BPA and DDT can affect the functioning of the steroid hormone based on their

concentration and strength in relation to the hormone. Hence, the impact would be dependent upon the potency of the xenoestrogen in comparison to the natural hormone. Mathematical models of concentration addition (CA) and independent action (IA) for fixed ratios of mixtures were used that predicted the dose-response relationship, and synergy between the two compounds was assumed. Then, predictions made were tested experimentally. The IA model defined the maximal effect of a xenoestrogen; whereas the CA model estimated concentrations of 2 xenoestrogens combined to yield a predetermined additive effect using regression analysis. According to the IA data, all 3 compounds activated human ERα in a dosimetric fashion, reaching maximal effects at 1.59, 1.65, and 0.45 for E2, BPA, and o, p'DDT respectively. This followed well with the experimental data. The maximal effects of BPA and E2 matched well at 1.65 and 1.59, the maximal effect obtained for o, p'DDT was low comparatively to the hormone at 0.45 and 1.59.

The predicted CA data for 2-compounds mix, regression yielded a shift in the dose-response graphs to lower concentrations that becoming more and more pronounced when the amount of E2 were consistently increased in the mix for both E2 and BPA as well as E2 and o, p'DDT, until the hormone completely over-shadowed the effects in each mixture. Then, changing gears by increasing the relative strength of each weak estrogen in the 2-compound mix showed that their effects at low concentrations (1:5000 molar ratios of E2 vs. BPA or o, p'DDT) were almost negligible, but, when each of the weak estrogenic compound was consistently increased while E2 was kept at a constant concentration (1:20000 molar ratios of E2 vs. BPA and o, p'DDT). The prediction curves

started to shift in favor of the weak estrogenic compound. The CA models were similar for E2 and BPA, but only over-lapped in low level effects for E2 and o, p'DDT. Upon testing this model experimentally, the E2: BPA mix corresponded completely with the predictions made by the CA mathematical model. The response of the mix far exceeded the 95% CI of the E2 regression line. In the case of E2: DDT mix the experimental effects observed were well-matched with the predicted models only in the low effect range (up to 0.45 absorbance levels), but neither model was accurately depicted for the high effect range. The researchers owed this to the low solubility of o, p'DDT which hindered its absorbance by the yeast cells. To further test this possibility, they added 2% DMSO in the E2: DDT mixture; as DMSO had previously shown to increase permeability of the yeast cell-wall, thereby increasing the solubility of o, p'DDT within the yeast system. With this mix there was a 40% increase observed in the maximal effect of o,p'DDT, and now the CA model prediction and the experimental were in sync until 1:50000 ratio of E2: o,p'DDT, or 1.2 absorbance levels after which the responses started to plateau off.

In all, these studies provided support that weak estrogenic compounds were able to add to the already strong effects of the endogenous sex steroid action. As this study provided external validity to the study that was conducted by Payne, Rajapakse, Wilkins, & Kortenkamp (2000), proposed that action of weak estrogens or xenoestrogens found in combination were additive in nature. The health implications of these additive effects of xenoestrogens should be seriously considered.

Resistance to Chemotherapy

Chemotherapeutic resistance can play a crucial role in breast cancer therapy, and thereby its survival. BPA has the property of being a chemo-resistant for breast cancer therapeutic agents even when it occurred in low (nanomolar) concentrations. LaPensee Tuttle, Fox, & Ben-Jonathon (2009) investigated this property of BPA with various chemotherapeutic agents (e.g., cisplatin, vinplatin, and doxorubicin) used for breast cancer by conducting cytotoxicity assays on ERα positive cell line T47D (estrogen responsive), and ERα negative line MDA-MB-468 (estrogen non-responsive). MTT cytotoxicity assay showed that the viability of ERα positive and ER negative cancer cells increased in a dose-dependent manner. Even at low dosage BPA (range: 1nM to 10nM) significantly ($P=<0.05$) protected both of the lines from the cytotoxicity mediated by doxorubin. Additionally, when the lines were subjected to BPA in the presence of ICI and PHTPP, blocking ERα and β receptors respectively, BPA still inhibited the action of the chemotherapeutic agents, exhibiting that this action occurs via other pathways besides just the classical ERα and β. Protein analysis by Western blots indicated an increased expression of anti-cell death proteins such as BcL-2, Bc1-xL, and Survivin upon BPA exposure indicative of an anti-apoptotic action.

Modulate VEGF

Experiments revealed that some xenoestrogens increased the production of VEGF, more so in the presence of high levels of ERα, thus increasing the angiogenic capabilities of the breast cells. Buteau-Lozano, Velasco, Cristofari, Balaguer, & Perrot-Applanat (2008) assessed whether xenoestrogens incorporated ERs in their ability to

regulate the secretion of this factor by performing dose-response experiments. The researchers exposed two breast cancer lines (i.e., MELN which over-express ERα and MELP with low levels of ERα) to various xenoestrogens (e.g., E2, BPA, DEHP, BBP, genestin, OP, and various phthalates) for 24 hours, and then quantified VEGF using immunoassay. Ethanol was used as the control.

For the MELN cells with high ERα expression, the VEGF expression patterns showed that E2 is the most sensitive, inducing VEGF production (two and a half time more, $p = <.05$) in extremely low amounts (10^{-10} M concentration). A similar induction was observed in the case of genestin, but with increased concentration (10^{-7} M, $p = <.05$). A significant increase (twice that of control, $p = <.05$) in VEGF secretion was also noted with dieldrin, BBP, OP, DEHP, and BPA although they did so at even higher concentrations ranging from 10^{-4} to 10^{-6} M. Contrastingly, some pesticides (e.g., vinclozin, atrazine, HCH), and phthalates (e.g., DIDP, DINP) did not produce any effect even with increased concentration. Whereas, for the MELP cells with low ERα expression, there was only a significant increase (twice that of control, $p = <.05$) with E2. All other xenoestrogens, the amount of VEGF secreted did increase, but to a lesser amount (one and a half times). Interestingly, *Her2* amplification also increases VEGF production, and once again it is possible that *Her2* is involved in these processes alongwith ER because it cross-communicates with it during tumorigenesis of the breast tissue.

To ascertain whether VEGF increase had occurred in the presence of ER, the different xenoestrogens were treated in the presence of ICI; which blocks ER. Results

showed a 50% to 70% reduction in VEGF secretion (p = .05) between the various xenoestrogens, suggesting that ERα is needed for its secretion. It was further determined if this activity was mediated by ER regulated kinases, the MELN cells were treated with various kinase inhibitors (e.g., SB2035580 inhibits MAPK, and Wortmannin inhibits PI3-K/Akt pathway). It was observed that different xenoestrogens used different kinases for modulating VEGF secretion in breast cancer. For example, for BBP, OP, and dieldrin, the VEGF secretion was reduced by the MAPK inhibitor and the PI-3/Akt inhibitor; whereas only MAPK inhibitor reduced VEGF in the case of genestin suggesting similar and different pathways are involved for different xenoestrogens. Interestingly, VEGF also modulated with the increase in *Her2* expression/copy numbers (Konecny et al., 2004; Ye & Lu, 2010), and once again the cellular endpoints reached with the applications of xenoestrogens overlapped with those observed when *Her2* is over expressed, thus, making it biologically plausible that this oncogene was being activated in this process.

Mediate Cellular Proliferation by Other Pathways (Androgenic Pathway)

Xenoestrogens, such as DDE utilized various cellular pathways and augmented breast cancer progression. Principally, it has been known that xenoestrogens used the estrogenic signaling pathway to produce cellular proliferation. Another direction explored by Aube, Larochelle, & Ayotte (2008) studied the effects of DDE on cellular growth by opposing the androgenic pathway using CAMA-1 cell-lines that expressed both ERα as well as androgen receptors (AR), and MCF-AR line that was genetically manipulated to express AR. Cell proliferation assays captured cellular growth. The cells were grown in DDE alone, or with E2 and Dihydrotestoterone (DHT). Steroid dependant gene

expression; such as ESR1, CCND1 (Cyclin D1), and AR was studied using RT-PCR or immunoblotting. Notably, an inverse concentration response relationship between increasing DHT (androgen) concentration and cell growth (coefficient β=0.887, p=< 0.001) was observed. When combined with E2, DHT decreased the proliferation response at 27% (p = <.05), 54% (*p* = <.001), and 60% (*p* = <.001) with 100, 500, and 1,000 picomolar concentrations of AR respectively. Additionally, DDE alone had the capacity to induce cellular growth response (3 to 3.5 times at 5 and 10uM respectively, *p* = <.001). Gene expression levels for E2 treated cells had up-regulated CCND1 mRNA (50% increase, *p* = <.01), but adding DHT (androgen) in nanomolar quantity (1nM) significantly decreased this expression (*p* = <.01). The CCND1 gene regulates the passage of cells from G1 (resting phase) to S (synthesis phase) in breast cell growth. E2 plus DHT markedly decreased ERα (>50% decrease, *p* = <.05), and CCND1 (~60% decrease, *p* = <.01) expression levels compared to adding E2 alone whereas AR expression was significantly heightened (~50% increase, *p* = <.01). Adding E2 alone decreased (28%) AR expression significantly (*p* = <.05). Results of this study bear evidence that DDE can significantly increase cellular growth of breast cancer line by modulating not only its estrogenic response, but also its anti-androgenic responses. Thus, more than a single pathway could be used by various xenoestrogens to induce cell growth.

A confirmatory study to this end was performed by Aube, Larochelle, & Ayotte (2011), where they studied the androgenic and estrogenic effects of organochlorine (OC) mixtures in human breast cancer lines using reporter gene assays. For the estrogenic

assay, MCF7 line was exposed to various concentrations (0.05-5 μM) of OC mixture for 24 hours in the presence of 1 nM estrogen (E2), and for the androgenic assay, the CAMA-1 line was exposed to various concentrations (0.01-10 μM) of OC mixture for 24 hours in the presence of testosterone (DHT). Receptor expression assays showed that both of these lines had equal amounts of the estrogen receptors (ERs), but CAMA-1 line had greater amount of the androgenic receptors (ARs) when compared to the MCF7 line. The results of the gene reporter assays showed that the OC mixtures induced the estrogenic pathway in the MCF7 line that had a lower expression of the androgenic receptors, whereas the OCs inhibited the androgenic pathway in the CAMA-1 line which had a higher expression of androgenic receptors compared to the MCF7 line.

Increase Intracellular ERα

La Rosa, Pellegrini, Totta, Acconcia, and Marino (2014) studied the effects of BPA, a synthetic xenoestrogen vs. Naringinin (Nar), a plant derived xenoestrogen on intracellular ERα levels when stimulated by estrogen (E2). Using MCF7 line, they performed dosimetric analysis on the ERα protein (mRNA) expression after stimulating the cells either with E2, BPA, and Nar for 48 hours. The mRNA expression on ERα was then assessed by Western blots. The results showed that while Nar prevented intracellular ERα from degradation, BPA promoted ERα degradation. Additionally, cell proliferation assays performed on both xenoestrogens, it was observed that BPA increases cell growth with highest proliferative index observed at 10^{-5} M concentration ($p=<.001$), whereas Nar decreases cell growth which is dose-dependent with significant decreases ($p=<.001$) occurring between 10^{-7} to 10^{-4} M concentrations compared to the control. Comparing the

proliferation rates with E2, it was noted that E2 also increased cellular growth, and BPA reached similar growth curves as E2 at 10^{-5} M concentration. Together, these experiments elucidated how synthetic xenoestrogens and plant derived xenoestrogens had activated and modulated ERα levels in opposing ways.

Xenoestrogens and Breast Cancer Risk: Population-based Studies

Population-based studies using Xenoestrogens have been conducted mainly on organochlorines and their derivatives or pharmaceutical estrogens. The data is confusing since most of these studies; especially with organochlorines have yielded negative results, but a handful of these studies were showing positive results also and cannot be ignored. In the following section, first, the studies performed using organochlorines and their derivatives will be described and assessed for their strengths and limitations, followed by the same for studies conducted using pharmaceutical estrogens.

Organochlorines

Demers, Ayotte, Brisson, Dodin, Robert, & Dowally (2000) assessed the risk of breast cancer initiation as well as cancer progression in relation to various organochlorines using case-control study design. Plasma concentrations of 11 chlorinated and 14 polychlorinated biphenyl congeners was measured in 315 newly diagnosed cases, and 526 controls matched on age and residence. Of the controls, 219 were gynecological disease-free hospital-based, and 307 were randomly chosen. Cases were stratified mainly by tumor-size and metastasis. Organochlorines levels were divided into tertiles, based upon their distribution found in the controls. Mean concentrations of the organochlorines

between the cases and the controls were similar, showing no increase in risk of disease initiation.

Statistical analysis was performed on cases classified by disease status to address if potential risk of disease progression is present. After confounding (age, parity, body mass index, residence, and breast feeding) adjustments, results indicate that the odds of having a more aggressive tumor was significant in women with increased exposure of the following: a) β-HCB (OR = 2.25, 95% CI = 1.12-4.51), b) *trans*-nonachlor (OR = 2.27, 95% CI = 1.11-4.65) showed an increased tumor size (≥2 cm) comparing the highest (4^{th}) to the lowest (1^{st}) exposures; whereas c) p-p'DDE (OR = 2.9, 95% CI = 1.43-5.91), d) oxychlordane (OR = 2.34, 95% CI = 1.10-4.97), and e) PCB congener 153 (OR=2.12, 95% CI=1.05-4.30) showed increased metastasis comparing 1^{st} and 3^{rd} exposure tertiles. Relationship between cancer aggressiveness and p-p'DDE was further dissected for dosimetric effects. Both, tumor progression characteristics increased in a dose-dependent fashion. The OR for 2^{nd} compared to 1^{st} tertile was 2.23 (95% CI = 0.94-5.77), and comparing the 3^{rd} to the 1^{st} generated an OR of 3.51 (95% CI = 1.41-8.73). Similarly, β-HCB (OR = 3.91, 95% CI = 1.47-10.35), and oxychlordane (OR = 3.22, 95% CI = 1.18-8.80) also showed an increased risk for higher levels of exposure.

A prospective case-control study by Hoyer, Jorgensen, Grandjean, & Hartvig (2000) suggested that repeat measurements of xenoestrogens like organochlorines provided for a more accurate method of breast cancer risk assessment compared to a single measure, and that multiple exposures to xenoestrogens can change the risk of breast cancer over time. Totally, 155 cases and 274 matched controls from Denmark

participated. Sera collected over a 5 year period was subjected to gas chromatography for isomers of DDT, total DDT, and PCBs. Trend analyses with two measurements yielded a significant dose-response relationship only with p, p'DDT and PCB138. More than a three-fold increase in breast cancer risk was noted only for p,-p'DDT (OR (1st exam) = 1.9, 95% CI = 0.9-4.3; OR (2nd exam) = 3.6, 95% CI = 1.1-12.2, Ptrend = 0.02), and for PCB congener 138 the risk for breast cancer increased by 2.5 times (OR (1st exam) = 1.4, 95% CI = 0.8-2.6; OR (2nd exam) = 2.1, 95% CI = 1.0-4.4, Ptrend = 0.04). No associations were observed with any other isomer. A significant dose-response relationship was noted only with p, p'DDT and PCB138.

Another study by Hoyer, Jorgensen, Rank, & Grandjean (2001) has shown that exposure to xenoestrogens does not necessarily lead to ER positive breast cancer. To evaluate the influence of organochlorines on breast cancer risk according the hormone receptor status, a case only study (n=161) was done. Tumor characteristics were obtained from the hospital. Paraffin-embedded sections showed 7:3 ratios of ER positive and negative tumors. Interestingly, even though a vast majority of the tumors were ER positive, but, a higher tumor stage (RR = 5.4, 95% CI = 1.8-15.9), size (RR = 4.6, 95% CI = 1.7-12.3), and metastasis (RR = 6.0, 95% CI = 2.1-16.9) were found to be significantly associated with ER negative tumors. For ER negative women, a 7-fold increased risk of developing breast cancer was noted for the highest levels of dieldrin exposure when compared to those with the lowest level (OR 1st vs. 4th quartile = 7.6, 95% CI = 1.4-46.1, Ptrend = 0.01), and the risk was two and a half times more with PCBs (OR 1st vs. 4th quartile = 2.6, 95% CI = 1.1-5.7, *Ptrend* = 0.02). This study indicated that

exposures to certain organochlorines increased the risk of developing ER negative cancers which represent a poor prognosis for the patients, because these tumors are larger and have a high metastatic grade. A limitation of this study was that the numbers of ER negative patients (n = 45) was small leading to insufficient power, and should be interpreted with caution.

Warner et al. (2002) used a historical cohort from the 1976 industrial accident in Seveso, Italy and studied the association between TCDD and breast cancer risk. Archival samples from 981 women between 11 to 40 years were studied. Cases were ascertained by in-person interviews and medical records. Biopsies were reviewed by a pathologist, and TCDD levels assessed by gas chromatography. A double-blinded study was conducted which added to the validity due to lack of bias. Hazard modeling was done on: a) categorical variables; where the categories of exposure were <20 parts per trillion (ppt), 20.1 to 44 ppt, 44.1 to 100 ppt, and >100 ppt, and b) continuous variables as $\log_{10}$ TCDD. Fifteen of the 981 women (15/981) developed breast cancer. Three (3/981) died due to it, and were not included. The average age at the time of explosion and diagnosis was 30 years (range: 14 to 39 years), and 45 years (range: 31 to 57 years) respectively. Therefore, it took almost 15 years for the disease to have occurred, thus marking its latent period. Median TCDD levels observed in the cases vs. controls were much higher (71.8 ppt vs. 55.1 ppt respectively). The continuous variable projected doubling of hazard rates for every 10 fold increment in exposure (e.g., from 10 ppt of exposure to 100 ppt of exposure) in TCDD exposure levels (HR = 2.1, 95% CI = 1.0 to 4.6, p = 0.05).

Again, a limiting factor of this study was the small number of cases present. However, an important point raised by the researchers was that the expected age-specific incidence rates for breast cancer for Seveso between 1988 through 1992 should be 11 cases, but they report 15, making the standardized incidence rates (SIR) higher than expected (1.36). Furthermore, if the three women who died were included, the SIR would be even greater than 1.36. Although, this study showed that acute xenoestrogen exposures like TCDD were significantly associated with breast cancer incidence, but it cannot provide for everyday low to moderate levels of exposures that most women are subjected to.

Aschengrau, Rogers, & Ozonoff (2002) investigated the association between perchloroethylene (PCE) found leaching from the drinking water-pipes into the water system, and the risk of developing breast cancer. They hypothesized that PCE acts as a genotoxic agent either directly or indirectly via metabolites, hence increased the risk of breast cancer. Cases comprised of 672 women diagnosed with breast cancer, and 616 controls matched for location and age. Exposure was an estimated amount of PCE entering residences by water-pipes. Measurements were based on a PCE leaching model; which relies on the rate of water flow estimated by pipe attributes and the amount of water that it can distribute called pipe-load. Geographic information systems (GIS) mapped the participants thus aiding locating participants with different pipe-line characteristics (e.g., diameter, composition, year installed). Only a small to moderate risk was observed for women that were exposed between 75^{th} percentile, and 90^{th} percentile exposure levels ($OR_{(adjusted)}$ = 1.3 to 2.8, and 1.5 to 1.9 respectively).

In 2002, Gammon et al. conducted a case-control study to evaluate the risk of breast cancer associated with various organochlorines with disparate estrogenic properties (e.g., p, p'DDT, p, p'DDE, dieldrin, PCBs). Cases (n = 415) were newly diagnosed with breast cancer, and controls (n = 406) were matched for age and residency. Like the previous study, this study also found only a small increment in risk between the highest vs. the lowest exposure groups for DDE (OR = 1.20, 95% CI = 0.76 to 1.90), and dieldrin (OR = 1.37, 95% CI = 0.69 to 2.72). Effect modifications could not be ascertained as the sample size became too small to investigate their effects.

Charlier et al. (2003) evaluated the risk of breast cancer with organochlorine exposure by measuring blood levels of DDT and hexachlorobenzene (HCB). All participants were White women. Cases (n = 159), and controls (n = 250) matched for smoking, age, residence, breast-feeding, menopause, and reproduction history. Mean DDT and HCB concentrations obtained by gas chromatography were significantly more in cases compared to controls (3.94 vs. 1.83 parts per billion(ppb) for DDT, p = <.0001; and 0.79 vs. 0.09 ppb for HCB, p = .0005 for cases and controls respectively). Notably, the blood concentrations of DDT and HCB were independent of the smoking status (50% vs. 44% respectively, p = .54), or residence (56% vs. 52% respectively, p = .66). Surprisingly, the ER status did not co-relate with DDT (r = 0.02, p = 0.08) or HCB concentrations (r = 0.09, p = .49). However, since all their data comes from White women only, the generalizability of the research conducted was limited, especially since another study (Rosenberg et al., 2008) indicated that interethnic variations can play an important role in breast cancer.

Another factor that played a role towards an increased susceptibility to breast cancer with xenoestrogenic exposure is age at which the exposure occurred. Cohn, Wolff, Cirillo, & Scholtz (2007) used a prospective case-control design and examined if p, p'DDT exposure in early adolescence (<14 years of age) increased breast cancer risk later during adult life. A 1:1 ratio of cases and controls (n = 258), were matched for age and residence. Commercial grade DDT with p, p'DDT (active ingredient), o, p'DDT (contaminant), and p, p'DDE (metabolite) were analyzed. A five-fold increased risk of breast cancer was found only for women that were less than 14 years of age during exposure (OR = 5.2, CI = 1.7 to 17.1, p = <.001). Further, only p, p'DDT showed an increased risk for breast cancer (OR = 2.9, CI = 1.1 to 8.0, p = .04). Once again, the possible mechanism indicated for p, p'DDT being associated with the increased risk is genotoxicity.

Xenoestrogens affected the biological nature of the fat tissue found in the nearby stroma to where the breast tumor occurs. This could bear important implications in the progression of breast cancer. Using a case only design, Munoz-de-Toro et al. (2006) examined the burden of organochlorines from various pesticides and PCBs in invasive breast carcinomas (n = 55). Gas chromatography measured organochlorine content, and IHC assessed biomarkers of breast cancer within the breast tumors plus the stroma surrounding it. Results showed that all the patients had increased levels of organochlorine residues with the highest values of DDE and β-HCH at 4,794 parts per billion (ppb), and 1,780 ppb respectively. Post-menopausal women had higher levels (≥2,600 ppb) of organochlorine concentrations in the surrounding stroma; and vice-versa for the pre-

menopausal women (<2600 ppb). A positive association between dietary fat intake and tumor growth (Fischer's exact, $p = .025$) was also revealed. As this study was performed in vivo, it brings about a real-time facet of the diverse effects that organochlorines can have on different cells that compose the breast tissue.

In a recent case-control study conducted by Boada et al. (2012), the researchers assessed the association between exposures to mixtures of organochlorines and the risk of developing breast cancer. The study was conducted in Spain (Gran Canaria Islands) using 103 healthy women and 121 women that were diagnosed with breast cancer. The organochlorine pesticides (OCPs) examined were *p,p′*-DDT, *o,p′*-DDT (DDT isomers), *p,p′*-DDE, *o,p′*-DDE, *p,p′*-DDD, and *o,p′*-DDD (DDT metabolites), aldrin, dieldrin, endrin, cyclodienes, and lindane. There was a statistically significant difference in the demographics of the study population, specifically, the mean age (58±11.7 vs. 45.3±13.8 years for controls, $p = <.001$), and the BMI of the cases as compared to the controls (27.7±4.8 vs. 26.3±4.3 kg/m^2 respectively, $p = .031$).

The results showed that median values of DDT were higher among the healthy controls compared to the women with breast cancer (217 vs. 153 ng/g of lipid respectively*;* $p = < .001$). But, vice-versa results were noted for DDE and DDD as their levels were higher among breast cancer cases compared to the healthy controls (DDE: 300 vs. 167ng/g lipid; DDD: 0.0 vs. 551 ng/g lipid, respectively*,* $p = <.001$ for DDE & DDD). Consequently, it follows that the body burden of total DDT residues were found to be significantly greater in women with breast cancer when compared to the healthy controls (979 vs. 665 ng/g lipid respectively, $p = <.001$). However, contrastingly, the total

cyclodienes and lindane burden was found to more in the healthy controls compared to the women suffering with breast cancer (91.4 vs. 80 ng/g lipid respectively, $p = .027$).

An interesting set of results were observed in this study, mainly, the two groups had a differences that were statistically significant body burden for different sets of OCPs between the case and the control groups, that is, none of the healthy controls had a combination of aldrin and DDE and DDD, and none of the women with breast cancer had a combination of lindane and endrin residues. Also, it was observed that the main ingredient of DDT (i.e., p, p'DDT) was detected in the serum of more than 70% of both the groups (cases and controls), but DDT has been banned in Spain since 1970s; similar to the USA. This indicates that DDT residues still exists even though it has been almost three decades since its effective ban.

Furthermore, their findings were comparable to those observed by Aube, Larochelle, & Ayotte (2011), where the researcher performed gene reporter assays after exposing MCF7 breast cancer cell line to mixtures of 15 different organochlorines; and found that DDT and its analogs caused cellular growth and division of the MCF7 cells.

Besides these studies that have found positive associations between organochlorine exposures and breast, there are others that have yielded a negative result. A list of these studies appears in Table 2.

Table 2

Epidemiological Studies that Resulted in No Association of Breast Cancer Risk to Organochlorine Exposures

Reference & Year	Type of Study & Cohort	OCs	# of Cases (Mean exposure)	# of Controls (Mean exposure)	OR (95% CI)	RR (95% CI)	Exposure Assessed	P - Value
Krieger et al. (1994)	Nested Study, San Francisco multiphase health exam participants (California)	DDE	150 (43.3 ppb ng/ml)	150 (43.1 ppb ng/ml)	1.33 (0.68 -2.2)		4^{th} vs. 1^{st} quartile	P_{trend} 0.43
van't Veer et al. (1997)	Case-Control, Hospital based (multicenter, Europe)	DDE	374 (1.35 µg/g)	374 (1.51 µg/g)	0.73 (0.44 -1.2)		4^{th} vs. 1^{st} quartile	P_{trend} 0.02
Lopez-Carillo et al. (1997)	Case-Control, Hospital Based (Mexico City)	DDE	141 (567.2 ppb)	141 (505.4 ppb)	0.69 (0.38 -1.2)		3^{rd} vs. 1^{st} 2^{nd} vs. 1^{st} quartiles	$P_{trend} \geq .05$ for both
Hunter et al. (1997)	Nested, Nurses' Health Study (NHS), Boston, Massachusetts	DDE & PCBs	DDE 236 (Median = 6.0 ppb) PCBs 230 (Median = 5.0 ppb)	DDE 236 (Median = 6.9 ppb) PCBs 230 (Median = 5.1 ppb)		DDE 0.72 (0.37 - 1.4) PCBs 0.66 (0.32 - 1.37)	5^{th} vs. 1^{st} Quintile for DDE & PCBs	P_{trend} 0.43 for DDE and PCBs
Helzlsouer et al. (1999)	Nested, Campaign Against Cancer & Stroke, Washington County, Maryland. Samples assessed in 1974 and 1989	DDE & 26 PCB congeners (PCBs)	346 (DDE, 1974 11.5 ng/ml) (DDE, 1989 7.9 ng/ml) (PCBs, 1974 4.9 ng/ml) (PCBs, 1989 2.1 ng/ml)	346 (DDE, 1974 13.6 ng/ml) (DDE, 1989 9.6 ng/ml) (PCBs, 1974 4.7 ng/ml) (PCBs, 1989 2.2 ng/ml)	DDE, 1974 0.5 (0.27-0.89) DDE, 1989 0.53 (0.24-1.17) PCBs, 1974 0.68 (0.36-1.29) PCBs, 1989 0.73 (0.37-1.46)		1974 5^{th} vs. 1^{st} quintile for DDE & PCBs 1989 3^{rd} vs. 1^{st} quintile for DDE & PCBs	DDE, 1974 P_{trend} 0.02 DDE, 1989 P_{trend} 0.08 PCBs, 1974 P_{trend} 0.13 PCBs, 1989 P_{trend} 0.15
^Zheng et al. (1999)	Case-Control, Hospital Based (Connecticut)	DDE & DDT	304 (DDE: 736 ppb) (DDT: 51.8 ppb)	186 (w/BBD) (DDE: 784 ppb) (DDT: 55.6 ppb)	DDE 0.9 (0.5-1.5) DDT 0.8 (0.5-1.5)		4^{th} vs. 1^{st} quartile for DDE & DDT	DDE P_{trend} 0.41 DDT P_{trend} 0.22

(table continues)

Reference & Year	Type of Study & Cohort	OCs	# of Cases (Mean exposure)	# of Controls (Mean exposure)	OR (95% CI)	RR (95% CI)	Exposure Assessed	P - Value
^Bagga et al. (1999)	Unmatched Case-Control study, Hospital Based, Kaiser Permanente, Woodland Hills (California)	DDT, DDE, DDD, & DDT + DDE + DDD	73 (Serum, DDT: 197.6 ng/g ^^DDE: 642.0 ng/g DDD: 21.7 ng/g DDT + DDE + DDD: 861.4 ng/g) (Lipid, DDT: 267.3 ng/g ^^DDE: 709.1 ng/g DDD: 24.0 ng/g DDT + DDE + DDD: 861.4 ng/g)	73 w/mammo-megaly (Serum, DDT: 231 ng/g ^^DDE: 693.6 ng/g DDD: 9.2 ng/g DDT + DDE + DDD: 934.3 ng/g) (Lipid, DDT: 261.3 ng/g ^^DDE: 800.0 ng/g DDD: 9.8 ng/g DDT + DDE + DDD: 1,071 ng/g)	DDT 1.05 (0.93-1.1) DDE 1.13 (0.8-1.6) DDT + DDE + DDD 0.90 (0.71–1.15)		Two-way t-test	DDT P = 0.42 DDE P = 0.50
^Zheng et al. (2000)	Case-Control, Yale-New Haven Hospital (cases & controls), Tolland & New Haven (controls) (Connecticut)	DDE & 9 PCB congeners (PCBs)	475 (DDE: 460.1 ppb) (PCBs: 733.1 ppb)	502 (DDE: 457.2 ppb) (PCBs: 747.1 ppb)	DDE 0.96 (0.67-1.36) PCBs 0.95 (0.68-1.32)		3rd vs. 1st tertile for DDE & PCBs	DDE P_{trend} 0.58 PCBs P_{trend} 0.44
^Stellman et al. (2000)	Case-Control, Two hospital based, Long Island, New York	7 OCPs & 14 PCB congeners (PCBs)	232 (OCPs: 1080 ng/g, 75th percentile) (PCBs: 458 ng/g, 75th percentile)	323 w/BBD (OCPs: 1094 ng/g, 75th percentile) (PCBs: 382 ng/g, 75th percentile)	OCPs 1.29 (0.8-2.0)		Highest magnitude of OR (1.29) observed in the middle tertile of OCPs	$P_{trend} \geq .05$ for OCPs & PCBs
Ward et al. (2000)	Case-Control, Farm workers, (Norway)	71 OCs, & 26 PCB congeners (PCBs)	150 (∑PCBs, serum 4.76 ng/g ∑PCBs, lipid 776.1 ng/g) (Range: ∑OCs, serum $0.5x10^{-3}$ to 7.9 ng/g ∑OCs, lipid 0.080 to 1230 ng/g)	150 (∑PCBs, serum 5.09 ng/g ∑PCBs, lipid 806.6 ng/g) (Range: ∑OCs, serum $0.5x10^{-3}$ to 8.23 ng/g ∑OCs, lipid 0.084 to 1260 ng/g)			Paired t-tests for ∑PCBs & ∑OCs ∑PCBs: Q4 = 0.5 ∑OCs: Range = 0.2 - 1.8	∑PCBs = 0.47 ∑OCs = ≥0.05

(table continues)

Reference & Year	Type of Study & Cohort	OCs	# of Cases (Mean exposure)	# of Controls (Mean exposure)	OR (95% CI)	RR (95% CI)	Exposure Assessed	P - Value
*Millikan et al. (2000)	Case-Control, Rural population-based, farm workers, AAW & WW (North Carolina)	DDE & PCBs	AAW 292 (DDE: 9.90 ppb ng/ml PCBs: 2.79 ppb ng/ml) WW 456 (DDE: 3.52 ppb ng/ml PCBs: 1.89 ppb ng/ml)	AAW 270 (DDE: 8.82 ppb ng/ml PCBs: 2.56 ppb ng/ml) WW 389 (DDE: 3.94 ppb ng/ml PCBs: 1.89 ppb ng/ml)	AAW DDE 1.47 (0.87-2.2) PCBs 1.74 (1.0 – 3.0) WW DDE 0.98 (0.67-1.4) PCBs 1.03 (0.68-1.6)		3^{rd} vs. 1^{st} tertile	$P_{trend} \geq .05$ DDE & PCBs in AAW* or WW
Laden et al. (2001)	Nested, Nurses' Health Study (NHS), Boston, Massachusetts	DDE, PCB congeners 118, 138, 153, 180 & ∑PCBs	370 Median (µg/g) DDE = 0.77 ∑PCBs = 0.54 Congener 118 = 0.9 138 = 0.9 153 = 0.11 180 = 0.07	370 Median (µg/g) DDE = 0.82 ∑PCBs = 0.54 Congener 118 = 0.07 138 = 0.09 153 = 0.11 180 = 0.08		DDE 0.82 (0.49-1.37) ∑PCBs 0.84 (0.47-1.52) PCB118 0.69 (0.39-1.22) PCB138 0.87 (0.5-1.5) PCB153 0.83 (0.47-1.48) PCB180 0.98 (0.55-1.75)	5^{th} vs. 1^{st} quintile for all	P_{trend} DDE = 0.15 ∑PCBs = 0.56 PCB118 = 0.67 PCB138 = 0.21 PCB153 = 0.26 PCB180 = 0.67
Raaschou-Nielson et al. (2005)	Nested, Danish Diet, Cancer & Health Cohort (Post-menopausal women only)	DDE & 31 other organo-chlorines	409 Mean Range (∑OCs: 3.3 - 639.0 µg/Kg lipids)	409 Mean Range (∑OCs: 3.3 - 686.3 µg/Kg lipids)		∑OCs 1.1 (0.7 - 1.7) DDE 0.7 (0.5 - 1.2)	4^{th} vs. 1^{st} quartile for ∑OCs & DDE	P_{trend} ∑OCs = 0.44 P_{trend} ∑DDE = 0.29

(table continues)

Reference & Year	Type of Study & Cohort	OCs	# of Cases (Mean exposure)	# of Controls (Mean exposure)	OR (95% CI)	RR (95% CI)	Exposure Assessed	P - Value
Rubin et al. (2006)	Case-Control, Native American Community-based (Alaska)	DDE & 28 congeners (∑PCBs)	63 (DDE: 8.6 ppb) (∑PCBs: 4.55 ppb)	63 (DDE: 7.36 ppb) (∑PCBs: 6.1 ppb)	DDE 0.42 (0.07-2.38) ∑PCBs 1.43 (0.46-4.47)		4th vs. 1st quartile for DDE & ∑PCBs	P_{trend} ≥0.05 for DDE & ∑PCBs
Gatto et al. (2007)	Nested, multi-center, Women's Contraceptive & Reproductive Experiences (CARE) Study	DDE & PCB congeners (PCBs)	355 (PCBs: 2.2 g/L (serum) 0.31g/g (lipid) (DDE: 9.9g/L (serum) 1.4g/g (lipid)	327 (PCBs: 2.0 g/L (serum) 0.31g/g (lipid) (DDE: 8.1g/L (serum) 1.3g/g (lipid)	DDE 1.02 (0.61-1.72) PCBs 1.01 (0.63-1.63)		5th vs. 1st quintile	P_{trend} DDE = 0.74 PCBs = 0.56
Iwasaki et al. (2008)	Nested Case-Control study, Japanese Public Health Center	p,p'DDT, p,p'DDE, HCB, & β-HCB	139	278	p,p'DDE 1.48 (0.70-3.13) p,p'DDT, HCB, & β-HCB <1.0		4th vs. 1st quartile	P_{trend} DDE = 0.25

Note. OCs=Organochlorines, OCPs=Organochlorine pesticides, AAW=African American Women, WW=White Women, ^=Controls with Breast Disease [Benign Breast Disease (BBD) or mammomegaly], ^^ =Mean differences in the amount of DDE found between the cases and the controls was statistically significant (p=.005 and .006 for serum and lipid, respectively), *=Study used race-specific Body Mass Index (BMI). In this study, stratification for BMI showed a positive association in the highest levels of BMI for AAW for total PCBs (OR=4.92, 95% CI=1.63-14.83) & DDE (OR=1.90, 95% CI=0.71-5.09). (Table was made by self using Microsoft Word, 2007).

A major shortcoming in several of the larger case-control studies that have yielded a negative association between organochlorine exposures and breast cancer risk (Zheng et al., 1999; Bagga et al., 1999; Zheng et al., 2000; and Stellman et al., 2000) is the usage of controls that suffer with breast disease; either benign breast disease (BBD) or mammomegaly (MM). A study by Dupont et al. (1994) reported that women that have had a history of BBD have a greater probability of developing breast cancer, and

enrolling a control population with a previous breast condition may have skewed the results towards the null by acting as possible confounders in these studies. Furthermore, in the study conducted by Bagga et al. (1999) the mean values of lipid and serum DDE between the controls and the cases were found to be significantly different (serum: 642 vs. 693.6 ng/g, P=.005; lipid: 709.1 vs. 800 ng/g, P=.006 respectively), however, the odds ratios were found to be non-significant. The use of unmatched controls for the study may have caused this discrepancy in the study result.

Interestingly, the study conducted by Millikan et al. (2000) overall did not show a positive association, but when the AAW were stratified using race-specific BMI, then the women with the highest BMI values were found to be at risk for breast cancer with exposures to PCBs and DDE. These facts may add up since xenoestrogens (organochlorines) are stored in the adipose (fat) cells in the body, so a person with higher BMI values may have a greater risk of developing breast cancer upon exposure to xenoestrogens.

Some case-control studies conducted on organochlorine (DDE) exposure for their associated risk for breast cancer in the 1990s (Djorveck et al., 1994; Falck, Ricci, Wolff, Godbold, & Deckers, 1992) had shown a positive association between the variables, but the sample size was too small (<20 subjects in each group). Falck et al. (1992) had enrolled 20 cases and controls, and the study conducted by Djorveck et al. (1994) the sample size consisted of only 5 cases and control subjects. In another study conducted in Canada by Dewailly et al. (1994), the researchers found a positive association of DDE exposures and breast cancer risk, but only in women with breast cancer that had a

positive ER status, but this study only had 18 cases and controls, of which 9 case subjects were ER-positive. Due to the extremely small sample sizes in these aforementioned studies the positive results will need to be viewed with extreme caution.

Pharmaceutical Estrogens

Gammon et al. (1999) utilized a case-control design to evaluate the relationship between the use of oral contraceptives (OCs) and *Her2* protein over-expression. *Her2* expression was assessed by IHC on paraffin-embedded tissue on a cohort of women between 20 to 44 years, with newly diagnosed invasive breast cancer. Information was obtained by in-person interviews from 509 cases and 462 controls. Confounders assessed were contraceptive use, body mass index (BMI), reproductive and family history. IHC experiments were successfully conducted only in 371 of the 509 cases. Among those that were tested, 159 (43%), cases showed an over-expression of *Her2* receptor, and these women had a more aggressive disease. Furthermore, when compared to controls, these women were mostly ER negative. The study had also demonstrated that the risk of having a *Her2* positive cancer is heightened by more than two-fold when contraceptive usage begins early (<18 years) (OR = 2.39, 95% CI = 1.08 to 5.30, $p = <.05$). A *Her2* positive status indicated that the *Her2* proto-oncogene found in two copies in each human cell (i.e., *Her2* negative state) had mutated into its oncogenic state, and resulted in the copy number increase/amplification of the *Her2* gene. In its mutated or oncogenic form in the breast tissue, this gene causes breast cancer with a more aggressive disease and high mortality rates (Hynes et al., 1994; Meng et al., 2004; Slamon et al., 1989). This study found that a significant amount of risk is associated with the use of oral contraceptives

when their usage had started at an early age (<18 years old), and the risk of having a *Her2* over-expressed tumor increased when the usage of contraceptives had begun during adolescence.

Von Hoften et al. (2000) conducted a case-control study in the Netherlands, where 309 histology confirmed cases of breast cancer were compared to 610 disease-free controls with respect to oral contraceptive use. Questionnaires were administered to study participants capturing information to oral contraceptive usage, medical and reproductive history, and demographic and behavioral data (e.g., weight, height, diet). The study found that women who used an OC for more than a decade had twice the risk of breast cancer (OR = 2.1, 95% CI = 1.1 to 4.0), nevertheless, it was non-significant. The authors noted, however, that 13.6% of cases and 9% of control participants were also using oral contraceptives. This may have skewed the OR values. Additionally, the type of oral contraceptive was not determined, which made it impossible to assess whether one oral contraceptive increased the risk of breast cancer more than the other.

To answer this question and reduce recall bias, Kumle et al. (2000) specifically designed a population-based prospective study. Women that were between the ages of 30 to 49 years from Norway and Sweden were randomly enrolled to study the associated risk of breast cancer in relation to the type of oral contraceptive preparation and the duration of its use in pre-menopausal women. The sample consisted of 103,027 women, 1008 of which had developed breast cancer. Exposure information was based on a questionnaire filled during enrollment. Contraceptive use was defined as the various time-periods an oral contraceptive was used and the type of contraceptive pill that was used. Summing all

of the time-periods yielded the duration of oral contraceptive usage. A significant interaction (p = .031) was noted between current use of oral contraceptive and age at follow-up, so the sample was further stratified into two age-groups: 30 to 39, and 40 to 49 years with respect to their possible menopausal status at the end of follow-up. Regardless of the type of oral contraceptive used, the current users were at an increased risk (RR = 1.6, 95%CI = 1.2-2.1). Similar risk estimates were noted for women using a combination of estrogen and progestin pills, as well as for those who used progestin pills alone (RR = 1.5, 95% CI = 1.0-2.0). Trend data compared women who had used oral contraceptives for more than five years to those that were never users showed that women that had used oral contraceptives for an extended period of time (≥5 years) were at an increased risk of developing breast cancer compared to those that had never used any oral contraceptives in their lives (p = .005).

Weiss et al. (2002) investigated the risk associated with hormone replacement therapy (HRT) regimens, recent use, and duration in relation to breast cancer in post-menopausal women between the ages of 35 and 64 years. Cases (n = 1,870) of breast cancer were histologically graded, and age and residence matched to the controls (n = 1,953). Exposures were stratified by estrogen replacement therapy (ERT), combined HRT, and ERT plus combined HRT. An increased risk of breast cancer was associated only with an HRT regimen that consisted of a progestin component, and had been used for five or more years. The risk of developing breast cancer was almost one and a half times more when combined HRT was used for five or more years (OR = 1.45, 95% CI = 1.09 to 1.91), and statistically significant (p = .01). Trend analysis also showed an

elevated risk with increased usage duration of the combined (P_{trend} = .003), and continuous combined HRT (P_{trend} = .01) regimens. Similarly, trend analysis between past and recent users demonstrated that combined as well as continuous combined HRT represents a significant risk (p = .04 and .03 respectively). No risk was noted with ERT. In this particular instance, the evidence suggested that progestin and the duration of therapy increased the risk of developing breast cancer. Importantly, this study suggested that all oral contraceptives (xenoestrogens) may not incur a similar level of risk of breast cancer and that they had different carcinogenic potential.

Althuis et al. (2003) examined the relationship between breast cancer risk and various strengths and types of oral contraceptives pills in women between the ages of 25 and 54 years. A sample of newly diagnosed breast cancer patients (n = 1,640) were compared to randomly selected controls (n = 1,492). Results showed that women who were younger than 35 years who had used birth control pills within five years of their diagnoses had the greatest risk of developing breast cancer (RR = 2.22, 95% CI = 1.4 to 3.5) compared to older women (35 to 44 years) (RR = 1.44, 95% CI = 1.30 to 1.80). More than 35 micrograms of EE was associated with a significant risk of breast cancer for women under 35 years of age (RR =3.62, 95% CI = 1.7 to 7.9) compared to women between the ages of 35 and 44 years (RR=1.52, 95% CI=.8 to 2.8, P_{trend} = <.01). When the type of hormone was examined, an increased risk was observed in women under the age of 35 years using high strength progestin (RR = 8.11, 95% CI = 2.1 to 31.6) and estrogen (RR = 2.56, 95% CI = 0.7- to .9) pills.

To take stock of the various studies conducted in this area of research,

Kahlenborn, Modugno, Potter, & Severs (2006) conducted a meta-analysis by pooled data from 34 studies and examined the risk of breast cancer with OC usage in younger women (less than 50 years old). The analysis included 14 hospital-based studies, 19 population-based studies, and 1 study that was a combination of both conducted in several countries around the world (e.g., Brazil, England, France, Taiwan, Sweden, Slovenia to name a few), thereby constituting a truly international sample with much genetic and environmental variation. Studies that were published in or after the 80s were taken into consideration for this analysis. Statistical analyses were done using the random effects model which incorporated the differential variance observed between various studies to estimate effects (Der Simonian & Laird, 1986). The major findings of this meta-analysis were:

1) An overall increase in risk of breast cancer was associated with the use of oral contraceptives (RR = 1.19, 95% CI = 1.09 to 1.29).
2) In 21 out of 23 (91%) retrospective studies, the risk of breast cancer was greater in women who had used oral contraceptives prior to their first full-term pregnancy (FFTP) (OR = 1.44, 95% CI = 1.28 to 1.62) compared to women who were on oral contraceptives after their first full-term birth (OR = 1.15, 95% CI = 1.06 to 1.26).
3) The risk was highest among who had used oral contraceptives for four or more years prior to their FFTP (OR = 1.52, 95% CI = 1.26 to 1.82).

Despite these compelling findings, the meta-analysis was limited for four (4) reasons. First, only crude (vs. adjusted) odds ratios were calculated, hence they did not adjust for

potential confounders; such as age at FFTP, menarche. Secondly, a survivor bias could be involved due to exclusion of women who had previously died from breast cancer and as such could not participate in the study. Such non-participation may have attenuated the value of the combined OR, thereby skewing the results towards the null hypothesis of no association between oral contraceptive use and breast cancer. Thirdly, in the random-effects model a population mean is calculated by the odds ratio where the population means are normally distributed. However, due to substantial variation of race and ethnicity (i.e., genetic variation) in the study population, some sub-populations could be at a greater risk for breast cancer; hence shifting the normal distribution of the population means (μ). Lastly, because there were retrospective case-control studies included recall bias is possible which could have been excluded if only prospective studies were used for the analysis.

In a more recent study, Rosenberg, Zhang, Coogan, Strom, & Palmer (2008) investigated whether there is an increased risk for women using the newer lower strength progestin and estrogen oral contraceptives compared to the original higher strength ones. The study also examined whether there was an increased risk associated with hormone receptor status and ethnicity, and focused specifically on White and Black women and contraceptive use. Drawing on a hospital-based participant pool comprised of 907 cases, and 1,711 controls between the ages of 25 to 67 years were interviewed. Eighty percent of the cases (n = 731) and sixty-seven percent (n = 1152) controls were Whites. Notably, although not statistically significant, Black women had a higher risk of developing breast cancer with each duration category of oral contraceptive use compared to White women.

Specifically, Black women had approximately five fold increased risk (OR = 5.3, 95% CI = 1.6 to 17.4) compared to White women (OR = 1.4, 95% CI = 1.0 to 1.9) with five to nine years of contraceptive use. Overall, the odds of developing breast cancer with OC use were highest for women who had previously used the pill for five to nine years (OR = 2.7, 95% CI = 1.7 to 4.5). Furthermore, the risk increased to one and a half times for women that had used OCs for 15 or more years when compared to those that has used it for less than a year (OR = 1.5, 95% CI = 1.2 to 1.8). These findings suggested that the risk of breast cancer associated with certain xenoestrogens could vary according to ethnicity and contraceptive use. Importantly, there may be a different genetic liability underlying breast cancer risk with exposure to oral contraceptives (xenoestrogens).

Summary of Past Research

This literature review has described how and why the *Her2* proto-oncogene is required for the normal growth and development of the breast tissue, and can mutate into a potent oncogene for breast cancer when activated (Slamon et al., 1989). Xenoestrogens are shown to interact with the ER, thereby increasing the normal estrogen levels found in a tissue (Bulaveya & Watson, 2004). *Her2* can cross communicate with ER, which in turn activates the PI-3K pathway for cellular signal transduction (Stoica et al., 2003). The oncogenic amplification of *Her2* also initializes the MAPK pathway, further impacting the re-localization of ER from the nucleus to the cellular cytoplasm and forming a positive feedback loop that continuously re-localizes the ER from the cytoplasm into the nucleus leading to uncontrolled proliferation of breast cells (Yang et al., 2004). It has been hypothesized that increasing the estrogenicity of the breast tissue with

xenoestrogens leading to mutation of the breast cancer genes found in 17q chromosomal loci; one of these genes is the *Her2* gene (Davis et al., 1997). In a study performed by Jenkins et al. (2009) on a rat model that developed breast cancer, an increased *Her2* and *Her3* levels and activity were observed when exposed to xenoestrogens. Importantly, a case-control study by Gammon et al. (1999) using IHC observed increased *Her2* expression levels in women with breast cancer with oral contraceptives (xenoestrogens), and these women were noted to have a more aggressive disease.

Laboratory studies on animal models, cell-lines, and bioassays have proved that although compounds mimicking endogenous estrogens are broadly categorized as xenoestrogens, but they have differential estrogenic potentials (Silva et al., 2007), and can differentially activate cellular signaling (Bulaveya et al., 2004). The activation of cellular signal transduction further resulted in nuclear activity of cell division processes (Recchia et al., 2004) that consequently activated cellular growth and proliferation (Mercado-Feliciano & Bigsby, 2008). The initiation of cellular activity had occurred even at low doses of some xenoestrogenic compounds (Maras et al., 2005). The increased cellular activity promoted changes in breast tissue morphology which can lead to breast carcinogenesis (Brown & Lamartinere, 1995).

Some of the population-based studies have indicated that organochlorine (xenoestrogen) exposures are a risk factor for breast cancer and its progression (Demers et al., 2000; Charlier et al., 2003) and attest to the aforementioned observations in the laboratory using cell-lines and animal models. A prospective study by Hoyer, Jorgensen, Grandjean, & Hartvig (2000) used a Danish cohort established a dose-response

relationship with p, p'DDT, and this feature of xenoestrogens has been observed in laboratory based research (Rajapakse, Ong, & Kortenkamp, 2001).

Exposures to xenoestrogens increased susceptibility to breast cancer, especially when these exposures occurred at an early age (<14 years old) (Cohn, Wolff, Cirillo, & Scholtz, 2007), and when exposed to even minute or acute amounts of single xenoestrogen during gestation or neonatal period could lead to breast cancer during adulthood (Murray, Maffini, Ucci, Sonnenschein, & Soto, 2007; Jenkins et al., 2009).

Epidemiological studies conducted on pharmaceutical estrogens (oral contraceptives) and their associated risk of breast cancer have suggested that the type of contraceptive, duration of their use, and recency of use can incur an increased risk of breast cancer (Van Hoften et al., 2000; Kumle et al., 2000; Weiss et al., 2002). The risk was found to be greater in women under the age of 35 years than those that are older (Althuis et al., 2003). This risk further increased especially in those women who had used a contraceptive pill for four or more years prior to their first full-term pregnancy (Kahlenborn, Modugno, Potter, & Severs, 2006). An increased risk was also found in the newer contraceptive pill that has a lower potency compared to the older ones, and although not statistically significant, but this risk was found to be more pronounced in African-American women when compared to White women (Rosenberg, Zhang, Coogan, Strom, & Palmer, 2008).

A population study indicated that accidental, acute exposures with dioxin were associated with a two-fold increase (HR = 2.1) in breast cancer risk for women with ten-times the levels of dioxin in their blood (Warner et al., 2002). In contrast, another study

reported only a moderate increase ($OR_{(>75percentile)} = 1.5$) in the risk of developing breast cancer with PCE exposures leaching from PVC water pipes that distributed water in Cape Cod homes in Massachusetts (Aschengrau, Rogers, & Ozonoff, 2002), and yet another study found no increased risk with exposures to organochlorines like DDE, dieldrin, total PCBs, and chlordane, although, a slight increase in risk (OR = 1.2) was noted when comparing the highest exposure levels (4^{th} tertile) with the lowest exposures (1^{st} tertile) (Gammon et al., 2002).

Overall, the population-based data was found only on limited xenoestrogens, was inconsistent and somewhat confusing since some studies indicated organochlorines to be a risk factor for breast cancer (Charlier et al., 2003; Warner et al., 2002), while others reported a minimal risk (Aschengrau, Rogers, & Ozonoff, 2002; Gammon et al., 2002), and yet others did not show any risk at all (Hunter et al., 1997; Snedkar et al., 2001). Additionally, some population-based studies showed an overall negative result, however, when the data was stratified then some sub-populations showed an increased risk for developing breast cancer when exposed to xenoestrogens over others (Hoyer, Jorgensen, & Grandjean, & Hartvig, 2001; Millikan et al., 2000; Rosenberg, Zhang, Coogan, Strom, & Palmer, 2008).

Most epidemiological studies have shown negative results between xenoestrogen exposures and the risk of developing breast cancer (Table 2), only some of the studies showed a positive association (Boada et al., 2012; Charlier et al., 2003; Cohn, Wolff, Cirillo, & Scholtz, 2007; Hoyer, Jorgensen, & Grandjean, & Hartvig, 2001; Hoyer,

Jorgensen, Rank, & Grandjean, 2000; Warner et al., 2002), but these studies simply cannot be ignored and discarded.

Implications of Past Research for Present Research

In conclusion, population-based studies have revealed that the relative risks (RR) for xenoestrogen exposures associated to breast cancer are small to moderate (Aschengrau, Rogers, & Ozonoff, 2002; Kahlenborn, Modugno, Potter, & Severs, 2006; Rosenberg, Zhang, Coogan, Strom, & Palmer, 2008; Weiss et al., 2002) . Nevertheless, these substances are environmentally ubiquitous and affect every woman in some way, suggesting that identifying a modifiable risk factor and controlling their sale and use by introducing policy level changes would end-up saving thousands of lives translating into a large public health impact. However, a key problem with small relative risks found in the epidemiological data is the translation of the risk estimates into actionable clinical as well as policy level decision making. One way to buttress the findings of the epidemiological data that could assist both clinical as well as policy decision making is by elucidating the biologic mechanism/s or processes by which xenoestrogens can promote carcinogenesis.

Additionally, although population-based studies have led to policy level changes, but in the case of xenoestrogens they leave many unanswered questions because the results of various studies were found to be inconsistent. The overall negative results of some may not be enough to close down the chapter on future research with xenoestrogens, because then there are also some studies that yielded positive results (Boada et al., 2012; Charlier et al., 2003; Warner et al., 2002), and yet others that were

positive only with effect modifications, but, in these studies the sample-sizes had become too small to answer the research question/s asked with much confidence (Hoyer, Jorgensen, Rank, & Grandjean, 2001; Kumle et al., 2000; Van Hoften et al., 2000). Furthermore, population-based studies have shown low reproducibility and were tremendously expensive making them difficult to pursue in further research (Brody, Tickner, & Rudel, 2005). Due to these reasons, no two epidemiological studies were found to be alike. Thus, even though there were only some studies that yielded positive results with xenoestrogen exposure and the risk of developing breast cancer; these studies simply cannot be over-looked.

Although some epidemiological studies yielded a positive association, but since these studies relied on self-reports or interviews for exposure categorization the précis exposures were difficult to be quantified using these techniques (Boada et al., 2012; Charlier et al., 2003; Demers et al., 2000). Whereas, studies performed using cell-lines, in vitro assays, and animal models have provided us with insights to the unique attributes of xenoestrogens all while using précis measurements (Aube, Larochelle, & Ayotte, 2011; Brody, Tickner, & Rudel, 2005.; Brown & Lamartinere, 1995., Jenkins et al., 2009; Johnson et al., 2012; Payne, Rajapakse, Wilkins, & Kortenkamp, 2000., Rajapakse, Ong, & Kortenkamp, 2001), and these studies provided biological validation (Jenkins et al., 2009; Johnson et al., 2012) to the epidemiological data (Gammon et al., 1999; Rosenberg, Zhang, Coogan, Strom, & Palmer, 2008).

Due to the chemically diverse nature that was observed in the xenoestrogens (Payne, Rajapakse, Wilkins, & Kortenkamp, 2000; Rajapakse, Ong, & Kortenkamp,

2001; Maras et al., 2005; LaPensee et al., 2009), it will not only be deemed important that the précis exposure measurements be evaluated, but also the metastatic/carcinogenic potential of the xenoestrogen be assessed in order to make policy-level changes (Snedkar et al., 2001). Assessing the carcinogenic potential will provide us with the lowest exposure levels of xenoestrogens that does not incur any breast cancer risk and is safe to use, and this information will be extremely important to make policy level changes for common household products (e.g., plastics).

In a commentary, Davis et al. (1993) hypothesized the role of oncogenic activation in the 17q loci due to increased estrogenicity exerted by xenoestrogenic exposures, and emphasized that this hypothesis requires to be evaluated carefully. *Her2*, a proto-oncogene found in the 17q loci in humans, is required for normal breast development, but becomes into a potent oncogene upon unregulated stimulation (Slamon et al., 1989., Tzahar et al., 1989). Of note, ER and *Her2* have been shown to cross-communicate with each other via signaling pathways (Stoica et al., 2003), making it biologically plausible that exposure to xenoestrogen/s activated *Her2* via this crosstalk, led to over-activation of *Her2*. This then resulted in excessive cellular proliferation and growth for tumorigenesis. Hence, it plausible that xenoestrogenic exposures maybe activating *Her2* pathway via this crosstalk that occurred between these two receptors and activated and over-activated *Her2*, this further resulted in excessive cellular proliferation and growth for tumorigenesis. Intriguingly, research also indicated that the cellular end-points upon xenoestrogenic exposures; such as ERK1/2 activity, cell growth, metastasis, and angiogenesis observed using biochemical assays are similar to those when *Her2*

oncogene copies increase or amplify during its oncogenic activation during breast carcinogenesis.

Some population data pointed to elevated *Her2* expressions with xenoestrogenic exposure (Gammon et al., 1999). However, this study was done only using IHC assay that have high inter-observer variability and low accuracy (Press et al., 2002; Varga, Noske, Ramach, Padberg, & Moch, 2013). Another study in which increased *Her2* oncogene was observed with persistent exposures to a single xenoestrogen was performed in an animal model (Jenkins et al., 2009), and not in a human model using sensitive techniques.

Interestingly, some population studies indicated that the phenotypic characteristics of the breast cell-type maybe a feature that could impact disease type (Gammon et al., 1999). Another study showed that women not only with an ER-positive status are impacted with xenoestrogen exposures, but those having an ER-negative status are also impacted, in fact the women with an ER negative status had a more aggressive disease when compared to women with an ER-positive status (Hoyer, Jorgensen, Rank, & Grandjean, 2001). These studies indicated that there may be a genetic liability involved with xenoestrogenic exposures which needs to be further dissected and understood in future research endeavors.

In all, past research has shown that xenoestrogens are a diverse group of chemicals with varying estrogenic potentials. They activated potent oncogenes; such as the *Her2* oncogene in the development of breast cancer. They incurred small to moderate relative risks for breast cancer upon exposures. Furthermore, they reacted differently in

different mammary cellular phenotypes (ER-positive and ER-negative). What has yet to be established in the literature is:

1) The carcinogenic potential of commonly used xenoestrogens with respect to their précis amount/s, duration, type.
2) Deciphering whether a specific cellular phenotype is more at risk of developing breast cancer upon xenoestrogenic exposures.
3) The mechanistic insights which provides biological validation of their carcinogenic properties, utilizing a technique with high reproducibility and a validated biomarker for breast cancer which can be used as tools for future research endeavors.

The design for the present study was chosen based upon a careful review of existing literature in the areas of breast cancer and xenoestrogens. The next chapter entails detailed discussions on the methodology, sample, instrumentation, and analysis used to conduct the study.

Chapter 3: Research Method

Introduction

This chapter includes a description of this study's design, sample, instrumentation, data analysis, and ethical considerations. It will entail a detailed discussion on the research design, instruments, materials, and protocols used in performing the experiments, and how the data generated were analyzed to answer the research questions asked.

Research Design and Approach

The purpose of this exploratory research study was to dosimetrically assess the carcinogenic potential of four commonly used synthetic xenoestrogens (i.e., BPA, DDT, EE, and NPH) with respect to the *Her2* gene expression. Further, the study further investigated whether specific cellular phenotypes of the mammary cell have greater susceptibility to oncogenic copy number increase. This was done using breast cancer cell-lines that differentially express the ER and *Her2* receptors. The xenoestrogens were applied in increments of their $\log_{10}$ ratios to examine the concentration at which xenoestrogens induced changes in *Her2* gene expression. To capture chronic low to moderate exposures that women are most likely subjected to everyday, the xenoestrogens were applied daily for a time-period of 7 weeks.

A RBD was appropriate for this study. In RBD, blocks of homogenous groups are further stratified into subgroups (Jefferey & Cooks, 2011; Piston, Gil-Humanes, Roderiguez-Quijano, & Barro, 2011; Rivera, Monsalve, Moran, & Suazo, 2013; Wu et al., 2013), and then the experimental design that the researcher wants to incorporate into

his or her project is implemented within each block. This is basically done in order to reduce noise due to variability present between each block, and doing so produces a better effect estimate overall than without its application. Because each cell line is phenotypically disparate, each line was stratified according to their receptor status (e.g., ER+/*Her2*+, ER-/*Her2*-). This design is analogous to a stratified design (Piston et al., 2011; Rivera et al., 2011; Trochim & Donnelly, 2007, p. 189-200; Wu et al., 2013). Then, for each line, the cells were randomly assigned to the case and control groups. The case flasks were exposed to xenoestrogens (i.e., different type, concentration, and duration), whereas the control flasks remained unexposed. This allowed comparison of differences in the *Her2* gene activity within each subgroup as well as overall when the synthetic xenoestrogen is applied (cases) as opposed to the nonexposed (control) group. This design ensured that the primary independent variables of interest—xenoestrogen type, concentration, and duration—could be precisely controlled and measured (Trochim & Donnelly, 2007, p. 187). The random assignment aided in making both groups (i.e., cases and controls) similar before treatment ensued, thereby increasing the internal validity of the study design (Trochim & Donnelly, 2007, p. 191).

In addition, to reduce researcher bias, all four lines were coded A, B, C, and D by a technologist not involved in the study as soon as they were received (Sackett, 1979). After the cell lines were treated to various exposure conditions, FISH experiments were conducted on interphase nuclei using the *Her2*/CEP17 probe-set (Abbott Molecular, Des Plaines, IL) to molecularly assess *Her2* gene copy number changes (i.e., increase in copy numbers or amplification) that incurred with the differential xenoestrogenic exposures.

FISH provides for the assessment of the *Her2* oncogenic copy number changes or amplification at the gene level (Ohlschlegel et al., 2014; Olsson et al., 2010). It also provides for greater accuracy and sensitivity of *Her2* testing, compared to other tests such as IHC that could be used to assess *Her2* values (Olsson et al., 2010; Press et al., 2002). Figure 2 shows the copy number patterns observed using the *Her2*/CEP17 probe set in a normal and *Her2* amplified mammary cell using a fluorescent microscope. In a normal mammary cell, two CEP17 signals (labeled in green) and two *Her2* gene copies (labeled in orange) are observed; whereas when there is an increase in the copy numbers or gene amplification, an increase in the number of signals is noted. Counting the number of copies using fluorescence microscopy, one can quantify the number of gene copies found in the sample and the control and calculate the differences between the two groups (Ohlschlegel, Zahel, Kradolfer, Hell, & Jochum, 2011; Ohlschlegel Zahel, Kradolfer, Hell, & Jochum, 2013; Olsson. Jansson, Holmund, & Gunnarson, 2013).

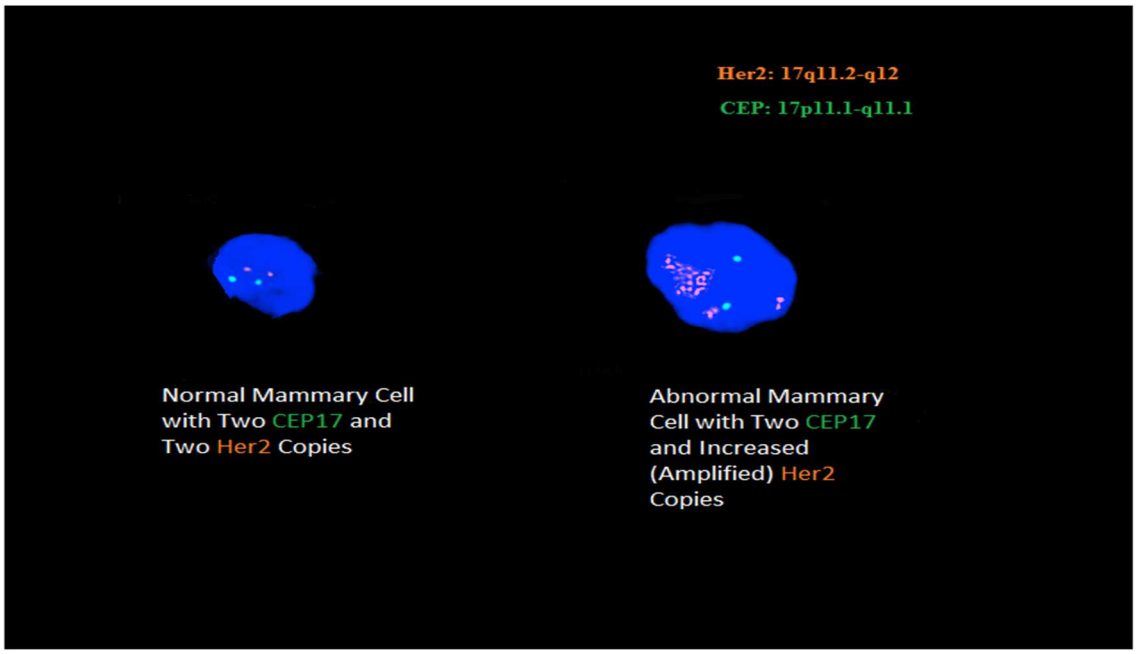


Figure 2. Normal and over expressed *Her2* gene. N example of a mammary cell with two orange signals represents normal *Her2* gene expression (left), and another with multiple orange signals shows copy number increase of the *Her2* gene (right). The green signals represent the centromeres of chromosome #17 on both cells. FISH was performed using the *Her2*/CEP17 dual probe kit from Abbott Molecular for *Her2* analysis on breast carcinoma tissue microarray (Image courtesy of the Cytogenetics Core Laboratory, Brigham and Women's Hospital, Boston, MA).

Setting and Sample

The study was laboratory-based. The research was conducted in the Cytogenetic Core Laboratory. This laboratory is a licensed laboratory of the Brigham and Women's Hospital (BWH) in Boston, Massachusetts.

Cell-Lines

The sample utilized human breast cancer lines, assessing the cell-line specific effect with the application of multiple and varied xenoestrogenic exposures. Because the xenoestrogens typically exert their effect by binding to ERs, and the ERs can further engage in cross-communication with *Her2* (Jung et al., 2012; Slamon et al., 2011; Yang,

Barnes, & Kumar, 2004), hence the breast cell lines for this study were selected on the basis of these two receptors types. As women can either have an ER- and *Her2*-positive or -negative receptor status, the cell-lines selected reflected all the different combinations of these two receptors. That is, ER+/*Her2*+, ER+/*Her2*-, and so on (Chang et al., 2006; Johnson et al., 2010; Wang et al., 2010).

ER-positive and -negative status: Normally, about 30% of breast cells express ER (alpha), but in breast cancer cells that are ER-positive almost all of the breast epithelial cells express the ER (alpha). Thus, in ER-positive breast cancer cell lines almost all the cells have been found to express high levels of ER (alpha), whereas the ER-negative indicates normal levels of ER (alpha) found in the breast epithelia (Allred et al., 2004; Johnson et al., 2010; Wang et al., 2010).

Her2-positive and -negative status: *Her2* gene makes the *Her2* protein. In normal breast epithelia only two copies of this gene is found; whereas in breast cancer cells that are *Her 2* positive too many copies of the *Her2* gene are found and the gene is known to be amplified, which results in its protein overexpression (Figure 2). The *Her2*-negative lines indicate normal levels of the gene copies (i.e., 2 copies) and the *Her2*-positive lines have increased gene copy numbers (Grushko et al., 2002). Additionally, the *Her2* protein overexpression can be tested using immunohistochemical (IHC) staining. This staining test can be negative (0, 1+ score), borderline (2+), or positive for *Her2* protein overexpression (3+) (Pinhel et al., 2012).

Considering all of the different combinations that can be obtained with these two receptors, the samples consisted of the following four breast cancer cell-lines:

- MCF7 line (ER-positive and *Her2*-negative) (Chang et al., 2006; Choi, Fan, Deng, Zhang, & An, 2012; Wang et al., 2010).
- BT474 (ER-positive and *Her2*-positive) (Garrett, Sutton, Kuba, Cook, & Artega, 2012; Johnson et al., 2010).
- MDAMB231 (ER-negative and *Her2*-negative) (Chang et al., 2006; Wang et al., 2010).
- SKBR3 (ER-negative and *Her2*-positive) receptor status (Chang et al., 2006; Garrett et al., 2012; Johnson et al., 2010).

As studies such as this one would be unethical to perform in a population-based setting, accounting for the all the different combinations of these two receptor types found in the mammary cell assisted in generalizability of the results obtained from the different data-sets. Further, it ascertained whether there are any differences in the risk levels that may occur with the different receptor phenotypes.

All of the above mentioned cell lines (Chang et al., 2006; Johnson et al., 2010; Wang et al., 2010) are human breast cancer lines that were obtained by American Tissue Culture Collection (ATCC) from patients that consented to the use of their biopsied materials. The cells from these biopsies were cultured, propagated, and frozen down right after the surgery by ATCC. The breast cancer cell lines are publicly known and used by researchers nationally and internationally for breast cancer research. ATCC is a well renowned bioresource center (BRC) that specializes in culturing and maintenance of cell lines. They also maintain patient data for each line.

The cell-lines were ordered from ATCC. Some advantages of procuring the lines from ATCC are: a) they have been procured and cultured immediately after biopsy, and b) these cell-lines have been frozen down at low passage levels, so the cells used in the experiments are not genetically far removed from the original tissue obtained from the breast cancer patient at the time of biopsy or surgery. This is important because high passage numbers (>40) can add stress to the cells due to their culturing environment, thus bringing about changes in their chromosomal constitution, cellular morphology, and response to external stimulus (ATCC, 2007).

To avoid application of any extraneous estrogen, all of the lines were maintained in media devoid of estrogens from extraneous sources; such as from media and the serum which is added to the media for maintaining the cells in culture. This process further aids in increasing the internal validity of the experiments. The media used did not contain any phenol red, because phenol red has estrogenic properties and can preferentially enhance the growth of cells expressing estrogen receptors (Berthois., Katzenellenbogen, & Katzenellenbogen, 1986; Aube, Larochelle, & Ayotte, 2011). The serum used was charcoal-stripped (Aube, Larochelle, & Ayotte, 2011, Buteno-Lozano, Velasco, Cristofari, & Perrot-Applanat, 2008; Maras et al., 2005; Valeron, Pestano, Luzardo, Zumbado, & Boada, 2009). The process of charcoal stripping utilizes filtering of the serum through charcoal for the removal of materials such as hormones (e.g., estradiol, progesterone, cortisol, testosterone and insulin) and growth factors (Life Technologies, Grand Island, NY). Both, the media and its serum were ordered from Gibco (Life Technologies, Grand Island, NY). Upon arrival, each line was coded (e.g., cell line 1, cell

line 2, etc) and these codes remained blinded to the researcher to reduce experimenter bias (Sackett, 1979). Flasks from each line were typically assigned to control (unexposed) and case groups randomly for the application of various exposure settings described later in this chapter.

Xenoestrogens

The xenoestrogens included in the study were obtained from Fischer Scientific; a company that provides biological chemicals and reagents. The following xenoestrogens were used for this research project:

1) DDT (catalog # 801175033196): is absorbed and retained by soil particles, and is being used as a malarial vector control in some countries (ATSDR, n.d.).
2) EE (catalog # 10166001): is an estrogen used contraceptive pills, but recently the EE part of the pill has been reduced to five times lesser amount (Chu, Zhang, Gentzchein, & Lobo, 2007).
3) BPA (catalog # S-509): is a chemical used in making plastics and resin (e.g., plastic containers, formula and soda bottles, plastic tubing, and dental sealants (Jenkins et al., 2009).
4) NP or NPH (catalog # AC41624-0010): used in industrial detergents and surfactants, and is added to many consumer products; such as pesticides, household cleaners, and cosmetics (Calafat et al., 2005).

For both, BPA and NP, in a sample consisting of 1,000 participants, it was observed that 95% (950 persons), and 51% (510 persons) had more than 0.1ug/L urine concentrations

of these two chemicals in their body (Calafat et al., 2005). These results indicate that most people are being exposed to at least 100ng/mL concentrations of BPA and NP.

Treatment/Exposures

For treatment with xenoestrogens, each cell-line were equally divided into two (2) sets of flasks, one set for single, short-term exposures and the second set for multiple, long-term exposures. Both of these sets of flasks were further divided according to their treatment conditions into A) Individual xenoestrogenic exposure, B) Combinatorial xenoestrogenic exposure, and C) Control flasks (unexposed).

FIRST SET OF FLASKS: Single, Short-Term Exposure Treatment Conditions

A. For individual xenoestrogenic exposure, one flask from each of the four breast cancer cell-lines were exposed to either BPA, EE, DDT, or NP/NPH in increasing concentration of $\log_{10}$ ratios with each xenoestrogen (i.e., .1nM; .01nM; and .001nM).
B. For combinatorial xenoestrogenic exposure, one flask from each line was exposed to .1 nM, .01nM, or .001nM concentration of all four xenoestrogens.
C. For control, one flask from each line (total of four flasks) remained unexposed.

All of the flasks (total = 64 flasks) were kept in the incubator at 37°C after their respective treatments they were harvested on the fifth day. This batch represented the single, short-term exposure.

SECOND SET OF FLASKS: Multiple, Long-Term Exposure Conditions

A. For individual xenoestrogenic exposure, one flask from each of the four lines was exposed to BPA, EE, DDT, or NP/NPH with increasing concentration of $\log_{10}$ ratios of each xenoestrogen (i.e., 0.1nM; 0.01nM; and 0.001nM).
B. For combinatorial xenoestrogenic exposure, one flask from each line was exposed to 0.1 nM, 0.01nM, or 0.001nM concentration of all four xenoestrogens.
C. For control, one flask from each line (total of four flasks) remained unexposed.

All of the flasks (total = 64 flasks) were treated with the aforementioned conditions daily for 50 days. The cells were maintained in the incubator at 37°C after the respective treatments, and then harvested. This batch represented multiple persistent exposures. A grand total of 128 flasks were obtained for the entire sample set consisting of the four breast cancer cell lines used with the various exposure settings. The following diagram (*Table 3*) schematically illustrates the various exposure settings for each of the four cell lines.

Table 3

Schematic Representation of Exposure Setting for Each Line

FIRST BATCH OF FLASKS: Single, Short-term Exposure (Total: 16 flasks / cell line)

BPA, .001nM	DDT, .001nM	EE, .001nM	NPH, .001nM	Combination, .001nM	Control (No exposure)
BPA, .01nM	DDT, .01nM	EE, .01nM	NPH, .01nM	Combination, .01nM	
BPA, .1nM	DDT, .1nM	EE, .1nM	NPH, .1nM	Combination, .1nM	

SECOND BATCH OF FLASKS: Multiple, Long-term Exposures (Total: 16 flasks / cell line)

BPA, .001nM	DDT, .001nM	EE, .001nM	NPH, .001nM	Combination, .001nM	Control (No exposure)
BPA, .01nM	DDT, .01nM	EE, .01nM	NPH, .01nM	Combination, .01nM	
BPA, .1nM	DDT, .1nM	EE, .1nM	NPH, .1nM	Combination, .1nM	

Total: 32 Flasks / Line
Grand Total: 128 Flasks for all 4 lines
Concentrations of Xenoestrogens used: .001, .01, and .1 nM (nanomolar)

The sample set of the four breast cancer cell lines (MCF7, BT474, MDA-MD-231, and SKBR5) was divided into four homogeneous groups, each group representing a cell-line (coded as 1, 2, 3 and 4). Each homogeneous group or cell line was randomly assigned to cases (exposed) and controls (unexposed) groups. Each cell line was further divided into two sets of flasks, the first batch received single, short-term exposure and the second multiple, long-term exposures. For each batch, the four xenoestrogens were applied individually and in combination (i.e., all four xenoestrogens) at concentrations of .001nM, .01nM, and .1nM respectively, while the control flask remained unexposed.

The treatment with xenoestrogens of the samples was done using micro-pipetors. These pipetors are manufactured and calibrated annually by Rainin (Rainin Instruments, CA) for precision and accuracy for laboratory usage. Then, the cells were harvested to obtain interphase nuclei. Slides were made from the cells obtained from each exposure setting on each of the four cell lines. FISH experiments were then conducted on the nuclei using the *Her2*/CEP17 (Abbott Molecular) probe-set. Signals from the FISH experiments were visualized using a fluorescent microscope, and counted using a cell-counter.

Instrumentation

FISH Technique

FISH is a molecular technique by which precise DNA sequences can be targeted within the genome, and this technique is currently being used in many areas of genetics and genomics. Using this technique one can target the entire genome of a particular species, whole chromosomes, specific regions of a chromosome or chromosomes, and single copy or multiple copies (gene amplification) of unique gene sequences, depending on the probes used in the experiment (Liehr, 2009, p. 26-28; Garimberti & Tosi, 2010). The technique is rapid, simple to implement, and offers great probe stability. The principles of this molecular technique applied are rather simple and straightforward. The protocol takes advantage of the fact that a DNA molecule consists of two homologous strands which can be denatured to a single-strand of DNA using heat. Under the right conditions, this single-strand can re-locate its homologue and re-build an exact replica of the initial double-stranded DNA molecule (Liehr, 2009, p.26; Garimberti & Tosi, 2010).

Specifically, DNA or RNA sequences from specific are labeled with fluorochromes (e.g., spectrum red, orange, green, or aqua). These fluorochromes act as the reporter molecules with which the gene can be visualized and counted using a fluorescent microscope. The labeled probe DNA and the sample DNA are then denatured using heat (Bishop, 2010; Garimberti & Tosi; 2010). The heat used for denaturation breaks the bonds of double-stranded DNA molecules of the sample and probe DNA, forming single-stranded DNA molecules. These single-strands of DNA are further hybridized together; which brings together the exact replica of the labeled probe to locate its homologue in the sample DNA placed on the slide. After washing the excess probe that maybe present on the slide, the specimen is screened for the presence or absence of the reporter molecule/s using a fluorescent microscope (Bishop, 2010; Wolff & Schwartz, 2005, p.455-458).

The FDA approved *Her2* probe kit (Abbott Molecular Inc., Des Plaines, IL) specifically hybridizes to the 17q11.2 to q12 chromosomal region, and each copy of the *Her2* gene is represented by the presence of a reporter molecule (Ohlschlegel, Zahel, Kradolfer, & Jochum, 2011; Ohlschlegel, Zahel, Kradolfer, & Jochum, 2013; Olsson, Jansson, Holmund, & Gunnarson, 2013; Wilking, Karlsson, Skoog, Hatscheck, Lidbrink, Elonberger, … Berg, 2011). Basically, all of the genetic information in the human genome is found in the nucleus of each cell in the human body. Within the nucleus the genes are tightly packed in structures called chromosomes, and humans have 23 pairs of chromosomes. Thus, all cells in the human body have a nucleus with 23 pairs of chromosomes that contains the genetic blueprint (Keagle, 2005, chp.2, p.9). Of the 23

pairs of chromosomes, there are 22 pairs of autosomes and a pair of sex chromosomes. The autosomal pairs are labeled as chromosome #s 1-22; whereas the sex chromosomes are designated as XX or XY for normal female or male respectively (Tharapel, 2005, chp.3, p.28). The *Her2* proto-oncogene is located in autosomal chromosome#17 of the human genome (Akiyama, Ogawara, Toyoshima, & Yamamoto, 1986; Slamon et al., 2011). Thus, a normal breast cell has two copies of the *Her2* gene, and more than two signals of the labeled probe would represent an increase in the copy numbers or an amplification of the *Her2* gene (Gutierrez and Schiff, 2011; McCormick et al., 2002). By hybridizing cases (exposure to xenoestrogens) and control groups (unexposed) with the *Her2* probe one can quantify the number of copies of the *Her2* gene present in each group. Then, the differences between mean ranks of the *Her2* signals between the cases and control groups were compared and assessed for significance for each cell line. Between the lines, differences were also examined by assessing the magnitude of change that had occurred for each line with treatment when compared to the controls.

Sensitivity, Specificity, and Accuracy of *Her2* FISH:

FISH is a tool that provides for molecular assessment of the *Her2* gene; specifically *Her2* copy numbers and amplification status. Research conducted (Press et al., 2002) used the *Her2* and CEP17 probes to measure its sensitivity and specificity projected these values as 95.4% (42/43 samples), and 98.6% (72/73 samples) respectively. Using the *Her2*/CEP17 probe set, FISH experiments conducted on a total of 117 samples correctly identified true positives and negatives in 114 samples, thus giving it an accuracy of 97.4% (114/117). Other researchers (Mass et al., 2005; Olsson, Jansson,

Holmund, & Gunnarson, 2013; Sauter, Bartlett, Slamon, & Press, 2007) have further validated the high predictive values obtained using *Her2*/CEP17 FISH probes, with sensitivity measures ranging from 95% -97% and sensitivity ranging from 97% -100% for the assessment of *Her2* copy number increase or amplification. Due to its high level of sensitivity the marking of *Her2* gene amplification, the *Her2/CEP17* FISH assay was optimal to use for this research project as it attenuated type I error rate. Type I errors occurs when a negative result is scored as a positive one, thus it is falsely positive. This type of an error can have significant consequences in the acceptance of the alternate hypothesis when it should actually have been rejected (Munro, 2005, p.88).

Probes for the FISH Experiments

The *Her2* assay kit from Abbott Molecular consists of dual probes: an alpha-satellite or repetitive sequence probe (CEP17), and a locus specific or unique sequence probe (*Her2*) (Burris, Rugo, Vukelja, Vogel, Borson, Limentani, … O'Shaughnessy, 2011; Fleming, Sill, Darcy, McMeekin, Thigpen, Adler, … Fiorica, 2010; Hanna, Ruschoff, Bilous, Coudry, Dowsett, Osamura, … Viale, 2014; Schoppman et al., 2010). This probe kit is FDA approved for breast cancer (Wulfkuhle et al., 2013). The alpha-satellite probe sequences are made of tandem repeats of 171 base pairs (bp) called alphoid monomers, and are anywhere from 0.1 to 5 Mega base (Mb) in length. The alpha-satellite probe hybridizes to sequences that are specific to the centromeric and pericentromeric region of the chromosome (Bishop, 2013Oliveira & French, 2005). The numbers of these alphoid monomers are found to vary in different chromosomes, and due to this property they can be tailored to a specific chromosome centromeric region. On the

other hand, the locus specific or unique sequence probes are generated from specific regions of the genome that contains a single gene or a set of contiguous genes either cloned into vectors (e.g., cosmids, yeast artificial chromosomes, bacterial artificial chromosomes) or obtained synthetically using sequence specific primers with polymerase chain reaction (PCR) (Wolff & Schwartz, 2005, p.455-457). The locus specific probes can vary in size from 1 Kilobase (KB) to >1Mb, and are used to examine gene deletions, copy numbers, and gene re-arrangements (Bishop, 2013; Oliveira & French, 2005; Salmon et al., 2011; Wolff & Schwartz, 2005, p.455-457).

In the *Her2*/CEP17 dual probe breast cancer assay kit from Abbott Molecular (Abbott Molecular, Inc., Des Plaines, IL, USA), the *Her2* probe labeled in spectrum orange hybridizes to region 17q11.2 to q12; which is where the *Her2* oncogene resides, and CEP17 probe labeled in spectrum green hybridizes to 17p11.1 to 17q11.1 (centromeric region of chromosome 17) (Ohlschlegel, Zahel, Kradolfer, Hell, & Jochum, 2011; Ohlschlegel, Zahel, Kradolfer, Hell, & Jochum, 2013; Olsson, Jansson, Holmund, & Gunnarson, 2013; Wilking, Karlsson, Skoog, Hatscheck, Lidbrink, Elonberger, … Berg, 2011).

Protocols for Harvesting Cell-lines and FISH Experiments

Harvesting of Cell-lines

After exposure to xenoestrogens, the cells were harvested with 75mM KCl (Potassium Chloride) for 20 min at 37°C, and then fixed in freshly prepared 3:1 methanol: acetic acid fixative (Ismail, Aly, Khaled, & Mohamed, 2009).

FISH on Interphase Nuclei

FISH was performed using the vendor (Abbott Molecular) instructions for this specific probe-set (Ohlschlegel, Zahel, Kradolfer, Hell, & Jochum, 2011; Ohlschlegel, Zahel, Kradolfer, Hell, & Jochum, 2013; Olsson, Jansson, Holmund, & Gunnarson, 2013; Wilking et al., 2011). In brief, sample slides were prepared from the cell-pellet obtained from the harvest. The slides were denatured using 70% Formamide at 72°C for five minutes, and dipped in 70% Ethanol for a minute at room temperature to remove all the Formamide from it. To remove all the water from the slide they were dehydrated by immersion in 90%, and 100% ethanol for two minutes each at room temperature, and dried on the slide-warmer at 50°C for two to five minutes. Then, 8 µl of the *Her2*/CEP17 probe was applied to the slides and covered with a glass cover-slip. This area was then sealed using a sealant, to ensure that the slides do not dry-out. Next, the slides were hybridized at 37°C for 14-18 hours in a humidified chamber. After this, to remove excess probes off the slides they were washed in 2X SSC/0.3% NP40 solution at 72°C for two minutes, and DAPI, a blue counter-stain was applied. The slides were then viewed under a fluorescent microscope and scored for orange (*Her2*), and green (CEP17) signals (Perez, Pess, Dueck, Jenkins, Kim, Chen, …Slamon, 2013). This protocol is also followed by the Cytogenetics Core Laboratory in BWH using this specific probe-set from Abbott Molecular.

FISH Analysis

Scoring of Interphase Nuclei

The scoring of the nuclei was done as suggested by the vendor (Abbott Molecular) for the *Her2*/CEP17 probe-set (Perez et al., 2013). According to the vendor, scoring 20 nuclei for each sample should be sufficient to study *Her2* copy number increase or amplification (Ismail, Aly, Khaled, & Mohamed, 2009; Press et al., 2002; Wolff, Hammond, Hicks, Dowsett, McShane, Allison, …Hayes, 2013). Ortiz de Solórzano, Santos, Vallcorba, & Garcia-Sagredo (1998) performed statistical analysis for the validation and data correction of automated FISH probe signals. Some of the limitations that they found with interphase FISH signals were: i) the nuclei can overlap, and so do the signals, ii) some organic residues could auto-fluoresce (i.e., background or noise), iii) there could be damage to the nuclei during slide preparation, and iv) differential probe hybridization on the slide. These can be summarized as systemic errors, as they all can result in false positives. In order to avoid these, and increase the accuracy and sensitivity of the test (97%), they emphasize the use of an internal control vs. an external control due to its assured accuracy to the chromosome of interest. As the *Her2* gene resides on chromosome 17, in this *Her2*/CEP17 probe-set, the centromeric probe for 17 (i.e., CEP17) acted as the internal control.

Both orange (*Her2*) and green (CEP17) signals on 20 randomly selected, well separated, intact nuclei were counted (Abbott Molecular) using a fluorescent microscope with dual band filters for orange and green wavelengths of light (Applied Imaging) and a cell counter (Perez et al., 2013). The scoring of all the nuclei was performed using a 100x

objective to avoid variability in FISH signal observation and analysis. The *Her2* gene was then quantified in its absolute value; that is total *Her2* copies observed per nuclei (Hanna et al., 2014). Equal numbers of *Her2* and CEP 17 signals signify the gain of an entire chromosome 17 (Grushco et al., 2002; Hegyi, Lonberg, Monus, & Mehes, 2013; Hanna et al., 2014; McCormick, et al., 2002).

Raw Data

The scoring of the nuclei was formatted in an excel-sheet that projected the frequency of the types of orange and green signals noted in each of the 20 nuclei for line with each of the exposure conditions utilized in the project. For each FISH experiment, the scoring was done manually. The signals were counted using a cell-counter. A representative FISH image of nuclei were captured for each exposure condition and stored in the imaging system of the Cytogenetics Core Laboratory located in BWH.

Statistical Analysis of Data

Descriptive Statistics

Descriptive statistics included medians, range, and frequencies. Tables and graphs (histograms and Q-Q plots) represented the *Her2* signals observed all of the lines after subjecting them to the differential exposure settings. From the raw data, tables were created for each cell-line that marked the percent change values of the *Her2* signals observed under each exposure condition and control (unexposed) groups for each xenoestrogen and cell line using short-term (single exposure) vs. long-term exposures (multiple exposures) and different concentrations (.001, .01 and .1nM). Percent changes observed between cases and controls for different concentrations and durations were

assessed. These tables and graphs allowed easy visualization and comparison of FISH signals observed for the varying exposure conditions applied. The tables and graphs were created using MS Word and Excel version 2007.

Inferential Statistics

The number of green and orange FISH signals found on the 20 individual interphase nuclei was treated as continuous variables (Press et al., 2002). Because the cell-lines were of non- normal distribution, non-parametric statistical tests were performed. Non-parametric tests are those that do not conform to any parameters and are thus distribution free (Munro, 2005, p.110-111). Mann Whitney U test; a non-parametric equivalent to the t-test analyzes differences between two groups, and Kruskal-Wallis is the non-parametric analog to the Analyses of Variance (ANOVA) is used to compare two or more groups (Cubash, Joffe, Hanisch, Schuz, Neugut, Karsdaedt, … Jacobson 2013; Munro, 2005, p.123). The Kruskal-Wallis test assessed the differences between all the groups in the blocks of the RBD; whereas the Mann Whitney U test assessed the differences between the cases and control groups (Schoppmann et al., 2010). Significance level will be at $p = .01$. In addition, to evaluate the magnitude of the *Her2* copy number gains between groups, the Incidence Rate Ratio (IRR) were also computed for *Her2*. The research questions and hypotheses are listed below for review.

Research question #1) Do increasing concentrations of synthetic xenoestrogens significantly increase the *Her2* copy numbers?

Outcome/Dependent Variable: *Her2* gene copy numbers

Predictor/Independent Variable: Concentrations of xenoestrogens (0.000nM or

control, .1nM, .01nM, .001nM) and Receptor types (ER and *Her2* positive and negative).

Null ($H_0$1): There will be no significant increase in *Her2* copy numbers with application with increasing concentrations of xenoestrogens.

Alternate ($H_1$1): There will be a significant increase in *Her2* copy observed with the application of xenoestrogens with increasing concentrations.

Hypothesis: It was hypothesized that increasing the concentrations of the xenoestrogens would increase *Her2* copy numbers. It will also do so for each cell line or receptor type.

Research question #2) Do the concentration at which the increments in *Her2* copy numbers become significant vary between the four xenoestrogens?

Outcome/Dependent Variable: *Her2* gene copy numbers

Predictor/Independent Variable: Xenoestrogenic exposures of BPA, NPH, DDT, and Estrogen using .1nM, .01nM, .001nM concentrations.

Null ($H_0$2): A significant increase in *Her2* copy numbers occurred at similar concentration levels for all four xenoestrogens (BPA, NPH, DDT, and Estrogen).

Alternate ($H_1$2): A significant increase in *Her2* copy numbers occurred at different concentration levels for all xenoestrogens.

Hypothesis: It was hypothesized that *Her2* expressions will significantly increase for the four different xenoestrogens at different concentrations.

Research question #3) Overall, is there a significant increase in the *Her2* gene copies between short-term (5 days) and persistent/long-term (50 days) exposures to the xenoestrogens?

Outcome/Dependent Variable: *Her2* gene copy numbers

Predictor/Independent Variable: Exposure duration (short-term: single, short-term vs. multiple, persistent), and Xenoestrogen (BPA, DDT, EE, NPH).

Null ($H_0$3): No significant increase will be found in *Her2* copy numbers between the short and long term applications of xenoestrogens.

Alternate ($H_1$3): A significant difference in *Her2* copy numbers will be found between short and long term xenoestrogenic exposures.

Hypothesis: It was hypothesized that increasing the duration of xenoestrogenic exposures would significantly increase *Her2* copy numbers overall and for each categorical xenoestrogen.

Research question # 4) Overall, does the *Her2* expression vary significantly with each specific receptor type (i.e., cell line) upon exposure to xenoestrogens?

Outcome/Dependent Variable: *Her2* gene copy numbers

Predictor/Independent Variable: Receptor types/Cell lines (ER+/*Her2*+, ER-/*Her2*-, ER+/*Her2*-, and ER-/*Her2*+) and Exposure durations (single, short-term vs. multiple, long-term).

Null ($H_0$4): *Her2* copy numbers will not vary significantly between the different receptor types/cell lines upon exposure to xenoestrogens.

Alternate ($H_1$4): A significant difference will be found in the *Her2* copy numbers between the different receptor types/cell lines upon exposure to xenoestrogens.

Hypothesis: It was hypothesized that differential *Her2* copy number increase will be noted between the different receptor types/cell lines when they are exposed to

xenoestrogens. However, each cell line would show significant *Her2* copy number gains with multiple, persistent exposures compared to single, short-term exposures.

Ethical Considerations

The administration of xenoestrogens to human participants would be unethical since they could prove to be harmful (Brody & Rudel, 2003). Various animal studies conducted using xenoestrogens have provided evidence of their harmful effects. In fish, Lee, Raisuddin, Rhee, Kim, & Lee (2008a; 2008b) saw an increase in the ras oncogene and p53, a tumor suppressor gene in various tissues; such as intestine, liver, gonads after treatment with BPA and NPH/NP. Another study performed on rats by Zoeller, Bansal & Parris (2005) found that exposure with BPA can increase the levels of thyroid hormone signaling in the brain thereby affecting brain function and activity. Experiments done by Pandey, Pandey & Sharma (2011) using EE on rat liver lead to its degeneration and necrosis (i.e., death), thus, pointing to its toxic effects on liver cells. A recent study on female rats by Canales-Aguire, Padilla-Camberos, Gomez-Pinedo, Salado-Ponce, Feria-Velasco & De Celis (2001) evaluated the effects of DDT on blood lymphocytes and mammary epithelia. Exposure to DDT induced genetic damage in both the lymphocytes and the mammary epithelial cells.

The Agency for Toxic Substances & Disease Registry (ATSDR) has classified DDT as a probable carcinogen (i.e., a cancer causing agent) (ATSDR, 2011). Additionally, the Office of Environmental Health Hazard Assessment California Environmental Protection Agency concluded that although more studies are required to

determine the carcinogenicity of NPH, but the information available from the studies conducted are a cause for concern (OEHHS, 2009).

The Belmont Report (1978) provides us with the basic framework of the ethical principles and regulations used for the protection of human participants in biomedical, behavioral, and experimental research. According to this report, there are three ethical principles that a researcher should abide by. Beneficence is one of the three principles of the report. Within the realm of this principle are formulated two rules. They are:

1) Do not harm. Initially, this rule was introduced for those in the medical profession, but was later incorporated into research by Claude Bernard. He clearly stated that one should not harm or injure any person regardless of the benefits their research may reap for others.
2) Maximize benefits and reduce possible harm.

In summary, careful consideration was given to the nature of the xenoestrogens used in this research project, and their possible effects. Because administration of xenoestrogens to human participants can prove to be harmful, it is unethical to use human subjects for this study. Thus, the only permissible way to perform these experiments was in a laboratory-based setting using xenoestrogens in differential exposures on cell-lines obtained from regulated resources. The following chapter has provided a brief overview of the protocols used and a detailed description of the results obtained from this study.

Chapter 4: Results

Introduction

The purpose of the current study was to quantitatively assess *Her2* copy numbers with FISH technology on four phenotypically disparate human breast cancer cell-lines that are ER- and *Her2*-positive or -negative lines (BT474, MCF7, MDA-MB-231, and SKBR3) after exposing them to differential exposures with four commonly used xenoestrogens (BPA, NPH, DDT, and EE), while the controls remain unexposed. *Her2* gene copy numbers for the cases and controls were counted and their differences evaluated for significance. Four research questions and hypotheses were addressed using a variety of statistical techniques. This chapter provides an overview of the protocols followed to conduct the experiments and summarizes the results of the descriptive and inferential analysis and assumptions used for this study.

Protocols Used

Harvesting and FISH

Four breast cancer cell lines (BT474, MCF7, MDA-MB-231, and SKBR3) were ordered from ATCC. Upon arrival, these lines were coded by someone not involved in this project, and grown in flasks (T-75s) at 37º C with 5% CO_2,using media recommended for their growth by ATCC. Once confluent, the cells from each line were passed into 32 smaller flasks (T-25s) using an enzyme, trypsin-EDTA (.05% for 5 minutes at 37 ºC), and grown for another 4 days. From this point on, all the cells were fed with charcoal-stripped media for the global removal of hormones. Doing this assisted in the removal of extrinsic hormones present in the serum. Then, flasks were randomly

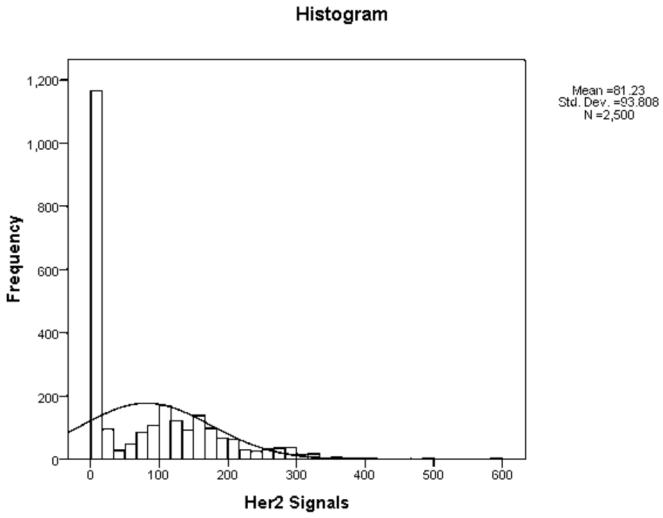
divided into case and control groups. The case flasks received the various treatments of xenoestrogens (BPA, DDT, EE, NPH), concentrations (.001, .01, and .1nM), durations (5 day single, and 50 days daily), while the controls remained without any treatment. After treatment, cells were prepared for FISH analysis by cytogenetic direct preparation after trypsinzation (rinsed with Hanks balanced salt solution to get rid of the media, then use trypsin-EDTA .05% for 5 minutes at 37 ºC) from cells plated in flasks. Cells were then exposed to a hypotonic solution (.075M potassium chloride) for 20 minutes at 37ºC and washed thrice with 3:1 methanol: acetic acid fixative at room temperature. Slides were made using the fixed cell pellets for each control and treatment condition. FISH experiments were performed on cells/interphase nuclei using the *Her2* breast cancer probe kit obtained from Abbott Molecular using manufacturer's instructions. As the hybridization time given by the manufacturer is a range, the hybridization time used for this project was 24 hours at 37ºC. The slides were counter stained with DAPI/antifade (Vectashield, Vector Laboratories, Burlingame, California); the dye is absorbed by the nuclei and it gives a blue color to the nuclei for scoring purposes. The success rate of 97.6% or 98% (125/128 experiments) was noted with the FISH results. Twenty nuclei were then counted for each case and control group using a fluorescent microscope (Zeiss AX-70 with CytoVision software) and cell counter.

Assumptions for Kruskal Wallis Test

Tests of Normality and Data Distribution

The data distribution of signals was found to be non-normal. Figure 3 shows the graph and statistics table of *Her2* signal distribution found with the normal curve overlay,

thus pointing to a non-normal distribution of data. A total of 2,500 nuclei were scored (N). Sixty nuclei for three exposure settings were not scored due to lack of hybridization in those cultures (missing values, N*). The mean and the median values are 81.2 and 30 signals respectively, and the standard deviation was found to be higher than the mean (93.8 signals). As the data set had a non-normal distribution, conducting non-parametric tests (Kruskal Wallis and Mann Whitney U) was optimal to derive better statistical inferences as these tests do not conform to parameters (e.g., normal distribution).



***Her2* Signals (All Lines)**		
N	Valid	2,500
N*	Missing	60
Mean		81.23
Median		30
SD		93.80
Range		596

Figure 3. Histogram of non-normal distribution of *Her2* for all lines. A normal distribution curve is shown in the overlay. Descriptive statistics of the *Her2* signals for all cell lines are in the table on the right; where N is the total number of cells scored and N* are the missing values due to no hybridization. A standard deviation (SD) of 93.8 was greater than the mean measure of 81.2 for the *Her2* signals observed in all the lines.

The histogram for each individual line with the normal curve overlay further confirmed that each of the four cell lines have a non-normal *Her2* signal distribution (Figures 4 a, b, c, and d). This descriptive data from the histograms also revealed that two of the lines had standard deviations that were above their means (MCF7 (ER+/Her2-);

mean: 5.7 and SD: 6.1, and MDA-MB-231 (ER-/*Her2*-); mean: 8.2 and SD: 11.4) as observed in Table 4.

Table 4

Descriptive Statistics for Histograms of Each Cell Line

Cell Line	Receptor	N (Valid)	N* (Missing)	Mean	Median	SD	Range
BT474	ER+/*Her2+*	620	20	165.5952	148	91.43026	567
MCF7	ER+/*Her2*-	640	0	5.71	3	6.211	64
MDA-MB-231	ER-/*Her2*-	640	0	8.22	4	11.479	92
SKBR3	ER-/*Her2+*	600	40	152.49	143	60.411	322

Note. N = Total cells scored, N* = missing values (no hybridization), SD = standard deviation

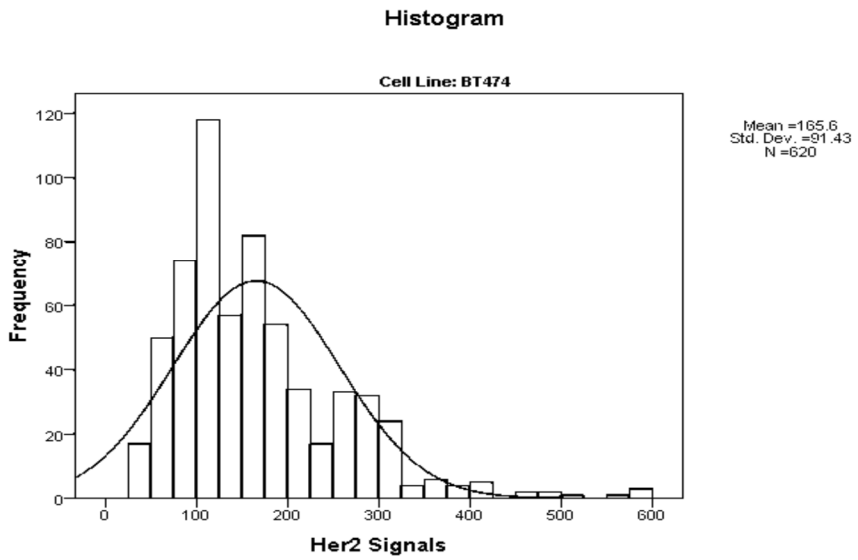


Figure 4a. Histogram of *Her2* Signals for Line BT474 (ER+/Her2+)

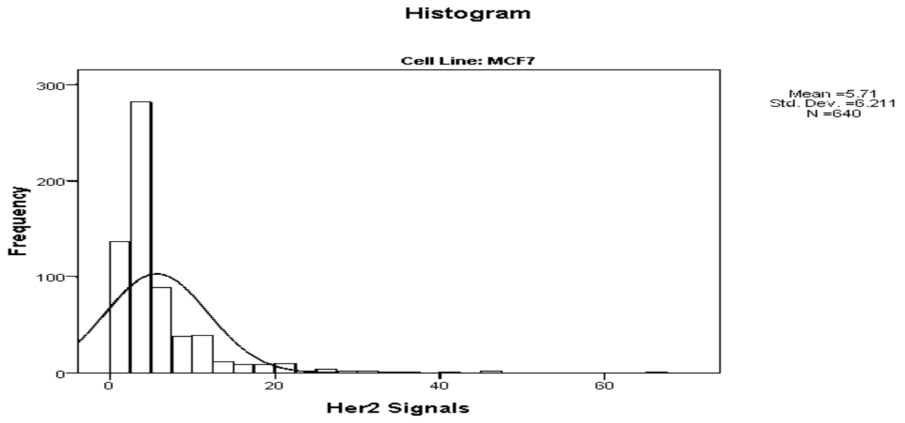


Figure 4b. Histogram of *Her2* Signals for Line MCF7 (ER+/*Her2*-)

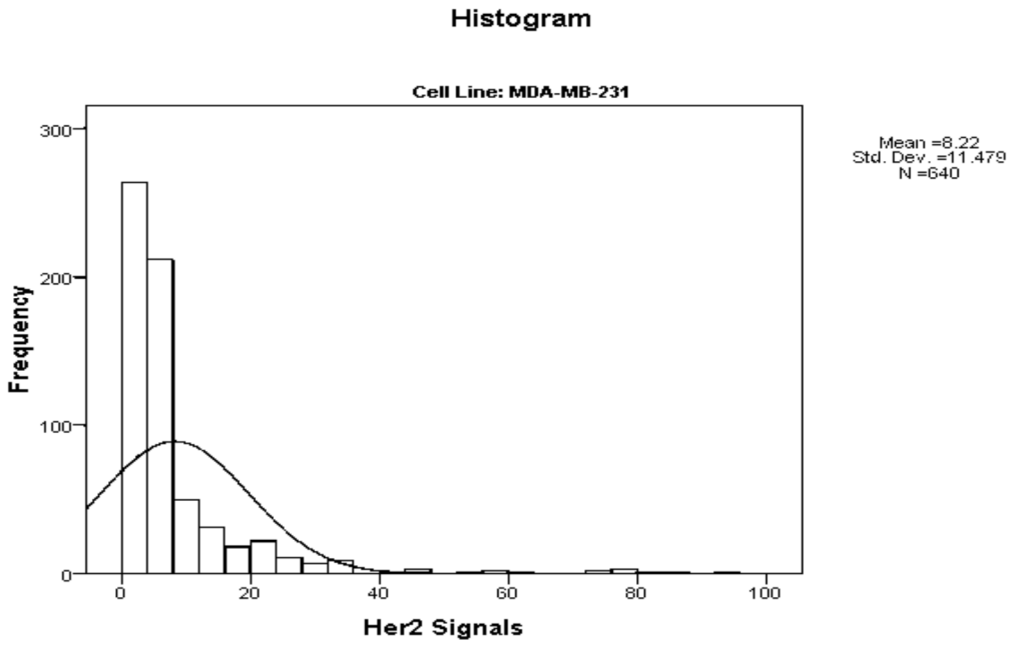


Figure 4c. Histogram of *Her2* Signals for Line MDA-MB-231 (ER-/*Her2*-)

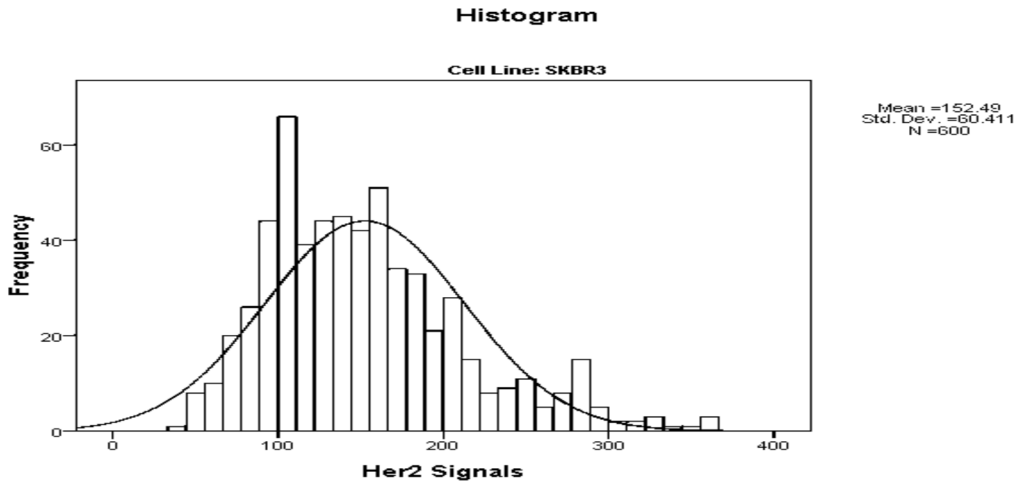


Figure 4d. Histogram of *Her2* Signals for Line SKBR3 (ER-/*Her2*+)

Figures 4 (a-d). Histograms of *Her2* signal distributions for individual line. A normal distribution curve is depicted in the overlay. All of the lines are observed with a non-normal distribution. Of note, the standard deviation is greater than the mean for two cell lines (MCF7 and MDA-MB-231). N denotes the number of cells scored and N* are the missing values due to no hybridization.

Because the histograms of line SKBR3 showed an almost normal distribution, I also graphed the Q-Q plots for the dependent variable (*Her2* signal counts), and performed the Shapiro-Wilk's test to further check for data normality. These Q-Q plots showed that each cell line was non-normally distributed (Figures 5 a, b, c and d). In the plots the normal expected values have been plotted in solid black lines and the *Her2* signal count values that were observed for each cell line in circles. It was noted that in each of the cell line the count values did digress off from the normal expected values of the solid black line. The Q-Q plots and the histograms also showed that the values of the *Her2* counts were spread out over a wider range in all the samples, but was more prominent for lines MCF7 (ER+/*Her2*-) and MDA-MB-231 (ER-/*Her2*-) hence lending

to standard deviations that were greater than the mean values found in these two lines. These widespread values of the dataset further suggested that their means will not be an optimal way to measure the central tendency.

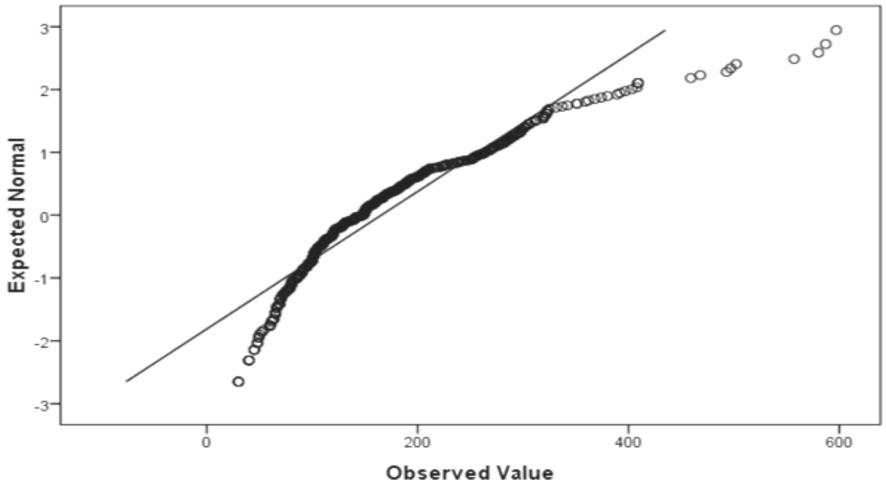


Figure 5a. Q-Q Plots of BT474 (ER+/*Her2*+)

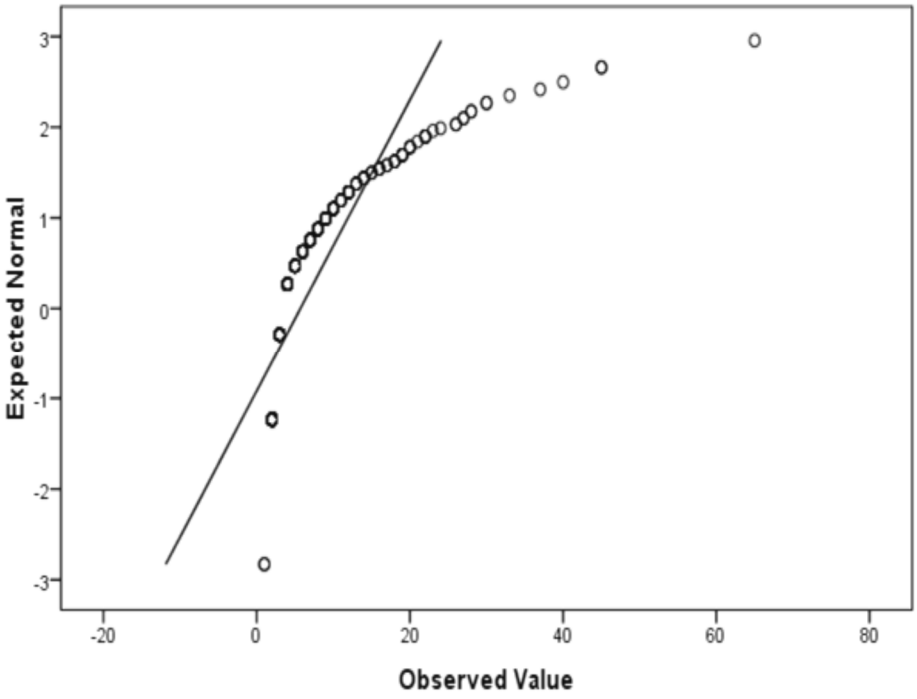


Figure 5b. Q-Q Plots of MCF7 (ER+/*Her2*-)

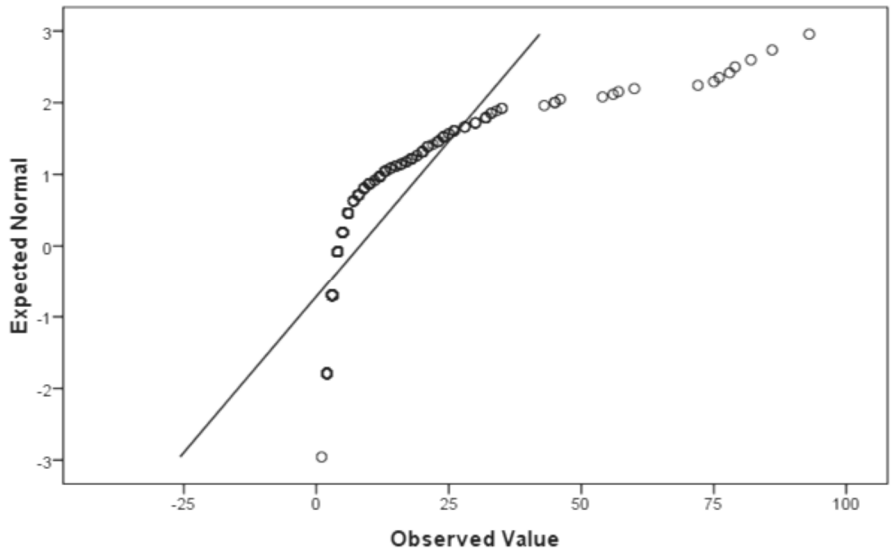


Figure 5c. Q-Q Plots of MDA-MB-231 (ER-/*Her2*-)

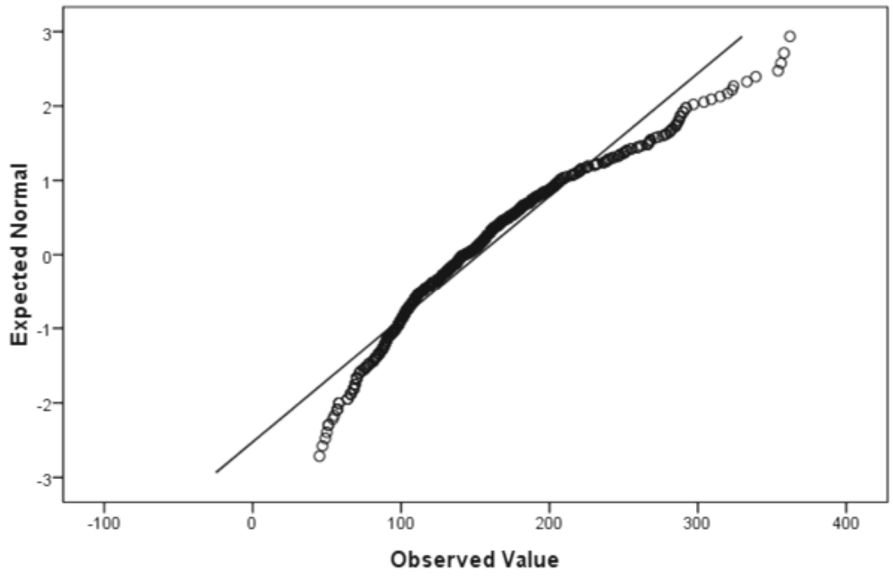


Figure 5d. Q-Q Plots of SKBR3 (ER-/*Her2*+)

Figures 5 (a- d). Normal Q-Q plots for individual cell line. The line in each plot shows the normal expected signals and the circles overlaying the expected values show each *Her2* signal observed in each individual line and their digression from normal expected *Her2* values, thus indicating that the cell lines do not have normal distribution.

The Shapiro-Wilk test of normality was conducted to assess normality of the dependent variable (*Her2* signal counts) for each cell line. The results of the test statistics are represented in Table 5.

Table 5

Shapiro-Wilk Test of Normality for Each Line

		Shapiro-Wilk		
		Statistic	**df**	**Sig.**
Her2 Signals	**BT474 (ER+/*Her2*+)**	0.894	620	**0.000***
	MCF7 (ER+/*Her2*-)	0.587	640	**0.000***
	MDA-MB-231 (ER-/*Her2*-)	0.51	640	**0.000***
	SKBR3 (ER-/*Her2*+)	0.949	600	**0.000***

df = degrees of freedom, Sig. = Significance, * and boldface denotes Significant values (p = <.05)

Null and Alternate hypothesis for Shapiro-Wilk test for normal distribution

Null (H_0): The data are normally distributed

Alternate (H_1): The data are not normally distributed

The Shapiro-Wilk test showed a *p* value of <.05 for each of the four lines (BT474, MCF7, MDA-MB-231 and SKBR3), thus the alternate hypothesis was accepted for each line.

Taken together, the results of the Shapiro-Wilk's test (*p* = <.05) (Razali & Wah, 2011) and a visual inspection of the histograms and the Q-Q plots showed that the *Her2*

signals were non-normally distributed in all four cell lines (BT474, MCF7, MDA-MB-231 and SKBR3). These sample characteristics further confirmed that non-parametric methods (e.g., Kruskal-Wallis and Mann Whitney U) were best suited for the statistical assessment of this data-set due to its non-normal distribution.

Homogeneity of Variances for Kruskal Wallis: Levene's Test

Another assumption besides non-normality of data for conducting Kruskal Wallis test is that the groups being compared should have homogeneous or similar type of variances amongst them. The test used for this is called Levene's test (Gastwirth, Gel & Miao, 2009). Hence this test was performed on the four different types of cell lines used in this project. Table 6 represents Levene's test statistics for the *Her2* values found on four cell lines.

Table 6

Test Statistics for Levene's Test

Individual Differences	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	3	.000	.000	**1.000**
Within Groups	3.16E+08	2496	126640.51		
Total	3.16E+08	2499			

Note. Levene's Test performed for homogeneity of variances on Her2 values found between the four cell lines (Sig. = 1.000 or p = >.05).

Hypothesis for Levene's test for distribution of variances between cell lines

Null (H_o) and alternate (H_1) hypothesis for the Levene's test:

H_o=the variances in *Her2* values between the four cell lines were similar

H_1=the variances in the *Her2* values between the four lines were dissimilar

The null (H_o) was accepted since the *Her2* values found on all four cell lines showed that the differences in their data variances were similar or non-significant (p = >.05 or 1.00).

Besides the non-normal distribution of the *Her2* counts that were observed in each line, the homogeneity of variances data also showed that the variances between each line was similar, hence the non-parametric tests for the statistical analyses were used for this dataset to compute the inferential statistics.

Descriptive Statistics for Xenoestrogen Categories, Concentrations, and Durations

The medians, range and standard deviation (SD) were calculated for *Her2* signals observed with the various xenoestrogens, concentrations, exposure durations and cell lines used. Since the data is non-normally distributed, the mean values were not included as it did not represent the central tendency of this dataset accurately. The range of *Her2* signals observed in each category (i.e., type of xenoestrogen used, concentrations, and exposures) were large (range: 1 to 597 signals). Interestingly, the lowest (3) and highest (579) *Her2* signals were noted in BPA. Albeit, the lowest *Her2* signals (3) observed were tied between control (no exposure), BPA, EE and NPH. The concentration *Her2* signal values ranged from one observed in control (no treatment) and .001nM concentration, and the largest value observed was 597 signals with .01nM concentration. Lastly, for the exposure duration, the lowest value was 1 signal noted using a single, short-term exposure and the highest value was 597 noted with persistent, long-term exposures. Lastly, the lowest value (1) *Her2* signal was noted in the two *Her2*- lines (i.e., MCF7: ER+/*Her2*-, and MDA-MB-231: ER-/*Her2*-), and the highest (597) *Her2* signals were

observed in BT474 line which is ER+/*Her2*+. A summary of the descriptive statistics is listed below in Table 7.

Table 7

Descriptive Statistics Summary of Her2 Signals with Different Xenoestrogens, Concentrations, Exposure, and Lines

Xenoestrogen	N	N*	SE	SD	Min	Q1	Median	Q3	Max
Control	160	0	4	50.54	1	3	25.5	79	210
BPA	440	40	5.88	123.38	1	3	14	152	597
DDT	480	0	4.29	94.06	2	5	62.5	128.75	497
EE	480	0	4.16	91.05	2	3	47.5	156	354
NPH	480	0	3.9	85.41	2	3	36.5	150	409
Combined	460	20	3.72	79.83	2	4.25	32	146.75	362

Concentration (nM)	N	N*	SE	SD	Min	Q1	Median	Q3	Max
Control	160	0	4	50.54	1	3	25.5	79	210
0.001	800	0	2.7	76.48	1	3	37	115	459
0.01	780	20	3.57	99.75	2	4	25	152	597
0.1	760	40	3.87	106.82	1	5	32	170	587

Exposure	N	N*	SE	SD	Min	Q1	Median	Q3	Max
Persistent	1260	20	3.12	110.9	2	5	50.5	180	597
Single	1240	40	1.89	66.54	1	3	11	112.75	315

Cell Line	N	N*	SE	SD	Min	Q1	Median	Q3	Max
BT474 (ER+/*Her2*+)	620	20	3.67	91.43	30	101	148	205.75	597
MCF7 (ER+/*Her2*-)	640	0	0.246	6.211	1	3	3	6	65
MDA-MB-231 (ER-/*Her2*-)	640	0	0.454	11.479	1	3	4	8	93
SKBR3 (ER-/*Her2*+)	600	40	2.47	60.41	40	107	143	183	362

Note. N = Total nuclei scored, N* = Missing values due to no hybridization, SD = standard deviation, SE = standard error, Min = minimum, Q1 = first quartile, Q3 = third quartile, Max = maximum, Control = Unexposed

Percentage Change Analysis Using Raw *Her2* Count Data

Cell Lines

For each cell line, the percent (%) *Her2* copy number increase noted varied with the type of xenoestrogen used individually or in combination. For BT474 (ER+/*Her2*+), a 50% increase was observed between the lowest value with combined exposure (14,293) and the highest value with BPA exposures (23,908). For MCF7 (ER+/*Her2*-), only a 25% increase was observed between the lowest values observed with DDT exposures (15,376) and highest were with EE exposures (19,840). Cell line MDA-MB-231 (ER-/*Her2*-), showed a 100% increment in *Her2* counts between the lowest values found with exposures to BPA (539) and highest values with exposures to DDT (1,629). Lastly, for SKBR3 (ER-/*Her2*+) line there was an 87.5% increase in *Her2* signals between the lowest counts noted with EE exposures (478) compared to the highest count (1,221) found with combined exposures to all four xenoestrogens.

In addition, the count values for all of the individual exposures to the four xenoestrogens in line MCF7 (ER+/*Her2*-) are within an 80 count range (478-557), except for combined exposures which is more than twice when compared to the highest value from the individual exposure range (557-1,221). In the case of line BT474 (ER+/*Her2*+), the count values for combined exposures are lower (14,293) compared to those found in individual xenoestrogenic exposures (range: 19,003-23,908). A possibility of this occurrence could be because the combined (.1nM) persistent exposures in this line yielded missing values (no hybridization). Table 8 represents the exact *Her2* counts

scored for each line with individual or combined exposures with BPA, DDT, EE, and NPH xenoestrogens.

Table 8

Her2 Count Values for All Four Lines with Individual or Combined Exposures to BPA, DDT, EE and NPH

Line	Receptor	BPA	DDT	EE	NPH	Combined
BT474	ER+/*Her2*+	23,908	22,551	19,849	19,003	14,293
MCF7	ER+/Her2-	557	510	478	553	1221
MDA-MB-231	ER-/Her2-	539	1629	879	681	1,064
SKBR3	ER-/Her2+	15,507	15,376	19,840	18,128	18,551

Note. ER+ /- = Estrogen receptor positive or negative respectively, *Her2+/- = Her2* receptor positive or negative respectively.

Exposure Concentrations & Durations

Comparing percent increase between each type of xenoestrogen at the highest concentration of .1nM (nanomolar) to the control group surprisingly showed that the lowest (42%) increment in *Her2* counts occurred with combined exposures to all 4 xenoestrogens. The percent increase in *Her2* counts were comparable for EE and DDT (75% and 72% respectively), and also the percent increase for BPA and NPH were nearly similar (65% and 63% respectively). The greatest increase in *Her2* copies were found to be with exposures to EE (75%). Table 9 represents these results.

Table 9

Percent Changes in Her2 for Each Xenoestrogen (.1nM) & Control

Xenoestrogen & Concentration (.1nM)	N	N*	Total (∑) *Her2* counts	Increase (%)
Control (Unexposed)	160		7467	
BPA	140	20	14755	65.60%
DDT	160		15798	71.63%
EE	160		16396	74.80%
NPH	160		14452	63.23%
Combination	140	20	11392	41.62%

Note. nM = nanomolar, N = total nuclei scored, N* = no hybridization, Total Her2 counts = sum of Her2 absolute count values for control and exposed groups. For each percent value, the sum of Her2 counts for each xenoestrogen was compared to the control group.

Comparing percent increase in *Her2* counts between each xenoestrogen exposure at the highest concentration (.1nM) with the control group for each cell line further showed a decline (2%) in *Her2* counts for combined exposures in BT474 (ER+/*Her2*+), however, this may have occurred due to missing values in combined exposures at .1nM concentrations (no hybridization). The greatest increase in *Her2* counts (121.6%) was noted in MCF7 (ER+/*Her2*-) line with combined exposures. For the individual xenoestrogens, the greatest increase for BPA, EE and NPH (97.8%, 87.5% and 80.3% respectively) all occurred in line BT474 (ER+/*Her2*+), and for DDT, the highest percent increase (117%) was found in MDA-MB-231 (ER-/*Her2*-) line. Of note, lines MCF7 and

MDA-MB-231 both have a *Her2* negative receptor status. These results are represented in Table 10

Table10

Percent Changes in Her2 Counts of Individual Xenoestrogen (.1nM) with Control in Each Line

Line & Receptor	Control & Xenoestrogen (.1nM)	N	N*	Total (∑) *Her2* Counts	Increase (%)	Decrease (%)
BT474	Control	40		3065		
(ER+/*Her2*+)	BPA	40		8937	97.85%	
	DDT	40		8297	92.10%	
	EE	40		7832	87.50%	
	NPH	40		7177	80.30%	
	Combination	20	20	3008		1.90%
MCF7	Control	40		151		
(ER+/*Her2*-)	BPA	40		199	27.42%	
	DDT	40		262	53.75%	
	EE	40		208	31.75%	
	NPH	40		249	49.00%	
	Combination	40		620	121.60%	
MDA-MB-231	Control	40		189		
(ER-/*Her2*-)	BPA	40		480	87.00%	
	DDT	40		722	117.00%	
	EE	40		362	62.80%	
	NPH	40		362	62.80%	
	Combination	40		561	99.20%	
SKBR3	Control	40		4062		
(ER-/*Her2*+)	BPA	20	20	5284	26.20%	
	DDT	40		6517	46.41%	
	EE	40		7994	65.22%	
	NPH	40		6664	48.51%	
	Combination	40		7269	56.6	

Note. N=total number of nuclei scored, N* = missing values, nm = nanomolar, no hyb = no hybridization, % = percent, Increase/Decrease = total percent increase or decrease found in Her2 counts compared to control (unexposed).

Comparing each line for the different durations of exposures used in the study (single vs. persistent exposures) at the highest concentration used (.1nM) with the controls (unexposed). An increase in *Her2* was found in single as well as persistent exposures. However, the percent increase was greater in single vs. persistent exposures in line SKBR3 (ER-/*Her2*+) for each categorical xenoestrogen assessed (single vs. persistent exposures: DDT 61% vs. 67.3%, EE 94% vs. 42%, NPH 78% vs. 25.3%, and Combined 70.4% vs. 48%). BPA could not be assessed due to missing values (no hybridization) for single exposure condition. Surprisingly, in the case of EE, it was noted that the percent gain in *Her2* copy numbers for persistent exposures was always lower compared to the single exposure in all four lines (BT474: 87.8% vs. 87.3%; MCF7: 36.2% vs. 29.3%; MDA-MB-231: 73% vs. 58.6%; SKBR3: 94% vs. 42% for single vs. persistent exposures respectively), even though in the case of BT474 line the difference was very little (.5%). The highest increments between single and persistent exposures were found in line MCF7 (ER+/*Her2*-) combined exposures (139.4 – 23.72 = 115.68%), followed by BT474 (ER+/*Her2*+) BPA exposure, and MCF7 (ER+/*Her2*-) DDT exposure (65.6% and 52% respectively). Notably, the greatest percent increment of *Her2* signals between single vs. persistent exposure occurred in MCF7 line which is *Her2*- in its receptor status. These results are represented in Table 11.

Table 11

Percent Changes in Her2 Counts between Individual Xenoestrogen (.1nM) & Controls for Single and Persistent Exposures

		Single Exposure (5 days)			***Persistent Exposure (50 days)***		
Line & Receptor	**Xenoestrogen**	**N**	***Her2* (.1nM)**	**Increase (%)**	**N**	***Her2* (.1nM)**	**Increase (%) & Difference**
BT474	Control	20	1175		20	1890	
(ER+/Her2+)	BPA	20	1945	49.2	20	6992	114.9 (65.7)
	DDT	20	2533	73.24	20	5764	101.2 (28)
	EE	20	3014	87.8^	20	4818	87.3
	NPH	20	2941	85.81^	20	4236	76.6
	Combined	20	2942	85.84	no hyb	N/A	N/A
MCF7	Control	20	52		20	99	
(ER+/Her2-)	BPA	20	56	7.4	20	143	36.4 (29)
	DDT	20	61	16	20	201	68 (52)
	EE	20	75	36.2^	20	133	29.31
	NPH	20	69	28	20	180	58.1 (30.1)
	Combined	20	66	23.72	20	554	139.4 (115.6)
MDA-MB-231	Control	20	54		20	135	
(ER-/Her2-)	BPA	20	95	55	20	240	56 (1)
	DDT	20	148	93.1	20	574	123.83 (30.73)
	EE	20	116	73^	20	246	58.26
	NPH	20	100	59.8	20	262	64 (4.2)
	Combined	20	141	89.23	20	420	103 (13.77)
SKBR3	Control	20	1439		20	2623	
(ER-/Her2+)	BPA	no hyb	N/A	N/A	20	5284	67.31
	DDT	20	2697	61^	20	3820	37.2
	EE	20	3986	94^	20	4008	42
	NPH	20	3280	78^	20	3384	25.33
	Combined	20	3001	70.4^	20	4268	48

Note. N = total nuclei counted, *Her2* = sum of *Her2* counts, .1nM = exposure concentration in nanomoles, no hyb = no hybridization, Increase = total percent (%) increase compared to control for single and persistent exposures, ^ = percent increase greater with single compared to persistent exposures, Difference = increase in *Her2* for persistent exposures compared to single exposure, ER- = estrogen receptor negative, ER+ = estrogen receptor positive, *Her2*+ = *Her2* positive, *Her2*- = *Her2* negative

An examination of percent changes that had occurred with the applications of each xenoestrogen (individually or in combination) from the lowest (.001nM) to the highest (.1nM) concentrations applied showed an increase in *Her2* counts regardless of the duration of application (5 days and 50 days), except for line MCF7 (ER+/*Her2*-) with single (5 days) combined exposures; where a percent decline was noted (15.4%) in *Her2* counts with increasing concentrations. However, the percent change was found to increase (difference: 138) when the combined exposures to xenoestrogens are applied persistently (50 days).

Interestingly, in line BT474, positive for both ER and *Her2*, all of the categorical xenoestrogens studied had a higher percent change with single, short-term exposure duration compared to persistent, long-term exposure duration. In this case, combined persistent exposures could not be ascertained or compared due to lack of hybridization of the *Her2* signals.

The greatest percent increase change of 315% was noted in line MDA-MB-231, negative for both ER and *Her2*, with combined persistent exposures, followed at 204% increase with combined persistent exposures in MCF7 line, which is ER-positive and *Her2-negative*. Also, the highest difference in percent change increase between single, short-term and persistent, long-term exposures was found in line MDA-MB-231 with combined persistent exposures at 250. Notably, both MDA-MB-231 and MCF7 lines are *Her2-negative.*

These data are represented in Table 12.

Table 12

Percent Changes in *Her2* Counts with Lowest (.001nM) and Highest (.1nM) Concentrations of Xenoestrogens

		Single Exposure (5days)						***Persistent Exposure (50 days)***				
Line & Receptor	**Xeno**	**N (.001)**	***Her2* (.001)**	**N (.1)**	***Her2* (.1)**	**Inc (%)**	**Dec (%)**	**N (.001)**	***Her2* (.001)**	**N (.1)**	***Her2* (.1)**	**Inc (%) & (Diff)**
BT474	BPA	20	1344	20	1945	44.7^		20	5534	20	6992	26.34
ER+/Her2+	DDT	20	1756	20	2533	44.24^		20	4463	20	5764	29.15
	EE	20	1895	20	3014	59.05^		20	3052	20	4818	57.86
	NPH	20	1782	20	2941	65.03^		20	3396	20	4236	24.73
	Comb	20	2447	20	2942	20.22		20	2501	0	NH	N/A
MCF7	BPA	20	47	20	64.47	37.17^		20	115	20	143	24.34
ER+/Her2-	DDT	20	59	20	61	3.38^		20	145	20	201	38.62
	EE	20	56	20	75	33.92		20	62	20	133	114 (80)
	NPH	20	52	20	69	32.69		20	76	20	180	136 (103)
	Comb	20	78	20	66		15.4	20	182	20	554	204 (138)
MDAMB231	BPA	20	93	20	95	2.15		20	85	20	240	182.35 (180)
ER-/Her2-	DDT	20	105	20	148	40.95		20	263	20	574	118 (77)
	EE	20	67	20	116	73.13		20	177	20	246	110 (37)
	NPH	20	72	20	100	38.88		20	91	20	262	187.91 (149)
	Comb	20	85	20	141	65.88		20	101	20	420	315 (250)
SKBR3	BPA	20	2757	0	NH	N/A		20	2867	20	5284	84.30
ER-/Her2+	DDT	20	2094	20	2697	28.79		20	2127	20	3820	78.6 (50)
	EE	20	2525	20	3986	57.14		20	2420	20	4008	65.61 (9)
	NPH	20	2690	20	3280	21.93		20	2709	20	3384	24.91 (2)
	Comb	20	1718	20	3001	74.67^		20	3018	20	4268	40.48

Note: Xeno = xenoestrogen, N = total nuclei counted, *Her2* = sum of *Her2* counts, .001 and .1 = nanomolar concentrations, % = percent, Inc & Dec = % increase & decrease, Comb = combined exposures, ^ = % increase greater in single exposure compared to persistent exposures, Diff = difference of increase found with persistent exposures compared to single exposure, NH = no hybridization, ER+/- = ER positive/negative, *Her2*+/- = *Her2* positive and negative.

Inferential Statistics & Hypothesis Testing

Hypothesis 1

Research question #1) Do increasing concentrations of synthetic xenoestrogens

significantly increase the *Her2* copy numbers?

Null ($H_0$1): There will be no significant increase in *Her2* copy numbers with application with increasing concentrations of xenoestrogens.

Alternate ($H_1$1): There will be a significant increase in *Her2* copy observed with the application of xenoestrogens with increasing concentration

The first hypothesis predicted that increasing concentrations of the four commonly used xenoestrogens (BPA, DDT, EE and NPH) would increase *Her2* copy numbers significantly with the log10 increase of concentration (.001, .01 and.1nM), and when each concentration is compared to the control (No Treatment) group. Kruskal Wallis test was performed for control (No Treatment) and all concentrations (.001nM, .01nM, and .1nM) of xenoestrogens applied to examine the relationship between the various concentrations of xenoestrogens as the predictor/independent variable and *Her2* copy number as the outcome/dependent variable. The result of the Kruskal Wallis omnibus model was significant (p = 0.000). Table 13 displays the results of the Kruskal Wallis.

Table 13

Kruskal Wallis Results Comparing *Her2* Copy Numbers Between Different Concentrations and Controls

Concentrations	N	Mean Rank	Chi-square	df	P
Control (No exposure)	160	783.53	89.728	3	**0.000***
.001nM	700	1177.7			
.01nM	840	1300.46			
.1nM	761	1294.27			
Total	2461				

Note: N = number of nuclei scored, df = degrees of freedom, P = asymptotic and 2-tailed, * and boldface numbers denote significant values (p = <.01)

Post-hoc pairwise analysis performed showed that the *Her2* copy numbers increased significantly (one-sided $p < .000$ or $p = <.05$) for all three concentrations (.001, .01 and .1nM) of xenoestrogens applied when compared to the control (No Treatment) group. Pairwise comparisons between the different groups of concentration showed that the *Her2* copy numbers significantly increased between .001nM and .01nM concentrations of xenoestrogens (one-sided $p = .008$ or $p = <.05$), and the null hypothesis ($H_0 1$) was rejected. However, the *Her2* copy number increase was not significant between .01nM and .1nM concentration (one-sided $p = .101$, or $p = >.05$) concentrations of xenoestrogens applied, and the null ($H_0 1$) was accepted in this case. Table 14 represents the results of the post-hoc pairwise tests respectively.

Table 14

Pairwise Comparisons for Increasing Concentrations (Control, .001nM, .01nM and .1nM)

Sample 1- Sample 2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig.
Control, 0.00nM-.001nM	-379.106	62.161	-6.099	0	**0***
Control, 0.00nM-.01nM	-495.672	61.696	-8.034	0	**0***
Control, 0.00nM-.1nM	-501.868	61.189	-8.202	0	**0***
.001nM -.1nM	-116.564	37.15	-3.138	0	**0***
.001nM-.01nM	-122.76	36.303	-3.381	0	**0***
.01nM-.1nM	6.196	35.501	0.175	0.861	1

Note. Control = Unexposed, nM = nanomolar, Sig. = P value, asymptotic, 2-tailed, Adj. Sig. = Adjusted P-values, 2-tailed, * and boldface numbers denotes significance (p = <.01), Comparisons of samples with increasing concentrations are marked in bold. Each row tests the null hypothesis that the distribution of *Her2* absolute values for Samples 1 & 2 is similar.

The box plot (Figure 6) below further displays the differences in *Her2* copy number expression observed between the different concentrations and the control.

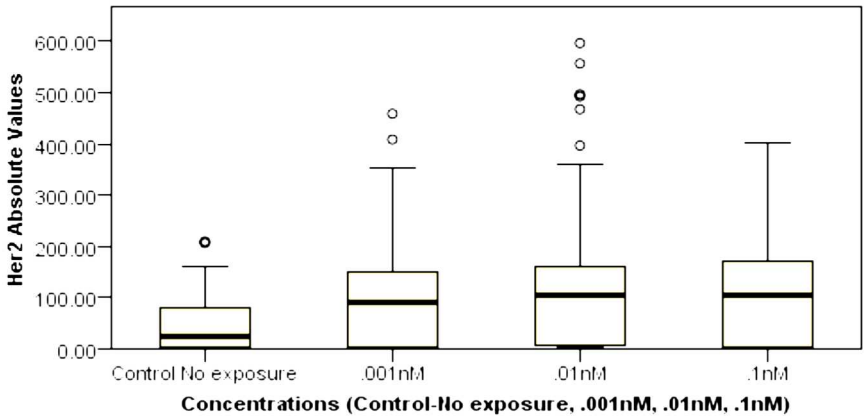


Figure 6. Box plots of *Her2* copy numbers with different concentrations. The concentrations used are .001, .01 and .1nM. The control group remained unexposed. Differences between each concentration when compared to the control is significant (p = <.01). There is also a significant increase in the *Her2* copy number between .001nM and .01nM (nanomolar) concentrations, however, this increase becomes non-significant (p = >.01) with the further increase in concentration from .01nM to .1nM.

To further explore if the above noted concentration gradient existed in every cell line, Mann Whitney tests were performed on each line using a similar gradient (i.e., Control to .001nM, .001nM to .01nM and .01nM to .1nM). It was noted that although for lines BT474 and MCF7 significant increase in *Her2* copy numbers does cap off at .01nM (p = >.01, single-tailed), this was not so for lines MDA-MB-231 and SKBR3. In both, MDA-MB-231 and SKBR3 lines the *Her2* copy numbers increase significantly (p = <.01, single-tailed) even from .01nM to .1nM concentrations of the xenoestrogenic application. Another variation noted in cell line MDA-MB-231 was that the *Her2* copy numbers do not significantly increase (p = >.01, single-tailed) between the Control and .001nM concentrations of xenoestrogenic exposures. These results are represented in Table 15.

Table 15

Mann Whitney Results for Increasing Concentrations of Xenoestrogen Application for Individual Line

Cell Line & Concentration	Receptors	N	Mean Rank	Sum of Ranks	MWU	Wilcoxon	Z	P (2-tailed)
BT474, Control	ER+/*Her2*+	40	59.39	2375.5	1555.5	2375.5	-6.1	**0.000***
BT474 .001nM		200	132.72	26544.5				
Total		240						
BT474 .001nM		200	172.47	34493.5	14393.5	34493.5	-4.85	**.000***
BT474 .01nM		200	228.53	45706.5				
Total		400						
BT474 .01nM		200	181.19	36238.5	16138.5	36238.5	-1.741	0.082
BT474 .1nM		180	200.84	36151.5				
Total							380	
MCF7, Control	ER+/*Her2*-	40	82.75	3310	2490	3310	-3.927	**.000***
MCF7 .001nM		200	128.05	25610				
Total		240						
MCF7 .001nM		200	185.92	37184	17084	37184	-2.602	**.009***
MCF7 .01nM		200	215.08	43016				
Total		400						
MCF7 .01nM		200	191.67	38333	18233	38333	-1.56	0.119
MCF7 .1nM		200	209.34	41867				
Total		400						
MDA-MB-231, Control	ER-/*Her2*-	40	102.24	4089.5	3269.5	4089.5	-1.91	0.056
MDA-MB-231 .001nM		200	124.15	24830.5				
Total		240						
MDA-MB-231 .001nM		200	185.04	37007.5	16907.5	37007.5	-2.768	**.006***
MDA-MB-231 .01nM		200	215.96	43192.5				
Total		400						
MDA-MB-231 .01nM		200	177.17	35433.5	15333.5	35433.5	-4.092	**.000***
MDA-MB-231 .1nM		200	223.83	44766.5				
Total		400						

(Table continues)

Cell Line & Concentration	Receptors	N	Mean Rank	Sum of Ranks	MWU	Wilcoxon	Z	P (2-tailed)
SKBR3, Control	ER-/*Her2*+	40	88.11	3524.5	2704.5	3524.5	-3.232	**0.001***
SKBR3 .001nM		200	126.98	25395.5				
Total		240						
SKBR3 .001nM		200	159.4	31879.5	11779.5	31879.5	-5.819	**.000***
SKBR3 .01nM		180	225.06	40510.5				
Total		380						
SKBR3 .01nM		180	155.61	28010	11720	28010	-4.538	**.000***
SKBR3 .1nM		180	205.39	36970				
Total		360						

Note. Control = No exposure, nM = nanomolar, N = number of nuclei scored, MWU = Mann Whitney U statistic, ER+/ER- = ER positive or negative respectively, *Her2*+/ *Her2*- = *Her2* positive and negative respectively, P = asymptotic and 2- tailed, * and boldface numbers denote significant values ($p = \leq .01$, 1-tailed).

Additionally, the incidence rate ratios (IRR) were analyzed using the negative binomial on the *Her2* count data, since the variances were larger than the mean (Ngatchou-Wandji and Paris, 2011). The control (No treatment) group was used as the reference (1.00). The results of IRR performed showed that the incidence of *Her2* copy number increase was 25% (IRR: 1.25, 95% CI = 1.13 to 1.38, $p = .000$), 58% (IRR: 1.58, 95% CI = 1.43 to 1.75, $p = .000$), and 96% (IRR: 1.96, 95% CI = 1.77 to 2.17, $p = .000$) more between the control and .001nM, .01nM, and .1nM concentrations of xenoestrogenic exposures respectively. These results showed that there was a steady increase in the *Her2* copy numbers from the lowest concentration of .001nM to .01nM and the highest concentration of .1nM of exposure concentrations when compared to the control group. The Null was thus rejected. These results are found in Table 16.

Table 16

Incidence Rate Ratios for Differential Concentrations of Xenoestrogens Compared to Control

Xenoestrogen Concentrations	Est.	Std. Err	Z	P-value	IRR	95% CI	
						LCI	UCI
No Treatment (Reference)					1.00		
(Dose)-0.001nM	0.22	0.05	4.29	**0.000***	1.25	1.13	1.38
(Dose)-0.01nM	0.46	0.05	8.80	**0.000***	1.58	1.43	1.75
(Dose)-0.1nM	0.67	0.05	13.01	**0.000***	1.96	1.77	2.17

Note. CI = confidence interval, % = percent, Z = z value, nM = nanomolar, Est. = estimated, LCI = lower confidence interval, UCI = upper confidence interval, Std. Err = standard error, ***** and boldface represent significant values (p = <.01), Control (unexposed) group was used as the reference. Scaled at 1.00

Hypothesis 2

Research question #2) Do the concentration at which the increments in *Her2* copy numbers become significant vary between the four xenoestrogens?

Null ($H_0$2): A significant increase in *Her2* copy numbers occurred at similar concentration levels for all four xenoestrogens (BPA, NPH, DDT, and Estrogen).

Alternate ($H_1$2): A significant increase in *Her2* copy numbers occurred at different concentration levels for all xenoestrogens.

The second hypothesis predicted that *Her2* copy numbers will increase significantly with similar concentrations of the four xenoestrogens applied. To examine this, I performed the Mann Whitney U (MWU) test to individually assess each xenoestrogen (BPA, DDT, EE, and NPH) applied at various concentrations applied (.001, .01 and .1nM) and compared to the control group for significance. The dependent

variable was *Her2* copies and the predictor variables were xenoestrogens at the different concentrations. The results of this test showed that for each category of xenoestrogen (BPA, DDT, EE, and NPH) there was a significant increase ($p = <.01$, single-tailed) in *Her2* copy numbers at the lowest concentration (.001nM) for all of the 4 xenoestrogens, applied individually or in combination. Because significant increases in *Her2* copy numbers were found to be at the same concentration (.001nM) for all categories of xenoestrogens applied, the null hypothesis ($H_0 2$) was accepted.

Although, significant increase ($p = <.01$, single-tailed) was observed for all 4 of the commonly used xenoestrogens at the lowest concentration (.001nM) of application, but interestingly in the case of BPA, the increase of *Her2* copy numbers for exposures at .01nM concentrations were not significant ($p = .018$, single-tailed or $p = >.01$). However, with the further increase in concentration (.1nM) the increase in *Her2* copy numbers did become significant once again ($p = <.01$, single-tailed). Table 17 represents the results obtained from the Mann Whitney U test ranks and test statistics respectively.

Table 17

Mann Whitney Results for Xenoestrogen/s Applied at .001, .01 and .1nM Concentrations and Controls

Xenoestrogen & Control	Concentration (nM)	N	Mean Rank	Sum of Ranks	MWU	Wilcoxon	Z	P (2-tailed)
BPA	0.001	160	174.43	27909.5	10570.5	23450.5	-2.708	**0.007***
Control	No exposure	160	146.57	23450.5				
Total		320						
BPA	0.01	140	161.64	22629	9641	22521	-2.09	0.037
Control	No exposure	160	140.76	22521				
Total		300						
BPA	0.1	140	164.58	23041.5	9228.5	22108.5	-2.645	**0.008***
Control	No exposure	160	138.18	22108.5				
Total		300						
DDT	0.001	160	175.39	28062	10418	23298	-2.891	**0.004***
Control	No exposure	160	145.61	23298				
Total		320						
DDT	0.01	160	180.8	28928.5	9551.5	22431.5	-3.942	**0.000***
Control	No exposure	160	140.2	22431.5				
Total		320						
DDT	0.1	160	188.24	30118	8362	21242	-5.374	**0.000***
Control	No exposure	160	132.76	21242				
Total		320						
EE	0.001	160	172.94	27670.5	10809.5	23689.5	-2.424	**0.015***
Control	No exposure	160	148.06	23689.5				
Total		320						
EE	0.01	160	181.17	28986.5	9493.5	22373.5	-4.016	**0.000***
Control	No exposure	160	139.83	22373.5				
Total		320						
EE	0.1	160	186.2	29792	8688	21568	-4.983	**0.000***
Control	No exposure	160	134.8	21568				
Total		320						

(table continues)

Xenoestrogen & Control	Concentration (nM)	N	Mean Rank	Sum of Ranks	MWU	Wilcoxon	Z	P (2-tailed)
NPH	0.001	160	172.39	27582	10898	23778	-2314	**0.021***
Control	No exposure	160	148.61	23778				
Total		320						
NPH	0.01	160	180.11	28817.5	9662.5	22542.5	-3.816	**0.000***
Control	No exposure	160	140.89	22542.5				
Total		320						
NPH	0.1	160	185.86	29737.5	8742.5	21622.5	-4.922	**0.000***
Control	No exposure	160	135.14	21622.5				
Total		320						
Combined	0.001	160	176.05	28168.5	10311.5	23191.5	-3.02	**0.003***
Control	No exposure	160	144.95	23191.5				
Total		320						
Combined	0.01	160	185.1	29616.5	8863.5	21743.5	-4.77	**0.000***
Control	No exposure	160	135.9	21743.5				
Total		320						
Combined	0.1	140	170.51	23871.5	8398.5	21278.5	-3.75	**0.000***
Control	No exposure	160	132.99	21278.5				
Total		300						

Note. N = total nuclei scored, MWU = Mann Whitney U statistic, P = asymptotic and 2-tailed, Total = sum of nuclei scored for the xenoestrogen (applied individually or in combination) and its control, ***** and boldface numbers denote significant values (p = <.01, single-tailed)

Hypothesis 3

Research question #3) Overall, is there a significant increase in the *Her2* gene copies between short-term (5 days) and persistent/long-term (50 days) exposures to the xenoestrogens?

Null ($H_0$3): No significant increase will be found in *Her2* copy numbers between the short and long term applications of xenoestrogens.

Alternate ($H_1$3): A significant difference in *Her2* copy numbers will be found between short and long term xenoestrogenic exposures.

The third hypothesis predicted that there would be a significant increase in *Her2* copy numbers between xenoestrogens applied for a single, short-term (5 days) exposure when compared to persistent, and long-term (50 days) applications. To test this hypothesis, I conducted the Mann Whitney U analysis for both durations of xenoestrogenic application (single, short-term and persistent, long-term) as the predictor/independent variables and *Her2* copy number as the dependent variable. The results of the analysis indicated that a significant increase of *Her2* copy numbers had occurred with the increasing durations of xenoestrogenic exposures (p = .000). Also, since the Mann Whitney U test results are two-tailed, thus the significance values for a single-tailed experiment can be divided by two. This would make the p-value even lower than .000 (p = <.000). Thus, the alternate hypothesis ($H_1 3$) was accepted. The results are presented in Table 18, and Figure 7 shows the representative box plot.

Table 18

Mann Whitney U Results of Her2 Copies for Short & Long-term Xenoestrogenic Exposures

Exposure Type & Duration	N	Mean Rank	Sum of Ranks	MWU	Wilcoxon	Z	P (2-tailed)
Single, short-term (5 days)	1240	1083.25	1343235.5	573815.5	1343235.5	-11.528	**.000***
Persistent, long-term (50 days)	1260	1415.09	1783014.5				
Total	2500						

Note. N = number of nuclei scored, MWU = Mann Whitney U statistic, P = asymptotic and 2-tailed, * and boldface numbers denote significant value (p = <.01).

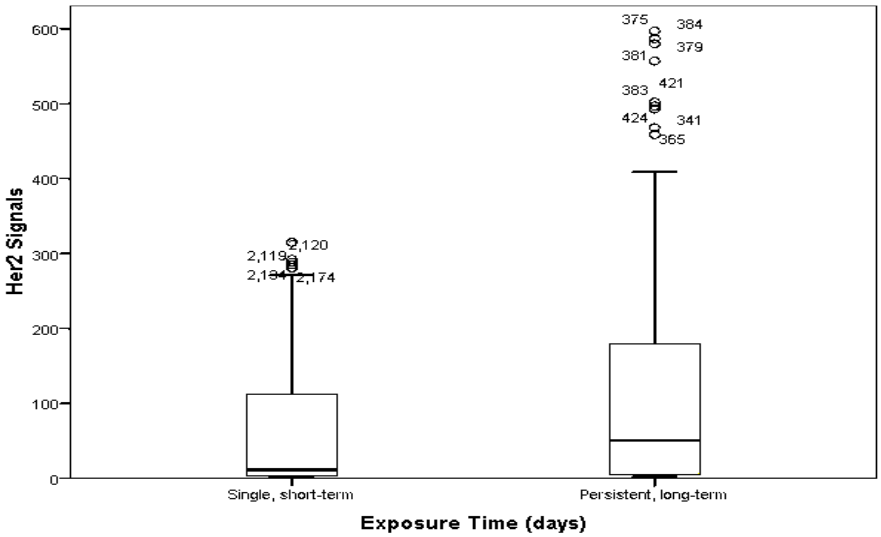


Figure 7. Box plot of *Her2* copies with short-term and long-term exposures. Significant (p = <.000) in *Her2* copies were observed with persistent, long-term (exposed daily for 50 days) when compared to single, short-term exposures (cultured for 5 days) with BPA, DDT, EE and NPH.

Incident rate ratios (IRR) of *Her2* copy numbers between single, short-term and persistent, long-term exposures were also calculated using negative binomial (Ngatchou-Wandji & Paris, 2011). Single, short-term exposure was used as the reference. These results found an 86% increase (IRR: 1.86, LCI = 1.78 and UCI = 1.94, p = .000*) in the incidence of *Her2* copy numbers with persistent, long-term exposures compared to single, short-term exposure durations.

To further explore whether the increase in *Her2* copy numbers occurs in some or all of the four xenoestrogens with their application duration, I conducted the Mann Whitney U test for each individual xenoestrogen that compared the *Her2* copy numbers between single, short-term exposure duration and persistent, long-term exposure duration. The results of the Mann Whitney U tests for each of the 4 commonly used xenoestrogens further showed a significant increase in the *Her2* copy numbers with increasing duration

of their use (p = .000). Thus, the alternate hypothesis ($H_1$3) was accepted for each individual xenoestrogen. The results of this research inquiry are presented in Table 19.

Table 19

Mann Whitney U Results of Her2 Copy Numbers for Single vs. Persistent Exposures of the Four Commonly Used Xenoestrogens

Xenoestrogen & Exposures	N	Mean Rank	Sum of Ranks	MWU	Wilcoxon W	Z	P (2-tailed)
BPA, single exposure	200	165.71	33142.5	13042.5	33142.5	-8.284	**0.000***
BPA, persistent exposures	240	266.16	63877.5				
Total	440						
DDT, single exposure	240	206.47	49552.5	20632.5	49552.5	-5.384	**0.000***
DDT, persistent exposures	240	274.53	65887.5				
Total	480						
EE, single exposure	240	218.16	52358	23438	52358	-3.542	**0.000***
EE, persistent exposures	240	262.84	63082				
Total	480						
NPH, single exposure	240	217.02	52084	23164	52084	-3.727	**0.000***
NPH, persistent exposures	240	263.98	63356				
Total	480						
Combinatorial, single exposure	240	207.11	49706.5	20786.5	49706.5	-3.948	**0.000***
Combinatorial, persistent exposures	220	256.02	56323.5				
Total	460						

Note. N = number of nuclei scored, Single exposure = cells were exposed to xeneoestrogen/s once and harvested on 5th day, Persistent exposure = cells were exposed daily with xenoestrogen/s and harvested on the 50th day, MWU = Mann Whitney U statistic, P = asymptotic and 2-tailed, ***** and boldface numbers denotes significant values ($p = \leq .01$).

Hypothesis 4

Research question # 4) Overall, does *Her2* gene expression vary significantly with each specific receptor type (i.e., cell line) upon exposure to xenoestrogens?

Outcome/Dependent Variable: *Her2* gene copy numbers

Predictor/Independent Variable: Receptor types/Cell lines (ER+/*Her2*+, ER-/*Her2*-, ER+/*Her2*-, and ER-/*Her2*+) and Exposure durations (single, short-term vs. multiple, long-term).

Null ($H_0$4): *Her2* copy numbers will not vary significantly between the different receptor types/cell lines upon exposure to xenoestrogens.

Alternate ($H_1$4): A significant difference will be found in the *Her2* copy numbers between the different receptor types/cell lines upon exposure to xenoestrogens.

The fourth hypothesis predicted that the magnitude or amount increase of the *Her2* copy numbers noted between the different receptor types found in the 4 lines when they are exposed to xenoestrogens would vary significantly. To test this hypothesis, I performed the Kruskal Wallis test using the 4 cell lines as the predictor/independent variables and *Her2* copy number as the outcome/dependent variable. These results were found to be significant for the receptor types found in the four cell lines (p = .000). The results from the Kruskal Wallis omnibus are represented below in Table 20.

Table 20

Kruskal Wallis Test for *Her2* Signals and Different Receptors

Cell Line	Receptors	N	Mean Rank	Chi-Square	df	P (2-tailed)
BT474	(ER+/Her2+)	620	1894.38	1888.416	3	**0.000***
MCF7	(ER+/*Her2*-)	640	578.08			
MDA-MB-231	(ER-/*Her2*-)	640	705.03			
SKBR3	(ER-/*Her2*+)	600	1884.23			
Total		2500				

Note: N = number of nuclei scored, df = degrees of freedom, P = asymptotic and 2-tailed, * and boldface numbers denote significant values (p = <.01).

Because the results of the Kruskal-Wallis omnibus were significant, post-hoc pairwise comparisons were conducted to check for similarities in *Her2* copy numbers between the different receptor types. The pairwise analyses found significant differences (adjusted $p = \leq .005$ for one-sided test, or $p = <.01$) for *Her2* copy numbers increments observed between all of the different pairs of receptor types found in the various lines, except for ER-/*Her2*+ (SKBR3) and ER+/*Her2*+ (BT474) lines where the distribution of *Her2* copies were similar (adjusted $p = .5$ for one-sided test, or $p = >.01$). The results from the post-hoc pairwise analysis for this hypothesis are represented below in Table 21.

Table 21

Post-hoc Pairwise Comparison of the Different Receptor Types and *Her2* Signals

Sample 1 - Sample 2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig.
ER+/*Her2*- and ER-/*Her2*- (MCF7 – MDA-MB-231)	-126.95	40.226	-3.156	0.002	**0.010***
ER+/*Her2*- and ER-/*Her2*+ (MCF7 – SKBR3)	-1,306.15	40.891	-31.943	0	**0.000***
ER+/*Her2*- and ER+/*Her2*+ (MCF7 – BT474)	1,316.30	40.549	32.462	0	**0.000***
ER-/*Her2*- and ER-/*Her2*+ (MDA-MB-231 – SKBR3)	-1,179.20	40.891	-28.838	0	**0.000***
ER-/*Her2*- and ER+/*Her2*+ (MDA-MB-231 – BT474)	1,189.35	40.549	29.331	0	**0.000***
ER-/*Her2*+ and ER+/*Her2*+ (SKBR3 – BT474)	10.152	41.208	0.246	0.805	1

Note: Sig = P-value, asymptotic significance (2-tailed), Std. Error = standard error, Adj. Sig = Adjusted P-values (2-tailed), * and boldface numbers denotes significant values ($p = \leq .01$ of adjusted significance).
Each row of the table tests the hypothesis that the distributions of sample 1 and 2 are similar.

To further elucidate the magnitude of increase found in the different receptor types, the IRRs were examined using the negative binomial model. Since ER+/*Her2*- (MCF7) line showed the lowest increase in its *Her2* copy number changes with exposures (single and persistent) to the various xenoestrogens; it was used as the reference line (1.00). The IRR results indicated that the incident rates of *Her2* copy number increase for both of the *Her2* positive lines regardless of their ER receptor status (BT474: ER+/*Her2*+ and SKBR3: ER-/*Her2*+) is 30 times greater than the reference which was *Her2* negative (95% CI, BT474: 28.81 to 32.59, and SKBR3: 27.88 to 31.58, $p = .000$). Additionally, the incidence rates of *Her2* for the line negative (or normal) for both ER and *Her2* receptors (MDA-MB-231: ER-/*Her2*-), showed a 44% increase (IRR: 1.44, 95% CI = 1.35 to 1.54, $p = .000$) in its *Her2* copy numbers when compared to the reference line. Because significant differences found were significant for all receptor types, the null ($H_0 4$) was rejected. The IRR results are represented in Table 22.

Table 22

Incidence Rate Ratios (IRR) of Her2 Copy Numbers between the Receptor Types

Receptor Type (Cell Line)	Estimate	Std. Error	Z-value	P-value	IRR	95% CI	
						LCI	UCI
ER+/*Her2*- (MCF7) (Reference)					1.00		
ER-/*Her2*+ (SKBR3)	3.39	0.03	107.42	**0.00000***	29.67	27.88	31.58
ER+/*Her2*+ (BT474)	3.42	0.03	108.99	**0.00000***	30.64	28.81	32.59
ER-/*Her2*- (MDA-MB-231)	0.37	0.03	10.68	**0.00000***	1.44	1.35	1.54

Note. CI = confidence interval, LCI = lower confidence interval, UCI = upper confidence interval, * and boldface denotes significant values (p = <.01).
MCF7 line was used as the reference line (1.00), because the lowest levels of *Her2* increments with single and persistent exposures to xenoestrogens were noted in this cell line.

To check whether the *Her2* gene copy numbers increased significantly for each line with increasing exposures durations to the 4 commonly used xenoestrogens, comparisons between the single vs. persistent exposures were made for individual lines using the Mann Whitney U test. Highly significant results ($p = <.01$) were observed between the single vs. persistent exposures for all 4 lines, hence the alternate ($H_1 4$) was accepted for all of the lines in this case. The results of the Mann Whitney U tests conducted for this hypothesis are displayed in Table 23 below.

Table 23

Comparison of Her2 Absolute Values for Single vs. Persistent Exposures in Individual Line

Cell Line and Exposures	Receptor Status	N	Mean Rank	Sum of Ranks	Mann Whitney U	Wilcoxon W	Z	P (2-tailed)
BT474, Single	ER+/*Her2*+	300	174.59	52377.5	7227.5	52377.5	-17.244	**0.000***
BT474, Persistent		280	414.69	116112.5				
Total		580						
MCF7, Single	ER+/*Her2*-	300	205.25	61574	16424	61574	-13.812	**0.000***
MCF7, Persistent		300	395.75	118726				
Total		600						
MDA-MB-231, Single	ER-/*Her2*-	300	256.35	76904	31754	76904	-6.37	**0.000***
MDA-MB-231, Persistent		300	344.65	103396				
Total		600						
SKBR3, Single	ER-/*Her2*+	260	235.79	61304.5	27374.5	61304.5	-6.088	**0.000***
SKBR3, Persistent		300	319.25	95775.5				
Total		560						

Note. N = total nuclei scored, Single = cells exposed to xeneoestrogen/s once and harvested on 5th day, Persistent = cells exposed daily with xenoestrogen/s and harvested on the 50th day, ER+ = Estrogen receptor positive, ER- = Estrogen receptor negative, Her2+ = Her2 receptor positive, Her2- = Her2 receptor negative, P = asymptotic and 2-tailed, * and boldface numbers denotes significant values ($p = \leq.01$).

Summary

Sample characteristics and data distribution using histograms, Q-Q plots and Shapiro-Wilk's tests of normality all showed non-normal distribution of the dependent variable (i.e., *Her2* signals) for all four cell lines. The data were also found to be highly dispersed, leading to a greater standard deviation (SD) than the mean values in two of the cell lines (MCF7 and MDA-MB-231). A Levene's test was conducted that further evaluated the similarity in the variances of the four lines which was non-significant. This meant that the differences in the variances between the four lines were similar. Since the assumptions for Kruskal Wallis analysis were cleared with the non-significant result of Levene's test, inferential statistics using this test were then performed.

The inferential analysis of this research study data supports hypothesis 3 and 4. Significant increase in the *Her2* copies were incurred with multiple, persistent exposures consisting of daily exposures of the xenoestrogens for 7 weeks compared to the single, short-term exposure cultured for five days. This held true for each categorical xenoestrogen (BPA, DDT, EE and NPH) applied individually or in combination, and for each receptor type (ER and *Her2* positive and negative). The various receptor types were found to be significantly different in their responses to the xenoestrogens and they were all found to incur significant increases in their *Her2* values compared to the reference line (MCF7, ER+/*Her2*-) that had the least amount of *Her2* copy number gain.

For hypothesis 1, a similar patterns of *Her2* copy number gains were noted (i.e., from control (unexposed) to .001nM to .01nM) with both the cell lines with ER positive status (BT474, ER+/*Her2*+; and MCF7, ER+/*Her2*-) as was found with the overall

exposures for all 4 lines. However, the patterns were dissimilar with respect to the other two lines. Here, one line (SKBR3, ER-/*Her2*+), the alternate was accepted, as in this line the *Her2* copy numbers did increase significantly (p = .000) with the control and each $\log_{10}$ increase in concentration (i.e., control to .001nM to .01nM to .1nM); whereas the other line (MDA-MB-231, ER-/*Her2*-) did show a significant increase in *Her2* copies (p = .000) between each categorical exposure concentration (i.e., .001nM to .01nM to .1nM), but a significant gain in *Her2* copy numbers did not occur between the control (unexposed) and .001nM exposure concentration. The alternate was accepted for line SKBR3 (ER-/*Her2*+) for all concentration gradients. For lines BT474 and MCF7, the alternate was accepted for concentration increase from control (unexposed) to .001nM to .01nM of exposures. Lastly, for MDA-MB-231 (ER-/*Her2*-) line, the alternate was accepted for concentration increase from .001nM to .01nM to .1nM of exposures.

With regards to hypothesis 2, although the alternate hypothesis supported a significant increase in the *Her2* copy numbers with dissimilar concentrations of the various xenoestrogens due to their disparate nature, but interestingly all four of the xenoestrogens studied (BPA, DDT, EE and NPH) were observed to significantly increase in their *Her2* values at the lowest nanomolar concentration of .001nM. This also held true for individual as well as combinatorial exposures. Thus, the null hypothesis was accepted in this case.

The following chapter has summarized the study, discussed social change implications of the study findings; presented the limitations of the study, pointed out future perspectives, and presented concluding remarks.

Chapter 5: Discussion, Conclusions, and Recommendations

Introduction

Xenoestrogens are substances with estrogenic properties, and repeated exposures with synthetic xenoestyrogens could chemically modulate the promotion and progression of breast cancer (Aube, Larochelle, & Ayotte, 2013; Brody & Rudel, 2003; Valeron, Pestano, Luzardo, Zumbado, & Boada, 2009). *Her2*, a proto-oncogene, is found in two copies in a normal mammary cell and is required for its normal development and function. However, it can mutate by amplification and become oncogenic (i.e., cancer-causing gene). Also for the normal functioning of the mammary cell, cross-communications occur between the ER and *Her2* receptors at the cell's surface, which further activates the *Her2* gene within the cell's nucleus (*Her2* gene expression) (Stoica et al., 2003; Yang, Barnes, & Kumar, 2004), and the nuclear ER (Jung et al., 2010; Montemurro, Di Cosimo, & Arpino, 2013). Hence, the perturbations of the ER with repeated extrinsic xenoestrogenic exposures could perturb the *Her2* proto-oncogene, thereby converting it to an oncogene. Animal models have demonstrated a connection between xenoestrogenic exposures and *Her2* gene activity; however, the carcinogenic potential influencing the expression of the *Her2* gene upon exposures to commonly used xenoestrogens has not been systematically examined.

Using molecular genetics techniques (FISH) with a case-control study design, this study assessed *Her2* gene expression with differential exposures to 4 commonly used household xenoestrogens (BPA, DDT, EE, and NPH) using 4 human breast cancer lines that were ER- and *Her2*-positive or -negative providing mechanistic insights to the

carcinogenicity of these xenoestrogens and evaluated their carcinogenic potential. Additionally, the study discerned cellular phenotypes more susceptible to aggressive disease with exposures to these xenoestrogens.

Summary and Interpretation of Findings

In a study conducted by Calfat et al. (2005) on 1,000 participants, the authors observed that 95% and 51% had more than 0.1microgram/Liter (µg /L) urine concentrations of BPA and NPH respectively, indicating that people are being exposed to at least 0.1 µg/L concentrations of BPA and NPH. In another study by Calafat et al. (2008) consisting of over 2,500 Americans, BPA concentrations ranging from 0.4 to 149 µg/L (mean=2.6 µg/L) were found in 92.6% of the study participants. The average levels of total BPA in male and female urine was 1.63 and 1.12 ng/ml (nanograms/milliliter) respectively. Additionally, Vandenburg, Maffini, Sonnenschein, & Soto (2009) estimated that adult human exposures to BPA ranged from <1 µg/Kg/day to 5 µg/Kg/day. Collectively, these data showed that human exposures to BPA ranged anywhere from 1.12 ng/ml to 5 µg /Kg/day.

In the current study, I applied xeneoestrogens in increasing $\log_{10}$ ratios of nanomolar concentrations (i.e., .001nM, .01nM, and .1nM), and observed that even the lowest concentrations (.001nM) of xenestrogenic applications significantly increased the *Her2* copy numbers when compared to the control (p = .000). Ad hoc pairwise comparisons found significant increase (p = .000) in every concentration category applied (.001nM, .01nM, and .1nM), when compared to the control group.

The incidence rate ratio (IRR) values further showed that the *Her2* copy number increase was almost twice as much (1.96, or 96% greater) with the applications of .1nM (highest) concentrations of xenoestrogen when compared to the control. The incidence of *Her2* increase was also found to have increased with increasing concentrations applied compared to the control. An increase of 25% (IRR: 1.25, $p = .000$) of *Her2* copy numbers was found in the lowest concentration of xenoestrogenic application, which steadily increased to 58% (IRR: 1.58, $p = .000$) at .01nM concentration of xenoestrogenic exposures, and capped off at 96% (1.96, $p = .000$) with the highest concentration (.1nM) of xenoestrogenic applications compared to the control. Comparing the three concentrations to the control group, there was a 33% increase (from 25% to 58%) in *Her2* copy numbers between .001 and .01nM concentrations, and a 38% increase (from 58% to 96%) observed in the *Her2* copies between .01 and .1nM concentrations of xenoestrogenic exposures. Thus, it can be inferred from Hypothesis 1 that *Her2* oncogene expression had increased even with nanomolar (nM) concentrations of xenoestrogenic applications compared to the control.

Interestingly, when comparing within the three different concentration groups, the pairwise comparisons found that that there was a significant increase ($p = .008$) in the *Her2* copy numbers ascending from .001nM to .01nM concentrations; however, a significant increase in the *Her2* copy numbers was not observed ($p > .05$) moving from .01nM to .1nM concentration. This further implied that the lowest concentrations could be more lethal in the case of *Her2* gene copy number gains and mutations of this gene.

Each line used in the study constituted of different types of receptors (ER and *Her2* positive or negative) and I further wanted to assess whether each line reacts differently to xenoestrogenic exposures. Thus, Hypothesis 1 also examined whether the increments in *Her2* copy numbers were similar or not for each receptor type with increasing concentrations of exposures to the 4 commonly used xenoestrogens. Interestingly, different patterns emerged for these lines. Both lines that were ER+ (i.e., BT474 and MCF7) showed a similar pattern as was observed with the overall concentrations; that is, *Her2* copies increased significantly until .01nM exposure concentrations to the 4 commonly used xenoestrogens and then they became non-significant (control to .001nM, and .001nM to .01nM, $p = .000$). However, for the lines that were ER- (i.e., MDA-MB-231 and SKBR3), the values in *Her2* copy numbers had increased significantly ($p = .000$) even from .01nM to .1nM concentrations of exposures to the 4 xenoestrogens. Also, for line MDA-MB-231, there was no significant increase in the *Her2* copy numbers found between the control and .001nM concentrations of exposures to the xenoestrogens.

Previous research has indicated that some xenoestrogens are slow-activators while others react quickly and are fast-activators of the cellular membrane (Bulaveya & Watson, 2004; Payne, Rajapakse, Wilkins, & Kortenkamp, 2000). Nuclear transcriptional assays performed that assessed their potency showed that some xenoestrogens were very weak (e.g., DDE), while others were somewhat weak (e.g., BPA), yet others were quite strong (e.g., DES) in their estrogenic activity (Silva, Scholze, & Kortenkamp, 2007). Together, these studies provided that even though xenoestrogens are categorically

grouped under one umbrella, they interacted differentially within biological systems. Also, because the biochemical nature of xenoestrogens is disparate, it follows that the carcinogenicity of xenoestrogens used individually as well as in combination may be quite different.

In this study, one way carcinogenicity of these four (i.e., BPA, DDT, EE, and NPH) commonly used household xenoestrogens was assessed was by examining the concentrations at which significant increase in *Her2* copy numbers initiated and when it plateaus off for each xenoestrogen. Keeping in mind lessons from the past research regarding the disparity of their biochemical nature, because some (e.g., DDT) are fast activators, whereas others (e.g., BPA and NPH) are moderate and slow activators respectively, it was proposed in Hypothesis 2 that the four xenoestrogens would significantly increase *Her2* copies at different concentrations of exposures when compared to the control. Further, there would be differences in concentrations at which individual vs. combinatorial exposures significantly increased in their *Her2* copy numbers.

Surprisingly, the Mann Whitney test statistics showed that not only did all four (BPA, DDT, EE, NPH) commonly used household xenoestrogens incurred significant (p = .000) increases in *Her2* copies at similar concentration, but this held true for individual as well as combinatorial xenoestrogenic exposures. Additionally, the significant increase in *Her2* copies was found at the lowest concentration (.001nM, p = .000) of application for each xenoestrogen, whether applied individually or in combination.

Studies conducted on xenoestrogens that assessed their carcinogenicity have been performed using cellular proliferation, reporter gene assays (estrogen and androgen receptor genes) and transcription arrays after short-term exposures (up to a week) with OCs and their derivatives (Aube, Larochelle, & Ayotte, 2011; Valeron Pestano, Luzardo, Zumbado, & Boada, 2009). However, breast cancer is observed to have a long latency period (Nadler & Zurbenko, 2013; Olsson, Baldetorp, Ferno, & Perfekt, 2003; Paez et al., 2012), but the aforementioned studies only studied short-term exposures ranging from 1 to 9 days.

In an effort to learn regarding the long-term effects of persistent applications of the 4 common household xenoestrogens on *Her2*, I subjected the 4 lines to 50 days of exposure with these xenoestrogens. Although the breast carcinogenesis latency period is anywhere from 20 to 30 years (Marsden, Wright, Carrier, Moroz, & Rowan, 2012; Nadler & Zurbenko, 2013), the breast cancer cell lines could only be treated for up to 50 days, because as per the ATCC culturing instructions, these cells may start to die after 8 weeks (56 days) of long-term culturing. Another caveat to long-term culturing of cells was the threat of losing all of the cells to contamination with various microbes (e.g., bacteria, mycoplasma, mold, yeasts, and viruses) (Life Technologies, n.d.). Due to these two reasons, for persistent, long-term exposures the four breast cancer cell lines were treated up to 50 days with the xenoestrogens. The results of the Mann Whitney test found that a significant increase did occur in the *Her2* copy numbers with persistent, long-term exposures (50 daily exposures) to the 4 xenoestrogens compared to the single, short-term (5 days) exposures ($p = <.000$).

Because highly significant values (p = <.000) were observed between the single, short-term exposures and persistent, long-term exposures, I next examined how much difference in the *Her2* copy number gains had occurred between these two exposure durations. Incidence rate ratios (IRR) were performed to compare differences in the magnitude of increase occurs between the single, short-term and persistent, long-term exposure durations. Almost twice as much (86% increase, IRR: 1.86, p = 0.000) *Her2* copy numbers were found with the persistent, long-term exposures with the 4 commonly used xenoestrogens compared to their single, short-term exposures. It can be inferred from this data that persistent low-level exposures occurring for even 7 weeks caused significant (p = .000) amounts of amplifications to the *Her2* oncogene. This finding is important since most women worldwide are being exposed on a daily basis to these common household xenoestrogens (Cohn, 2011; Darbre & Charles, 2010; Inifo-Nunez, Herreros, Eucinas, and Gonzalez-Bulnes, 2010; Kuch, Metzer, & von der Trenck, 2010; Qui, Suri, Bi, Sheng, & Fu, 2010).

Individual xenoestrogens have disparate characteristics even though they all have estrogenic properties (Payne, Rajapakse, Wilkins, & Kortenkamp, 2000; Silva, Scholze, & Kortenkamp, 2007; Aube, Larochelle, & Ayotte, 2008), therefore Hypothesis 3 of this study further classified whether the differences in single vs. persistent exposures significantly increased *Her2* copies in some or all of the 4 commonly used xenoestrogens. Mann Whitney tests were conducted with each xenoestrogen applied individually or in combinatorial exposures compared the *Her2* copy numbers between single, short-term (single exposure cultured for 5 days) and multiple, persistent exposures (daily exposures

cultured for 50 days). The results revealed that multiple, persistent exposures to all 4 commonly used xenoestrogens (BPA, DDT, EE and NPH) regardless of whether they were applied individually or in combination had significantly ($p = .000$) increased the *Her2* copy numbers when compared to single, short-term exposures.

A sentinel population study by Gammon et al. (1999) has indicated that exposures to contraceptive pills lead to aggressive breast cancer and that these women had an increased *Her2* receptor status. Additionally, patient data also demonstrated that when there is amplification of the *Her2* oncogene, then the patient prognosis related to a more aggressive type of breast cancer with disease progression, tumor invasion, fewer disease-free days, and worse survival outcomes lending to its poor prognostic value (Baselga & Swain, 2009; Gutierrez & Schiff, 2011; Johnson et al., 2012; Lindemann et al., 2007; Slamon et al., 2011).

The 4 cell lines used in this study were ER and *Her2* positive or negative. Pairwise analysis conducted on all of the different receptor types showed that the distribution of *Her2* copy numbers were significantly different for all of the lines except those that already had a *Her2* positive status (BT474 with ER+/*Her2*+ status, and SKBR3 with ER-/*Her2*+ status). Comparing the 4 lines, it was noted that MCF7 line with ER+/*Her2*- receptors had the least amount of *Her2* copy number increase, and was used as the reference. Both of the *Her2*+ lines regardless of the status of the ER receptor (BT474 and SKBR3) showed a 30 times greater *Her2* copy number increment compared to the reference, implying that women with a *Her2* positive status are highly susceptible to cancer progression with exposures to even nanomolar concentrations of these four

commonly used xenoestrogens. Importantly, even for the MDA-MB-231 line with ER-/*Her2*- status, it was observed that a significant increase of 44% in the *Her2* copy numbers (IRR: 1.44, $p = 0.000$) occurred when compared to the MCF7 line with ER+/*Her2*- status. Because the ER-/*Her2*- status is typically found on a normal mammary cell, this statistic can have important mechanistic implications, since it can be inferred from these results that the *Her2* negative expression had mutated via amplification and became *Her2* positive with exposures to nanomolar concentrations of the 4 commonly used xenoestrogens (BPA, DDT, EE, and NPH). Additionally, these results indicated that the increase in *Her2* oncogenic expression is multiplicative between the various receptors found in these 4 cell lines.

I also performed Mann Whitney analysis on each line (ER and *Her2* positive or negative), that compared the *Her2* copy numbers between single, short-term exposures (cultured for 5 days) with the multiple, persistent exposures (daily exposures for 50 days) for individual line. The increase in the *Her2* copy numbers was found to highly significant ($p = .000$) in each of the 4 lines regardless of their receptor status (i.e., ER and *Her2* positive or negative).

Breast carcinogenesis occurs with the activation of oncogenes or the inactivation of TSGs (Lee & Muller, 2010; Pitot & Dragon, 1993). Exposures to chemicals and hormones including xenoestrogens can trigger the activation of oncogenes (Brody, Tickner, & Rudel, 2005; Davis et al., 1993; Montemurro, Cosimo & Arpino, 2013) provided the theoretical construct of this study. All of the results of this study validated that exposing human mammary cells to the 4 commonly used xenoestrogens (BPA, DDT,

EE and NPH) leads to the mutation (i.e., copy number increase/amplification) of the *Her2* gene located on the long-arm of chromosome 17 in the human genome.

A pathway to breast carcinogenesis (Davis et al., 1993; Soto & Sonnenschein, 2010) hypothesized that xenoestrogens increased the estrogenicity in a mammary cell above normal levels and this led to mutation of genes located in 17q loci. Although, this study did not check for increases in the estrogen in mammary cells after exposing them to xenoestrogens used in the study, however, the results confirmed that xenoestrogenic exposures mutated the *Her2* gene located in 17q (17q11.2-17q12) via copy number gains/amplification.

Implications for Social Change

Breast cancer is the main cause of death for women worldwide with mortality rates reaching 522,000 women in 2012 (Ferlay et al., 2014; IARC, 2013). It is the second leading cause of cancer-related deaths for women in the U.S. (ACS, 2015; SEER, n.d.). Currently, an estimated 2.9 million women are living with a history of breast cancer in the United States alone (ACS, 2014; SEER, n.d.). Breast cancer care ranks the highest amongst all cancer care expenditures, accounting for $18.1 billion of annual healthcare cost in the United States alone (NCI, 2015a).

Synthetic xenoestrogens are partially being blamed for increase in breast cancer incidence (Aube, Larochelle, & Ayotte, 2011; Buteau-Lozano, Velasco, Cristofari, Balaguer, & Perrot-Applanat, 2008; Davis et al., 1997). In the case of breast cancer, synthetic xenoestrogenic exposures are now being researched as potential risk factors, and are considered as a modifiable lifestyle factors (Aube, Larochelle, & Ayotte, 2011;

Rudel, Attfield, Schifano, & Brody, 2007). Synthetic xenoestrogens are found in commercial products, and are easily available (e.g., herbicides, plastics, pesticides, contraceptives) to women in all societies worldwide (Cohn, 2011; Darbre and Charles, 2010; Inifo-Nunez, Herreros, Eucinas, & Gonzalez-Bulnes, 2010; Kuch, Metzer, & von der Trenck, 2010; Qui, Suri, Bi, Sheng, & Fu, 2010; Vogel, 2009).

This research has provided a GEI model that predicts the carcinogenic potential of 4 commonly used synthetic xenoestrogens (BPA, DDT, EE, and NPH) and how exposures to these xenoestrogens affected the *Her2* oncogenic expression, a biomarker of breast carcinogenesis. Using précis dosage analysis, this study clearly demonstrated that exposures to all 4 xenoestrogens even at .001 nanomolar (nM) concentrations resulted in significantly increasing the *Her2* oncogenic expression, regardless of whether these 4 xenoestrogens were applied individually or in combination. Also, this study found that with the increase in the concentrations from .001 to .1nM, the incidence of *Her2* copy number increase is almost twice as much (96% increase), indicating that even low-level increase in concentrations of these 4 synthetic xenoestrogens greatly impacted the *Her2* oncogenic expression levels.

The study further indicated that highly significant increase in *Her2* copies occurred not only with daily, persistent (chronic exposures) to all 4 xenoestrogens (BPA, DDT, EE, NPH), but also with all 4 receptor types (ER and *Her2* positive or negative). Highly significant increments in *Her2* oncogenic expression was found to occur (86% more) with persistent exposures to the 4 synthetic xenoestrogens within 7 weeks compared to a single exposure for five days. Although, these values are a little less than

two fold, but these persistent exposures accounted for only 7 weeks; whereas due to their ease of availability most women worldwide are constantly being exposed to these commonly used xenoestrogens for all or most of their lives (Cohn, 2011; Darbre and Charles, 2010; Inifo-Nunez, Herreros, Eucinas, & Gonzalez-Bulnes, 2010; Kuch, Metzer, & von der Trenck, 2010; Qui, Suri, Bi, Sheng, & Fu, 2010; Vogel, 2009).

Additionally, this study provided direct biological evidence to the altercations of the *Her2* oncogene upon exposures with synthetic xenoestrogens. The study demonstrated that the *Her2* mutated by the amplification of its copy numbers with exposures to the 4 commonly used synthetic xenoestrogens. Taken together, these results established the 4 commonly used xenoestrogens as a risk factor for breast carcinogenesis, and this information should be useful in making policy level decisions to curtail the sale of these synthetic xenoestrogens and encourage the use of alternate chemicals. These results have provided valuable information for advocacy groups to educate and empower women regarding the ill health effects even with low-level exposures to these xenoestrogens.

Because most women around the world are constantly exposed to these synthetic xenoestrogens due to their omnipresence, modifying these risk factors should have a great public health impact even though they only account for low levels (less than twice) of risk (Aube, Larochelle, & Ayotte, 2011; Brody, Tickner, & Rudel, 2005; Johnson et al., 2012). Reducing these risk factors of breast cancer would not only affect women worldwide by reducing its incidence and mortality, but doing so would also translate in

reducing the indirect costs that the disease incurs on family and extended family members, such as lost work days and wages.

The cell line data showed that women with a *Her2* positive (*Her2*+) status are at a very high risk (~30 times greater than *Her2*- status) of cancer progression with nanomolar levels of exposures to these synthetic xenoestrogens. If these results are corroborated in human mammary tissue samples, these findings may have implications for women with breast cancer with a greater risk for disease progression, especially since added *Her2* amplifications does lead to a more aggressive disease type, with disease progression, poor prognosis, and lower survival rates (Baselga & Swain, 2009; Gutierrez & Schiff, 2011; Johnson et al., 2012; Lindemann et al., 2007; Slamon et al., 2011).

For women with breast cancer under the age of 40 (young women), the *Her2* expression (*Her2*+) has been found to be significantly higher compared to those that are 65 years or older (11.1 *vs.* 9.4 respectively; $p = .0001$) (Assi, Khoury, Dbouk, Khalil, Mouhieddine, & ElSaghir, 2013). An increased proportion of ER-/*HER2*+ with high tumor grade occur in younger women (Anders et al., Collins et al., 2012). Younger women with breast cancer also had higher mortality rates compared those older than 40 years (18.3% vs. 12.1% respectively, $p = .001$). Although women with breast cancer under the age of 40 years constituted a small proportion (worldwide 146,660 incident cases in 2008) of the total, this was a significant burden not only on women but society as well since they presented with a more aggressive disease (Assi et al., 2013). Azim et al. (2012) demonstrated that there is an age-related differential in the gene expression associated with MAPK and PI3K growth factor signaling found especially in younger

women, thus heightening their risk for aggressive breast cancer. Xenoestrogens and *Her2* are known to use these specific signaling pathways (Jung et al., 2010; Lemmon & Schlesinger, 2010; Serra et al., 2011). This research study has also demonstrated that the *Her2* oncogenic expression increased even with nanomolar exposures to commonly used xenoestrogens. Taken together, these results indicated that the risk associated for aggressive breast cancer with low-level exposures to xenoestrogens would be far greater for younger women compared to those that are over the age of 40 years. These findings have implications for targeted advocacy, monitoring and early intervention/s for women under the age of 40 years.

Importantly, this study demonstrated that even with ER and *Her2* negative status (MDA-MB-231 line) a significant increase (44% more) had occurred in their *Her2* copy numbers, marking a shift in their *Her*2 status from *Her2* negative to *Her2* positive (*Her2*+) at nanomolar levels of exposures and with increasing concentrations (i.e., .001nM to .01nM to .1nM) to commonly used synthetic xenoestrogens. Because, ER and *Her2* negative status is found in a normal mammary cell, these results indicate that even in a cell that has normal *Her2* gene expression, the expression can change or mutate via *Her2* gene amplification upon low-levels of exposures to these xenoestrogens and become *Her2* positive and oncogenic; which ultimately would lead to breast carcinogenesis. These findings can be generalized to all women worldwide since normal levels of *Her2* or *Her2* negative (*Her2*-) gene status is found in a normal mammary cell. These findings further reiterate that even at nanomolar levels of exposures of these 4 commonly used synthetic xenoestrogens are risk factors for breast carcinogenesis. These

findings further reiterated that even at nanomolar levels of exposures of these four commonly used synthetic xenoestrogens they are indeed risk factors for breast carcinogenesis, and that its population-attributable risk for breast cancer is significant. This information can be used by public health and policy-makers to impose policies that will curtail the ease of availability and use of these xenoestrogens. Additionally, breast cancer advocacy groups, cancer agencies, public health nurses, cancer forums and foundations should use this information to advocate, educate and inform women against the use of these commonly found xenoestrogens due to their imposed risks even when used in low concentration levels. Health educators should use this information to teach the general public; especially young girls and women about the health risks with exposures to these commonly used xenoestrogens, thereby making the consumers privy to this information so they can make healthier choices when buying substances that contain these xenoestrogens.

Limitations

Although this study provided précis measurements of each categorical xenoestrogen and the durations of their exposures, however, due to ethical reasons this study was conducted using breast cancer cell lines from ATCC, bioresource center. Therefore, this study did not have any data on other risk factors (e.g., genetic predisposition of BRCA1 and 2, parity, age at menarche, lactation, reproductive history, smoking, breast-feeding, diet, and alcohol) involved in breast carcinogenesis (Aube, Larochelle, & Ayotte, 2011; Buteau-Lozano, Velasco, Cristofari, Balaguer, & Perrot-Applanat, Davis & Sieber, 1997). Including all of these risk factors and studying how

they interacted with exposures to some of the commonly used xenoestrogens would further enhance our knowledge regarding risk factor assessment and interventions against this disease.

Only ER and *Her2* positive and negative cell lines were selected for studying the effects of these xenoestrogens, mainly because xenoestrogens are thought to exert their effects via the ER (Mercado-Feliciano & Bigsby, 2008; Stoica et al., 2003), then the ER cross-communicate with *Her2*, and these two receptors are known to be the main drivers of breast carcinogenesis (Gutierrez & Stoica, 2011). But, other receptors, such as the insulin growth factor receptors, progesterone receptors, and androgenic receptors are also found in the mammary cell. Thus, the generalizability of this study is limited to the interactions of ER and *Her2* receptors only with exposures to the 4 commonly used xenoestrogens.

Lastly, because of the threat of losing the cell lines to either contamination or cell death due to viability of cells in long-term culturing (ATCC; Gibco, Life Technologies), this study was only be conducted for 7 weeks in order to study the long-term effects of the 4 commonly used synthetic xenoestrogens. Because breast cancer has a long latency period that could last up to 28 years (Marsden, Wright, Carrier, Moroz, & Rowan, 2012; Nadler & Zurbenko, 2013; Paez et al., 2011), this study mimicked persistent long-term exposures by daily exposing the cell lines to the four commonly used xenoestrogens for 50 days and examine their effects on *Her2* gene expression.

Future Perspectives

Based on the findings of this study, and due to the high reproducibility of FISH, it would be beneficial to use FISH technology in the future to study direct gene expression on fresh tissue samples using a hospital-based case-control design to study the effects of commonly used xenoestrogens and incorporating other breast cancer risk factors (e.g., genetic predisposition, smoking, alcohol, diet, lactation, age at menarche). Here, the patient exposures to the xenoestrogens can be assessed using gas chromatography or mass spectrometry (Boada et al., 2012; Charlier et al., 2003; Cohn, Wolff, Cirillo, & Scholtz, 2007; Hoyer, Jorgensen, Grandjean, & Hartvig, 2000, Hoyer et al; 2000; Hoyer, Jorgensen, Rank, & Grandjean, 2001; Warner et al., 2002). Although *Her2* is an important biomarker of breast carcinogenesis, using FISH technology for future studies would allow the incorporation of other genes in conjunction with *Her2*, such as p53, a TSG also known to be involved in breast carcinogenesis and could be affected with exposures to xenoestrogens (Davis et al., 1993; Soto and Sonnenschein, 2010)).

Because breast cancer is the main cause of death for women worldwide (IARC, 2013), and it is the second leading cause of death in women in the United States (CDC, 2014), using the aforementioned study design to perform survival analysis will also aid in providing important data and insights regarding breast carcinogenesis and mortality due to exposures to these xenoestrogens.

Conclusion

Breast cancer is still a public health concern (Aube, Larochelle, & Ayotte, 2011; Valeron, Pestano, Luzardo, Zumbado, & Boada, 2009). This study has biological

underpinnings and provides mechanistic insights that exposures to the 4 commonly used xenoestrogens (BPA, DDT, EE and NPH) incur changes in the *Her2* oncogene, a biomarker of breast carcinogenesis and aggressive disease via its copy number increase/gene amplification.

All the results of this study provide direct evidence of changes incurred to the *Her2* oncogene even with low-levels (nanomolar concentrations) of exposures to all 4 xenoestrogens, and that *Her2* copies increase significantly with daily exposures within short time span of 7 weeks (persistent, long-term exposures). A normal mammary cell expresses only two copies of the *Her2* gene and is *Her2*- (Grusko et al., 2002). Importantly, this data also indicates that *Her2* negative mammary cells can become *Her2* positive with exposures to synthetic xenoestrogens. This phenomena was observed in the lines that were *Her2* negative (MDA-MB-231 and MCF7), but with xenoestrogenic exposures their *Her2* copy numbers increase significantly ($p = <.000$), thereby shifting their *Her2* status from Her2-negative to Her2-positive. As *Her2* negative (*Her2*-) status can be generalized to all women worldwide, this data should help regulatory agencies to recognize the potential risk posed by even the low-levels of exposures to these xenoestrogens and apply the precautionary principle, either by substituting these chemicals with others that are not harmful to health or banning their sale to curtail their usage.

The epidemiology of *Her2* suggests that an increased proportion of ER-/*Her2*+ breast cancer with high tumor grade is found in younger women (<40 years) compared to those that are older (Anders et al., Collins et al., 2012). This study also discerned that the

women with a *Her2* positive status are at the highest risk of disease progression to a more aggressive disease type with exposures to these xenoestrogens. Cancer advocacy groups, and public health nurses should inform and educate these women with the potential risks with exposures to these 4 commonly used xenoestrogens and breast cancer progression. Furthermore, because women with a *Her2* positive status are at the greatest risk of disease progression with exposures to these commonly used xenoestrogens, they should regularly be monitored for their *Her2* levels even if their *Her2*status changes with therapy.

In all, this research improved our understanding of the molecular mechanisms underlying breast cancer pathogenesis and progression with exposures to the 4 commonly used xenoestrogens. Future breast cancer prevention efforts should benefit from this study in the following ways; first, this study will bring about awareness of the importance of avoiding or reducing exposures to the 4 commonly used xenoestrogens, and; second, by increasing our understanding that some women due to their greater genetic based sensitivity would need increased medical surveillance in order to intervene before the occurrence of a more serious disease condition. Because these xenoestrogens are found all over the world and the two receptor types incorporated in the study design are common to all women around the globe, these findings would help not only our immediate community in the fight against breast cancer, but our global community as well.

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