

AN ANALYSIS OF ESTROGEN METABOLISM AND BREAST CANCER RISK

by

Stacy Monique Lloyd

BS, Prairie View A&M University, 2004

MPH, University of Pittsburgh, 2007

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This dissertation was presented

by

Stacy Monique Lloyd

It was defended on

June 30, 2009

and approved by

Committee Member:

Robert Ferrell, PhD, Professor
Department of Human Genetics
Graduate School of Public Health
University of Pittsburgh

Committee Member:

Susanne Gollin, PhD, Professor
Department of Human Genetics
Graduate School of Public Health
University of Pittsburgh

Committee Member:

Seymour Garte, PhD, Professor,
Department of Environmental and Occupational Health
Graduate School of Public Health
University of Pittsburgh

Committee Co-Chair:

Emanuela Taioli, MD, PhD, Professor
Department of Epidemiology
Graduate School of Public Health
University of Pittsburgh

Dissertation Advisor:

Eleanor Feingold, PhD, Professor
Department of Human Genetics
Graduate School of Public Health
University of Pittsburgh

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Breast cancer is the most common noncutaneous form of cancer among women in the US. In recent years, the overall mortality rate has declined, yet there still exists a significant racial disparity in the incidence and mortality between African American and Caucasian women. While numerous hypotheses have been proposed to explain this difference, few offer a biological explanation.

There is a well established association between estrogens and breast cancer risk, and the ratio of two estrogen metabolites, 2-hydroxyestrone (2OHE₁) and 16 α -hydroxyestrone (16OHE₁), has been implicated as a marker of breast cancer risk.

Many studies have also assessed the relationship between endogenous estrogens and mammographic density. Mammographic density is one of the strongest predictors of breast cancer risk, but the mechanism by which it influences this risk remains unknown. Nonetheless, few have examined mammographic density in relation to the 2OHE₁:16OHE₁ estrogen metabolite ratio (EMR).

Research suggests that the *Cytochrome P450 1B1 (CYP1B1)* gene may also mediate breast cancer risk, as this gene is very active in estrogen metabolism. In fact, the Leu432Val polymorphism has reportedly been associated with urinary levels of the 2OHE₁:16OHE₁ EMR.

The objective of this study was to investigate some of the relationships found among the 2OHE₁: 16OHE₁ EMR, *CYP1B1* Leu432Val polymorphism, mammographic density, race, and

breast cancer risk. The 2OHE₁:16OHE₁ EMR was associated with both breast cancer risk and the *CYP1B1* Leu432Val polymorphism, yet, no association with breast cancer risk and this polymorphism was observed. This suggests that if the *CYP1B1* Leu432Val polymorphism alters breast cancer risk, it does so through variations in the 2OHE₁:16OHE₁ EMR. When taking race into account, no association between mammographic density and the 2OHE₁:16OHE₁ EMR was observed. In culture, evidence was found to suggest that the 2OHE₁:16OHE₁ EMR is influenced by subcellular effects or other intrinsic factors (i.e. genetic variation), as passage number was the only significant contributor to the 2OHE₁:16OHE₁ EMR.

The results of this study have great public health significance, as it provides a better understanding of the risk factors, including racial differences, and etiology of breast cancer, which will ultimately lead to better prevention and treatment for all women.

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PREFACE

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~Stacy Monique Lloyd, MPH, PhD

1.0 INTRODUCTION

Breast cancer is a complex disease influenced by both genetic and environmental factors. It is the second most diagnosed cancer among women in the United States, and is the second leading cause of cancer-related deaths (American Cancer Society, 2009; National Cancer Institute, 2008b). While breast cancer affects women of all racial/ethnic groups, women of African descent suffer disproportionately higher mortality rates (American Cancer Society, 2008a). Given the unknown etiology of this disease, the focus of numerous investigations is on estimating and reducing the risk of developing breast cancer in all populations. Scientists have now identified several risk factors that influence the likelihood of developing breast cancer. These include: increasing age, age at first full-term birth, early menarche, late menopause, mammographic density, family history, obesity, HRT use, Oral Contraceptive (OC) use, alcohol consumption, and physical inactivity (American Cancer Society, 2007; Key, Verkasalo, & Banks, 2001). To date, there are only two known genes, with variable penetrance, that confer an autosomal dominant transmission of Hereditary Breast Cancer, *BRCA1* and *BRCA2*. However, the large majority of breast cancer is sporadic in nature, and occurs as a result of numerous somatic mutations in low-penetrant genes. Nonetheless, epidemiological studies have established an association between estrogens and breast cancer risk, as the large majority of these risk factors are hormonally driven.

There are three naturally occurring types of estrogen: estradiol, estrone, and estriol, all of which are under the control of the *Cytochrome P450* superfamily of genes (Johnson, 2008; Marieb, 2009). The parent molecule, 17- β estradiol, is oxidized into three distinct catecholestrogens, 2-hydroxyestrone (2OHE₁), 4-hydroxyestrone (4OHE₁), and 16 α -hydroxyestrone (16OHE₁), all of which are catalyzed by the *CYP1B1* gene (Badawi, Cavalieri, & Rogan, 2001; Cribb et al., 2006; Lord, Bongiovanni, & Bralley, 2002; Mueck, Seeger, & Lippert, 2002; Tsuchiya, Nakajima, & Yokoi, 2005). Previous research has shown that 2OHE₁ is anti-estrogenic, and as such, has been found to decrease cell proliferation, bind with reduced affinity to the estrogen receptor (ER), and gives rise to other anti-estrogenic molecules. Conversely, 16OHE₁ has exhibited pro-estrogenic properties, such as increasing cell proliferation and activating the ER (Seeger, Wallwiener, Kraemer, & Mueck, 2006; Vandewalle & Lefebvre, 1989; Zhu & Conney, 1998). It is hypothesized that the ratio of these two catecholestrogens may strongly influence breast cancer risk, and that women who metabolize estrogens principally through 2OHE₁ are at a decreased risk of developing breast cancer, while those who metabolize estrogen primarily through 16OHE₁ are at an increased risk of developing breast cancer. Therefore, one goal of this research was to assess the association of the 2OHE₁:16OHE₁ EMR and breast cancer risk; as well as to explore the relationships between this ratio and the *CYP1B1* Leu432Val polymorphism, mammographic density, and race. The aims of this study were: 1) to explore the relationship between the urinary 2OHE₁:16OHE₁ EMR and the role of the *CYP1B1* Leu432Val polymorphism in a case-control population of Caucasian women; 2) to explore the relationship between the serum 2OHE₁:16OHE₁ EMR and mammographic density in a cohort of healthy, pre- and postmenopausal African American and Caucasian women; and 3) to determine

the 2OHE₁:16OHE₁ EMR in normal and tumor breast epithelial cell lines derived from African American and Caucasian women.

The results of these analyses will allow us to determine the relationship between the 2OHE₁:16OHE₁ EMR and breast cancer risk, while assessing the role the *CYP1B1* Leu432Val polymorphism plays in this association. We will also be able to evaluate any racial differences that may exist in the 2OHE₁:16OHE₁ EMR and the factors that may contribute to this ratio, which may explain the disparity in incidence and mortality between African American and Caucasian women. Our findings will also add to the body of knowledge regarding intracrinology, the local production of hormones, by examining whether racial differences, differences in cell type, and estradiol treatment can be detected in culture, providing a new methodology in which to evaluate potential breast cancer biomarkers.

1.1 BACKGROUND AND SIGNIFICANCE

Breast cancer is defined as, “An uncontrolled growth of cells in the lobules (glands responsible for milk production) and ducts (tube-like projections that connect the lobules to the nipples) of the breast” (American Cancer Society, 2008c). Breast cancer has become a significant public health challenge due to the complexities in prevention, risk reduction, and appropriate intervention methods. After non-melanoma skin cancer, breast cancer is the most frequently diagnosed cancer among women in the United States, accounting for more than one in four cancer diagnoses (American Cancer Society, 2007; National Cancer Institute, 2008b). Furthermore, breast cancer is the second leading cause of cancer-related mortalities in women,

preceded only by lung cancer (National Cancer Institute, 2008b). While breast cancer affects women of all racial/ethnic groups, the disparity facing African American women presents an even greater challenge in the quest to prevent and adequately treat women affected with this disease; therefore making the elimination of health disparities in breast cancer a goal of Healthy People 2010 (United States Department of Health and Human Services, 2009).

1.1.1 Incidence, Prevalence, and Mortality

The Surveillance Epidemiology and End Results (SEER) program reported that the age-adjusted breast cancer incidence rate for women of all races in 2006 was 123.8 per 100,000 women. As of January 2006, there were 2,533,193 women living with a history of breast cancer. This included women with active disease, and those with no sign of disease. The age-adjusted breast cancer mortality rate in 2006 was 24.5 per 100,000 women of all racial/ethnic groups. The SEER program also predicts that there will be 192,370 new cases, and 40,170 deaths due breast cancer in the year 2009 (Surveillance Epidemiology and End Results, 2009). When these results were stratified by race, African American women had a considerably lower incidence rate when compared to Caucasian women, 117.7 versus 127.8 per 100, 000, respectively. However, the mortality rates of African American women were much higher than Caucasian women, 33.0 versus 23.9 per 100, 000 in 2005 (Surveillance Epidemiology and End Results, 2009).

Broad surveillance of all cancers began in 1975 to illustrate overall trends in incidence rates. The American Cancer Society (ACS) reported that from 1975 to 1980, the incidence rates of breast cancer were relatively stable. From 1980 to 1987, the incidence of breast cancer increased dramatically, 3.7% per year, and in the years from 1987-2001, these rates continued to

increase at a nominal 0.5% per year. Yet, from 2001 to 2004, breast cancer incidence rates dropped considerably at a rate of 3.5% per year (American Cancer Society, 2007).

It is believed that the sudden increase in breast cancer diagnoses that occurred from 1980 to 1987 was largely due to the development of mammography. This allowed improved screening and increased early detection of impalpable tumors. The moderate increase observed in the 1990s indicates the frequent use of mammography, increasing obesity rates, and the use of HRT; the latter two being known risk factors for breast cancer. Nonetheless, the rapid decline in incident cases from 2001 to 2004 is likely due to the mass discontinuance of HRT in 2002, after the Woman's Health Initiative randomized clinical trial was terminated prematurely because participants exceeded the expected risk for developing breast cancer (American Cancer Society, 2007).

1.1.2 Etiology

Cancer is defined as a group of more than 100 diseases, in which a single, abnormal cell begins to divide without control (Surveillance Epidemiology and End Results, 2009). This response is due to cellular damage, which may occur when a gene, or part of a gene, is deleted, a chromosome is translocated, or due to DNA damage that results in a truncated protein. These abnormal cells continue to divide through the expression of oncogenes, and the loss and/or mutation of tumor suppressor genes. Oncogenes are derived from the transformation of proto-oncogenes, which encode growth factor proteins, and promote normal cell growth when appropriately stimulated (Surveillance Epidemiology and End Results, 2009). The function of tumor suppressor genes is to improve the neoplastic process by regulating basic cell function and

the cellular environment, controlling cell cycles, cell proliferation, differentiation, apoptosis, and DNA repair. However, when proto-oncogenes are mutated or overly expressed, this activates oncogenes, and cells begin to grow in an uncontrolled manner. When mutated or inactivated, tumor suppressor genes lose the ability to prevent this uncontrolled growth (Turnbull & Hodgson, 2005). Cancer cells usually form a tumor, and possess the ability to travel to other parts of the body via the bloodstream and lymphatic system, replacing normal tissue (American Cancer Society, 2008c). While scientists work effortlessly to identify the cause of this disease, the exact etiology of cancer, and site-specific cancers, such as breast cancer, remains unknown.

1.1.3 Risk Factors

Although the etiology of cancer is unknown, researchers have identified several key risk factors that have been found to increase an individual's risk for developing breast cancer. Some behavioral risk factors include obesity, the use of HRT, OC use, alcohol consumption, and physical inactivity. Some non-modifiable risk factors include increasing age, age at first full-term birth, early menarche, late menopause, mammographic density, and family history (American Cancer Society, 2007; Key et al., 2001). With the exception of mammographic density, alcohol consumption, physical inactivity, and family history of breast cancer, the remaining risk factors influence the likelihood of developing breast cancer by increasing one's lifetime exposure of breast tissue circulating estrogens (American Cancer Society, 2007). While the biological mechanism in which mammographic density influences breast cancer risk is unknown, positive associations with other hormonally driven risk factors for breast cancer have been observed (e.g. premenopause, late age at first birth, and nulliparity); therefore, it has been

postulated that the relationship between mammographic density and breast cancer risk is also influenced by estrogen exposure (N. Boyd et al., 2009; Vachon, Kuni, Anderson, Anderson, & Sellers, 2000). Moreover, the relationship between breast cancer risk and alcohol consumption is also unclear, as several biological mechanisms have been proposed. It has been reported that alcohol consumption may interfere with the function of the essential nutrients of fruits and vegetables that are thought to be cancer-protective (Singletary & Gapstur, 2001). Evidence also suggests that the risk of breast cancer related to alcohol consumption may be mediated by genetic factors. Significant gene-environment interactions were found between alcohol consumption and two glutathione S-transferase genes (*GSTM1* and *GSTT1*) that play an active role in detoxifying both endogenous and exogenous toxic substances (Park et al., 2000). Additionally, polymorphisms in genes responsible for alcohol metabolism, specifically, alcohol dehydrogenases that are responsible for oxidizing ethanol to acetaldehyde, have been also been investigated to assess the association between alcohol and breast cancer risk, yet these results have been inconsistent (Boffetta & Hashibe, 2006; Freudenheim et al., 1999; Hines et al., 2000; Singletary & Gapstur, 2001). The risk of breast cancer associated with physical inactivity is directly related to the effects of sedentary behavior (e.g., obesity and hormonal and energy imbalances), but must be inferred from studies examining the protective effects of physical activity on breast cancer risk. These include maintaining energy and hormonal balance, and increasing energy expenditure, as well as weight control (American Cancer Society, 2007; National Cancer Institute, 2009).

Formal linkage analyses of rare families with multiple members with breast cancer have proven the existence of an autosomal dominant transmission of breast cancer predisposition. These studies have identified two genes, *BRCA1* and *BRCA2*, with variable penetrance as one

cause of Heredity Breast Cancer (National Cancer Institute, 2008b). These genes function as tumor suppressors, and are involved in homologous DNA repair, maintaining genomic stability, transcriptional regulation, and cell cycle control (Brooker, 2009; National Cancer Institute, 2008b; Thull & Vogel, 2004). The Breast Cancer Information Core (BIC) database has registered more than 200 germline mutations in *BRCA1*, and over 100 mutations in *BRCA2* that are associated with breast cancer susceptibility (Breast Cancer Information Core, 1998).

BRCA1 is located on chromosome 17q12-21, and contains 24 exons that encode a protein of 1,863 amino acids (National Cancer Institute, 2008b; Petrucelli, Daly, Bars-Culver, & Feldman, 1998). It is estimated that the prevalence of *BRCA1* mutations in the general population is between 1:500 and 1:1000 (Petrucelli et al., 1998). Malone et al. (2006) conducted a population-based case-control study to assess the role of *BRCA1* in African Americans and in women between the ages of 35 to 64 years. The authors observed that *BRCA1* mutations were found in 2.4% of cases when compared to 0.04% of controls. Among cases, the prevalence of *BRCA1* mutations decreased with increasing age ($p < 0.001$), and was twice as frequent in Caucasians when compared to African Americans, 2.9% versus 1.4%, respectively ($p < 0.05$). Yet, the frequency of *BRCA1* mutations was substantially more common among cases of Jewish ancestry, 10.2% ($p < 0.001$). The frequency of *BRCA1* mutations also varied by family history, when comparing those with and without a first-degree relative with breast cancer; 5.6% and 1.9%, respectively (Malone et al., 2006).

BRCA2 is located on chromosome 13q12.3, and contains 27 exons that encode 3,418 amino acids (National Cancer Institute, 2008b). The prevalence of *BRCA2* mutations in the general population is unknown, but Malone et al. (2006) found that *BRCA2* mutations were more frequent among women with a younger age at-onset of breast cancer: 4.0% of cases aged 35 to

44 and 1.5% of cases aged 45 to 64 ($p = 0.003$), respectively (Malone et al., 2006). When examining the frequency of *BRCA2* mutations and family history, Malone, et al. (2006) observed that cases with three or more relatives with breast cancer, and in those with a diagnosis before age 45, had a much higher proportion of *BRCA2* mutations, when compared to those with no family history (10.7%, $p = 0.004$; 7.4%, $p = 0.002$, respectively). *BRCA2* mutations were also more common among families with a history of both breast and ovarian cancer (Malone et al., 2006).

Germline mutations in *BRCA1* and *BRCA2* are associated with an estimated 60% lifetime risk of developing breast cancer (National Cancer Institute, 2008b). However, mutations in these genes do not account for all hereditary breast cancer cases. Cancer syndromes, such as Li Fraumeni, Cowden Syndrome, Peutz-Jeghers Syndrome, and Ataxia-telangiectasia, all present significant risks for developing breast cancer as well (Table 1) (National Cancer Institute, 2008b; Thull & Vogel, 2004).

Nonetheless, investigators have concluded that sporadic cancer predisposition is largely polygenic, and occurs as a result of somatic mutations in numerous low-penetrant genes. It is presumed that these low-penetrant genes would exhibit a small effect on breast cancer risk, however, in combination with other genetic loci and environmental risk factors, they could significantly modify breast cancer risk (National Cancer Institute, 2008b; Thull & Vogel, 2004). These genetic alterations are fairly common, and are termed polymorphisms, as the particular gene or locus occurs in several “forms” in at least 1% of the population (Table 2) (National Cancer Institute, 2008b).

Table 1. Highly Penetrant Cancer Syndromes and Breast Cancer Risk¹

Syndrome	Gene(s)	Genetic Defect	Population Incidence	Penetrance	Lifetime Risk of Breast Cancer
Li Fraumeni Syndrome	<i>TP53</i>	Tumor suppression/DNA repair	Rare	90-95%	97%
Cowden Syndrome	<i>PTEN</i>	Protein phosphatase activity	1/200,000	90-95%	25-50%
Puetz-Jeghers Syndrome	<i>STK11</i>	Tumor Suppression/DNA repair	1/200,000	95-100%	31%
Ataxia Telangiectasia	<i>ATM</i>	DNA repair	1/30,00 to 1/100,000	100%	52-60%
Hereditary Diffuse Gastric Cancer	<i>CDH1</i>	E-cadherin	Unknown/rare	90%	39% lobular breast cancer
Bloom Syndrome	<i>BLM</i>	DNA repair	Unknown/rare	100%	150-300%
Werner Syndrome	<i>WRN</i>	RecQ Helicase/DNA repair	~ 1/200,000	90%	Unknown
Xeroderma Pigmentosa	<i>XPA, XPC, ERCC, DDB2, ERCC4, ERCC5, and POLH</i>	DNA repair	1/100,000	100%	Unknown
Nijmegen Breakage Syndrome	<i>NBS1</i>	Nibrin/DNA repair	Rare	100%	Unknown
Fanconi Anemia	<i>PALB2, BRIP1, BRCA2, FANCA, FANCB, FANCC, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCL, FANCM</i>	DNA repair	1/360,000	100%	Unknown

¹ Citations in appendix a

Table 2. Low Penetrance Breast Cancer Susceptible Variants²

Locus	Gene	Reference SNP/ Mutation
10q26	<i>FGFR2</i>	rs2981582 rs1219648
16q12	<i>TNRC9</i> <i>LOC643714</i>	rs3803662 rs3803662
2q35		rs13387042
5p12	<i>MRPS30</i>	rs10941679
5q11	<i>MAP3K1</i> <i>MGC33648</i> <i>MIER3</i>	rs889312
2q33	<i>CASP8</i> <i>TRAK2 (34)</i> <i>ALS2CR12</i> <i>ALS2CR2</i> <i>ALS2CR11</i> <i>LOC389286</i> <i>LOC729191</i>	rs1045485
8q24		rs13281615
11p15	<i>LSP1</i> <i>TNNT3</i> <i>MRPL23</i> <i>H19</i> <i>LOC728008</i>	rs3817198
26q22-q31	<i>CYP17</i>	Promoter T → C (T1931C)
10q26	<i>CYP19</i>	Polymorphic (TTTA) repeat in intron 5 C → T in exon 10
2p22-21	<i>CYP11B1</i>	rs1056836/ Leu432Val
22q13.1	<i>CYP2D6</i>	2367delA (A allele); Intron 3 G → A (G1934A) (B allele; Del Lys281 (C allele); 17.5-kb deletion (D allele); Frameshift Premature stop at residue 544
19	<i>EDH17B2</i>	Exon 6 A → G (Ser312Gly)
11q22-q23	<i>PGR</i>	Alu repeat insertion in intron G V660L
4p15.1	<i>PPARGCIA</i>	C1835T; Thr612Met
15q24.1	<i>CYP11A1</i>	3801T → C 3205T → C IL462Val Thr461Asp
1p13.3	<i>GSTM1</i>	
22q12.1	<i>CHEK2</i>	1100delC I157T IVS2 + 1G → A R145W S428F
22q11.21	<i>COMT</i>	Val158Met

² Citations in appendix b

Epidemiological studies of breast cancer have established that hormonal factors, sex hormones in particular, also play a vital role in the etiology of disease (Key et al., 2001). This premise is supported when reviewing the risk factors associated with disease, and confirms that the risk of breast cancer is elevated in those with early menarche and late menopause, therefore increasing endogenous estrogen exposure. Furthermore, animal studies have also demonstrated that estrogen and progesterone exposure promote the development of some mammary tumors. Lastly, increased exogenous hormone exposure in the form of contraceptives and HRT, also increases breast cancer risk (European Society of Human Reproduction and Embryology, 2004).

Another significant contributor to breast cancer risk is mammographic density. Mammographic density is a heritable measure of breast composition, and is the proportion of fibroglandular elements (i.e., stroma and epithelium) to total breast area (N. Boyd et al., 2009; Dite et al., 2008; Vachon et al., 2000). Breasts that are composed of 75% dense tissue are classified as being “mammographically dense,” while those composed exclusively of fat, are identified as having “no measurable dense tissue” (Haiman et al., 2003). Furthermore, numerous studies have reported that women with “mammographically dense” breast have a four to six times relative risk of developing breast cancer (Aiello et al., 2005; N. Boyd et al., 2009; Tamimi, Byrne, Colditz, & Hankinson, 2007; Tamimi, Hankinson, Colditz, & Byrne, 2005; Warren, 2004; Warren et al., 2006; Woolcott et al., 2009). The literature suggests that mammographic density decreases with age, pregnancy, menopause, and increasing BMI (N. Boyd et al., 2009; N. F. Boyd et al., 2009). Mammographic density has also been shown to be altered by exogenous hormone use and menstruation, making it a useful biomarker of risk, as it is presumed that a change in density may reflect a change in breast cancer risk (Warren, 2004).

1.1.4 Prevention and Screening

In an effort to reduce the burden of breast cancer, the National Cancer Institute (NCI) and the ACS recommend several screening options. The most commonly used screening methods to detect breast cancer include:

1. Breast Self Exam: Physical, self examination of the breast, recommended for women in their 20's.
2. Clinical Breast Exam: Performed by a physician or other health professional, which consist of a careful physical examination of the breast and area under the arm for lumps or other unusual findings. This is recommended for women in their 20's and 30's as part of a regular health exam.
3. Mammograms: X-ray of the breast capable of detecting impalpable tumors, and ductal carcinoma *in situ* (abnormal cells in the lining of the breast duct). Mammograms are recommended for women over the age of 40 every one to two years (American Cancer Society, 2008b; National Cancer Institute, 2008a).

1.2 CLASSIC ENDOCRINOLOGY

The endocrine system is one of the 11 major organ systems of the body that consists of a compilation of integrated glands and organs, such as:

- Hypothalamus;
- Pituitary Gland;
- Pineal Gland;
- Thyroid Gland;
 - Parathyroid Gland;
- Adrenal Gland;
- Pancreas;
- Ovaries; and
- Testes (Foster, 2008; Surveillance Epidemiology and End Results, 2009).

These glands and organs function in the release of biologically active hormones, and play a vital role in the body's growth, metabolism, and sexual development. The hormones secreted by this system are transported into the blood, where they then travel to their target tissues, where specific receptors await to receive them (Foster, 2008).

Hormones are classified into two groups, peptides and steroid hormones. Peptide hormones are generally produced as large proteins, and are synthesized by the anterior pituitary gland, thyroid gland, parathyroid gland, or pancreas, whereas, steroid hormones are produced by the adrenal gland, ovary, or testis (Kletter, 2008).

There are five major classes of steroid hormones: progestagens, glucocorticoids, mineralcorticoids, androgens, and estrogens. Each of these steroid hormones regulates gene expression, by binding and activating receptor molecules that function as transcription factors (Berg, Tymoczko, & Stryer, 2002). For this study, we will only focus on the direct synthesis and action of estrogens.

Estrogen biosynthesis is catalyzed by the *Cytochrome P450* superfamily of genes that encode a number of enzymes that are directly involved in drug and steroid metabolism (Genetics Home Reference, 2006; Nebert & Russell, 2002). The primary site for estrogen biosynthesis is in the theca cells of the ovary. The synthesis of estrogen starts with the formation of cholesterol, a 27-carbon lipid molecule that is a vital component of the plasma membrane (Figure 1) (Albert et al., 2002; Berg et al., 2002). The next step involves the removal of a 6-carbon unit to form pregnenolone. This process is carried out through the *CYP11A1* gene, which encodes the enzyme cholesterol desmolase, otherwise known as P450 side chain cleavage (P450_{scc}), and adrenocorticotrophic hormone (ACTH), a polypeptide synthesized by the anterior pituitary gland (Berg et al., 2002; GeneCards, 2008). From pregnenolone, progesterone is derived via the catalyzation of 3 β -hydroxy steroid dehydrogenase (Burger, 2002; Olson, Bandera, & Orlow, 2007). Then, an intermediate progesterone is produced, 17 α -Hydroxyprogesterone, from the hydroxylation of C-17 of progesterone, through the 17 α hydroxylase enzyme coded by the *CYP17A1* gene. Another enzyme of the *CYP17A1* gene, 17/20 lyase, a derivative of 17 α hydroxylase, catalyzes the cleavage of carbons 20 and 21 to yield androstenedione. From androstenedione, two products are formed:

1. Testosterone, via the 17 β -hydroxy steroid dehydrogenase enzyme; and
2. Estrone (an estrogen), after cleavage of carbon 20, and the aromatase activity encoded by the *CYP19A1* gene.

Utilizing the same aromatase activity of *CYP19A1*, testosterone produces another estrogen, estradiol (Berg et al., 2002; Burger, 2002; Olson et al., 2007).

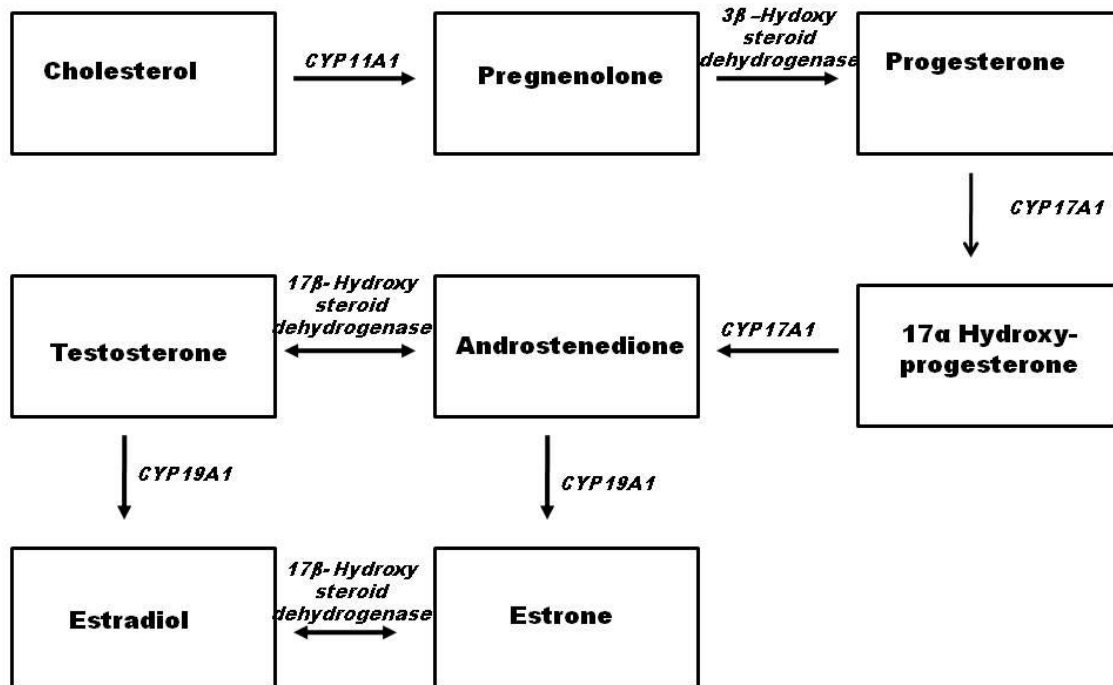


Figure 1. Classic Endocrinology and Associated Genes³

There are three naturally occurring types of estrogen: estradiol, estrone, and estriol. Estradiol (17 β -estradiol), the parent hormone, is the most abundant of the three, being present in both males and females (Johnson, 2008; Marieb, 2009). The metabolism of estradiol takes place in the liver, and is essentially the same in both sexes (Mueck et al., 2002; Zhu & Conney, 1998). Estradiol is metabolized almost exclusively through oxidation. The first step takes place at

³ Adapted from “Androgen Production in Women,” 2002, *Fertility and Sterility*, 77, p. S3-S5. Copyright 2002 by American Society for Reproductive Medicine.

Adapted from “Biochemistry”, 2002, *The Biosynthesis of Membrane Lipids and Steroids*, p. 735-736. Copyright 2002 by W.H. Freeman and Company.

Adapted from “Variants in estrogen biosynthesis genes, sex steroid hormone levels, and endometrial cancer: a HuGE review,” 2007, *American Journal of Epidemiology*, p. 235-245. Copyright 2006 by the Johns Hopkins Bloomberg School of Public Health.

position C-17, and results in the oxidation of estradiol to estrone by 17 β -hydroxysteroid dehydrogenase. This step is reversible, but the formation of estrone is favored. At this point, further metabolism occurs via two separate pathways (Mueck et al., 2002). The A-ring of estrone is hydroxylated and produces two catechol estrogens, 2-hydroxyestrone (2OHE₁) and 4-hydroxyestrone (4OHE₁); while the D-ring is hydroxylated to form one catechol estrogen, 16 α -hydroxyestrone (16OHE₁), which is later metabolized to estriol (Clemons & Goss, 2001; Mueck et al., 2002). These two pathways are formed by two separate enzyme systems; the A-ring is hydroxylated by *CYP1A1*, *CYP1A2*, and *CYP1B1* genes, while the D-ring is hydroxylated by *CYP1B1*, *CYP2C19*, *CYP1A1*, and *CYP3A5* (Badawi et al., 2001; Cribb et al., 2006; Tsuchiya et al., 2005). Once formed, these metabolites, 2OHE₁, 4OHE₁, 16OHE₁, and estriol, can no longer be reduced back to estrone, and are the most active in the human metabolic process (Figure 2) (Mueck et al., 2002).

Each estrogen metabolite is biologically distinct, and has various functions. Estriol is the primary circulating estrogen during pregnancy, and experiences an exponential increase after thirty-four weeks gestation. This surge has been observed at two to four weeks before the onset of term labor, while the absence of estriol was noted among women who presented for induction at forty-two weeks gestation. These results support the theory that estriol is important in the initiation of labor in humans (Yeast & Lu, 2007).

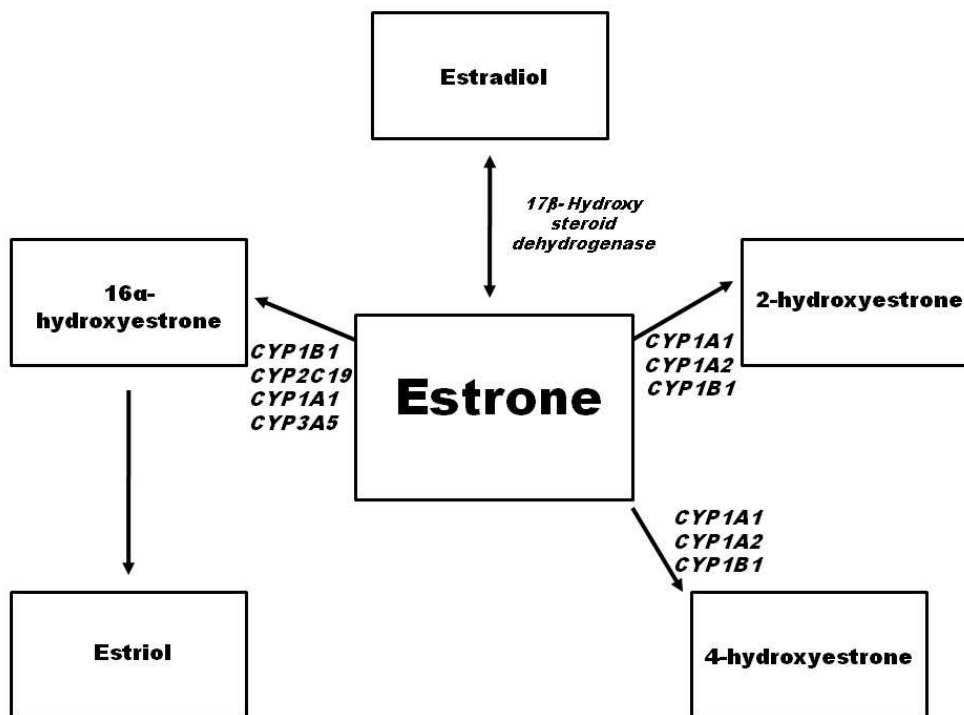


Figure 2. Estradiol Catabolism⁴

The remaining metabolites, 2OHE₁, 16OHE₁, and 4OHE₁ have been implicated as markers of breast cancer risk. Nevertheless, 4OHE₁ has been reported to be less stable than 2OHE₁ and 16OHE₁ (Mueck & Seeger, 2007). The remaining metabolites, 2OHE₁, 16OHE₁, and

⁴ Adapted from "Estradiol Metabolism and Malignant Disease," 2002, *Maturitas*, 43, p. 1-10. Copyright 2002 by Elsevier Science Ireland Ltd. Adapted with permission of the author.

Adapted from "Oxidative Metabolism of Estradiol," 1960, *The Journal of Biological Chemistry*, 11, p. 3104-3107. Printed in the USA.

Adapted from "Cytochrome P450-mediated metabolism of estrogens and its regulation in human," 2005, *Cancer Letters*, 227, p. 115-124. Copyright 2005 Elsevier Science Ireland Ltd.

Adapted from "Role of Polymorphic Human Cytochrome P450 Enzymes in Estrone Oxidation," 2006, *Cancer Epidemiology, Biomarkers, & Prevention*, 15(3), p. 551-558. Copyright 2006 American Association for Cancer Research.

Adapted from "Role of Human Cytochrome P450 1A1, 1A2, 1B1, and 3A4 in the 2-, 4-, and 16α-Hydroxylation of 17β-Estradiol," 2001, *Metabolism*, 50(9), p. 1001-1003. Copyright 2001 W.B. Saunders Company.

4OHE₁ have been implicated as markers of breast cancer risk. Nevertheless, 4OHE₁ has been reported to be less stable than 2OHE₁ and 16OHE₁ (Zhu & Conney, 1998).

Furthermore, less than 5% of estrogens become 4OHE₁ (Service, 1998). It is for this reason we have chosen to focus our attention on 2OHE₁ and 16OHE₁.

2OHE₁ and 16OHE₁ have contrasting biological properties (Lord et al., 2002). 2OHE₁ is capable of binding to the estrogen receptor (ER), but with reduced binding affinity (Zhu & Conney, 1998). This anti-estrogenic metabolite has been shown to decrease cell proliferation by 20 to 30% in cultured breast cancer cell lines (Vandewalle & Lefebvre, 1989). This anti-estrogenic metabolite has been shown to decrease cell proliferation by 20 to 30% in cultured breast cancer cell lines (Zhu & Conney, 1998). On the other hand, 16OHE₁ is a potent estrogenic molecule that functions by activating the ER, and increases proliferation of cultured breast cancer cells by 40% (Seeger et al., 2006; Zhu & Conney, 1998). In cells treated with 16OHE₁, there was also a 30% to 50% increase in *BCL-2*, an anti-apoptotic marker, and a 30% to 40% decrease in *Cytochrome C*, a pro-apoptotic marker (Seeger et al., 2006).

The catabolism of 2OHE₁ produces other anti-estrogenic metabolites, whereas 16OHE₁ is strongly estrogenic, and is believed to be a potential carcinogen. Therefore, the ratio of 2OHE₁ and 16OHE₁ regulates the proportion of anti-estrogenic molecules to estrogenic ones. Thus, it is hypothesized that women who metabolize estrogen primarily through the 2OHE₁ pathway have a lower risk of developing breast cancer, while those who metabolize estrogens primarily through the 16OHE₁ pathway are at an increased risk of developing breast cancer.

1.3 ESTROGENS AND BREAST CANCER

The association between estrogens and breast cancer risk was first demonstrated by Beatson in the late 19th century. He discovered the growth-stimulating properties of estrogen on breast tumors, and that tumor growth could be reduced by the removal of the ovaries (T. H. Lippert, Seeger, & Mueck, 2000). Given that estrogens are released directly into the bloodstream, it is not surprising that many studies have observed an association between elevated blood-estrogen levels and breast cancer risk. This finding is prominent in postmenopausal women, and is likely due to increased body weight, as aging and menopause are associated with weight gain, central adiposity, and physical inactivity, all risk factors for breast cancer (Baglietto et al., 2008; Gruber, Tschugguel, Schneeberger, & Huber, 2002; Yager & Davidson, 2006). BMI has also been reported as being associated with breast cancer risk among postmenopausal women. As little to no estrogen is synthesized in the ovaries, androgens, estrogens precursor molecules, must be converted to estrogens in extragonadal tissue, such as the liver, muscle, and skin; however, the majority of estrogens are produced in adipose tissue (Baglietto et al., 2008; Clemons & Goss, 2001). It is for this reason that obesity is a major risk factor for postmenopausal women. Consequently, obese postmenopausal women have a higher risk of developing breast cancer, than do non-obese postmenopausal women (Clemons & Goss, 2001). Other contributors to the association between elevated blood-estrogens and breast cancer risk include the large number of risk factors that contribute to the cumulative exposure of estrogen (e.g., OC use, HRT use, age at menarche, age at first full-term birth, and age at menopause) (Yager & Davidson, 2006). However, this association in premenopausal women is unfounded, as there is a lack of sufficient evidence to draw a definitive conclusion.

Although breast cancer in young, premenopausal patients seem to be more aggressive, poorly differentiated, exhibit rapid proliferation and vascularization, are estrogen receptor (ER) and progesterone receptor (PR) negative, and has a higher frequency of bone micrometastases, few studies have addressed the association of circulating sex steroids in these women (Aebi & Pagani, 2007; Hankinson & Eliassen, 2007). This is due in large part to fluctuating hormone levels during the menstrual cycle (Hankinson & Eliassen, 2007). However, analyses that have assessed this association are plagued by small sample size (Eliassen et al., 2006; Hankinson & Eliassen, 2007). In a recent study using the Nurse's Health Study II data, Eliassen, et al. (2006) found that women with high total follicular estradiol and free follicular estradiol concentrations demonstrated a significantly increased risk of breast cancer (RR for total follicular estrogen concentrations = 2.1, 95% CI 1.3 – 4.1, p for trend = 0.08; RR for free follicular estradiol = 2.4, 95% CI 1.3 – 4.5, p for trend = 0.01) (Eliassen et al., 2006).

Despite the disparity facing African American women, endogenous hormone concentrations in this population are rarely studied. It is known that African American women age 40 and older have a lower risk of developing breast cancer compared to Caucasian women. Yet, the risk is much greater for African American women under the age of 40, although their mortality rate exceeds that of Caucasians at every age (American Cancer Society, 2008a). Women of African descent are more likely to be diagnosed with breast cancer at a more advanced stage (which includes some stage III and stage IV tumors), have larger, estrogen-negative, high grade tumors, as well as lymph node involvement; all of which are poor prognostic predictors of survival (Breastcancer.org, 2008; Chlebowski et al., 2005; Curtis, Quale, Haggstrom, & Bindman-Smith, 2008). However, the ACS reported that the proportion of

African Americans that underwent breast cancer screening in 2004 nearly matched that of Caucasians (Table 3) (American Cancer Society, 2008a).

Table 3. Use of Breast Cancer Screening Examination

Breast Cancer Screening Method	African Americans	Caucasians
Mammogram	59.4	58.6
Clinical Breast Exam	64.3	65.6
Mammogram & Clinical Breast Exam	51.2	52.2

While the reasons behind these differences remain unknown, many have suggested that socioeconomic factors, such as a lack of access to health care and inadequate treatment after diagnosis are possible explanations. Nonetheless, Wojcik, Spinks, & Optenberg (1998) conducted a retrospective study of breast cancer patients diagnosed and treated in the United States military equal-access medical care system (United States Department of Defense), to evaluate survival differences between African American and Caucasian women. The authors observed that while African American participants fared better than African Americans nationally, participants still suffered higher mortality rates than did Caucasian participants (24.77% versus 18.09%) (Wojcik, Spinks, & Optenberg, 1998). These results were duplicated in a more recent study that also indicated that the mortality difference between African Americans and Caucasians was increasing in the United States Department of Defense healthcare system (Jatoi, Becher, & Leake, 2003). Therefore, access to healthcare, or a lack thereof, does not explain the mortality disparity facing African American women. Yet, when Pinheiro et al. (2005), assessed potential racial differences in premenopausal hormone concentrations, the authors observed that African Americans expressed 18% higher estradiol levels (170 pg/ml versus 144 pg/ml; $p < 0.01$), 17% higher free estradiol levels (2.1 pg/ml versus 1.8 pg/ml; $p <$

0.01), and 11% lower sex-hormone binding globulin (63 nmol/L versus 71 nmol/L; $p = 0.05$) when compared to Caucasians (Pinheiro, Holmes, Pollak, Barbieri, & Hankinson, 2005). While more research is needed in this area, findings such as these may assist in explaining the differences in breast cancer morbidity and mortality in African American populations.

Estrogens have also been examined in relation to mammographic density; as increases in mammographic density increase breast cancer risk. It is believed that the risk of developing breast cancer in relation to mammographic density may be due to the cumulative exposure of breast stroma and epithelium to sex hormones (N. Boyd et al., 2009). Nonetheless, few studies have addressed the effects of endogenous estrogens on mammographic density, and focus primarily on the effects of exogenous hormone use, specifically postmenopausal hormone use. However, these studies all demonstrate that hormone therapy consisting of estrogen and progesterone increases mammographic density (Boyd et al., 2005; Vachon et al., 2000; Warren, 2004; Wellejus et al., 2005; Ziv et al., 2004). In fact, in a cross sectional analysis of postmenopausal women, not using HRT, a statistically significant positive association between percent mammographic density and serum levels of estrone ($p = 0.013$), estradiol ($p = 0.008$), and bioavailable estradiol ($p = 0.017$) was observed (Greendale et al., 2005). Conversely, the anti-estrogen, Tamoxifen, has been reported to decrease mammographic density (Ziv et al., 2004). One study sought to compare the effects of the ER modulator, Raloxifene, and a low dose of transdermal estradiol on mammographic density in a group of postmenopausal women. The authors observed an increase in dense area in both groups, however, the increase by estradiol obtained statistical significance (Raloxifene: baseline – 0.16, 2 year. follow-up – 0.18; Estradiol: baseline – 0.16, 2 year. follow-up – 0.2, $p < 0.05$) (Nielsen et al., 2009).

Variations in mammographic density have also been assessed in various racial/ethnic groups, to determine if these differences account for the disparity in breast cancer risk experienced by minority populations. However, the results of these studies are inconsistent. Some studies report that mammographic density does not vary by race, while others cite African Americans as having significantly higher or lower breast density when compared to Caucasian or Asian American women. In fact, one study found that after adjusting for BMI, African Americans were more likely to have a higher breast density when compared to Caucasian women (OR = 1.4; 95% CI 1.2 – 1.6). This association became stronger after adjusting for BMI, reproductive and hormonal factors, in women age 65 and younger (ages \leq 45 years: OR = 1.7; 95% CI 1.2 – 2.3; ages 46 – 55 years: OR = 1.3; 95% CI 1.0 – 1.7; ages 56 – 65 years: OR = 1.7; 95% CI 1.2 – 2.3) when compared to women age 65 and over (OR = 1.1; 95% CI 0.7 – 1.6) (El-Bastawissi, White, Mandelson, & Taplin, 2001). Conversely, in a study by del Carmen, Hughes, Halpern, et al. (2003), African American women had a much lower mammographic density compared to Caucasian, Latina, and Asian American women (African American: 2.43; Caucasian: 2.69; Latina: 2.65; and Asian American: 3.09). This finding persisted, even after adjusting for age, BMI, and variations in measuring mammographic density (African American adjusted mean density: 2.54; Caucasian adjusted mean density: 2.66; $p = 0.0006$; Latina adjusted mean density: 2.67; $p = 0.0139$; Asian American adjusted mean density: 2.79; $p = 0.0372$) (del Carmen et al., 2003). The results presented here further demonstrate the complexity involved in understanding the underlying mechanisms of the racial/ethnic differences in breast cancer risk, and also exhibit the necessity for further research.

1.4 INTRACRINOLOGY

As previously mentioned, hormones are secreted via the organs and glands of the endocrine system. They are then transported through the blood, where they act on their target tissues to elicit a response from specific receptors. As the systematic name implies, this type of hormone action is called, “endocrine” (Sasano, Suzuki, Miki, & Moriya, 2008). However, several studies have demonstrated the ability of peripheral organs to synthesize their own estrogen. In fact, approximately 75% of estrogens in premenopausal women, and nearly 100% of the estrogens produced in postmenopausal women, are synthesized in extragonadal tissues, utilizing circulating androgens. This local production of hormone is termed, “intracrine,” as the hormone derived exerts its effects within the cells in which it is produced (Foster, 2008; Sasano et al., 2008).

The production of estrogens is tissue-specific. The ovary, which is the primary source of estrogen in premenopausal women, produces mostly estradiol, while the placenta produces estriol, and adipose tissue generates estrone. Estrone is the predominant form of estrogen in postmenopausal women, and the parent substance of 2OHE₁ and 16OHE₁ (Miettinen, Isomaa, Peltoketo, Ghosh, & Vihko, 2000; Mueck et al., 2002; Sasano, Suzuki, Nakata, & Moriya, 2006; Simpson et al., 1994). Although the main circulating estrogen in postmenopausal women is estrone, it exists primarily in its sulfated form as estrone sulfate (E1S), via estrogen sulfotransferase (EST) (Miettinen et al., 2000; Sasano et al., 2006; Suzuki, Moriya, Ishida, Ohuchi, & Sasano, 2003). EST belongs to a superfamily of steroid sulfotransferases that function in converting estrogens to biologically inactive, estrogen sulfates. E1S is then converted to estrone, via steroid sulfotransferase (STS) (Pasqualini & Chetrite, 1999; Sasano et

al., 2006). This pathway is entitled the “sulfatase pathway”(Figure 3) (Pasqualini & Chetrite, 1999).

As mentioned previously, the “aromatase pathway” catalyzes the conversion of androgens to estrogens in the ovary. Yet, this pathway is also present in breast tissue. In fact, immunolocalization revealed aromatase activity in the epithelial cells of the ducts of the terminal ductal lobular units (TDLU) of normal breast tissue. In situ hybridization also revealed aromatase mRNA in the adjacent stroma cells of the TDLU, as well as in epithelial cells lining cysts, and in the stroma cells of the parenchyma outside of the TDLU. Furthermore, aromatase activity was observed in the cytoplasm of epithelial cells and in the adjacent stroma cells of breast tumor tissue (Brodie, Lu, & Nakamura, 1997). Quantitatively, aromatase activity has been detected in more than 70% of breast tumors (Miettinen et al., 2000; Suzuki et al., 2003). When compared to non-malignant tissue, malignant ones expressed significantly greater levels of aromatase mRNA (Suzuki et al., 2003).

However, STS activity has been detected in 90% of breast tumors, in contrast to aromatase activity, that is expressed in only 70% of tumors (Foster, 2008; Suzuki et al., 2003). This increased activity could potentially result in a 10 fold greater production of estrone via the sulfatase pathway rather than the aromatase pathway in breast tissue, leading to a greater generation of 2OHE₁ and 16OHE₁. Furthermore, Real Time-Polymerase Chain Reaction (RT-PCR) assays have observed significantly larger amounts of STS activity in breast cancer tissue than in normal breast (Foster, 2008). Considering the evidence presented, EST and STS play vital roles in maintaining biologically active estrogens in tissue (Sasano et al., 2008).

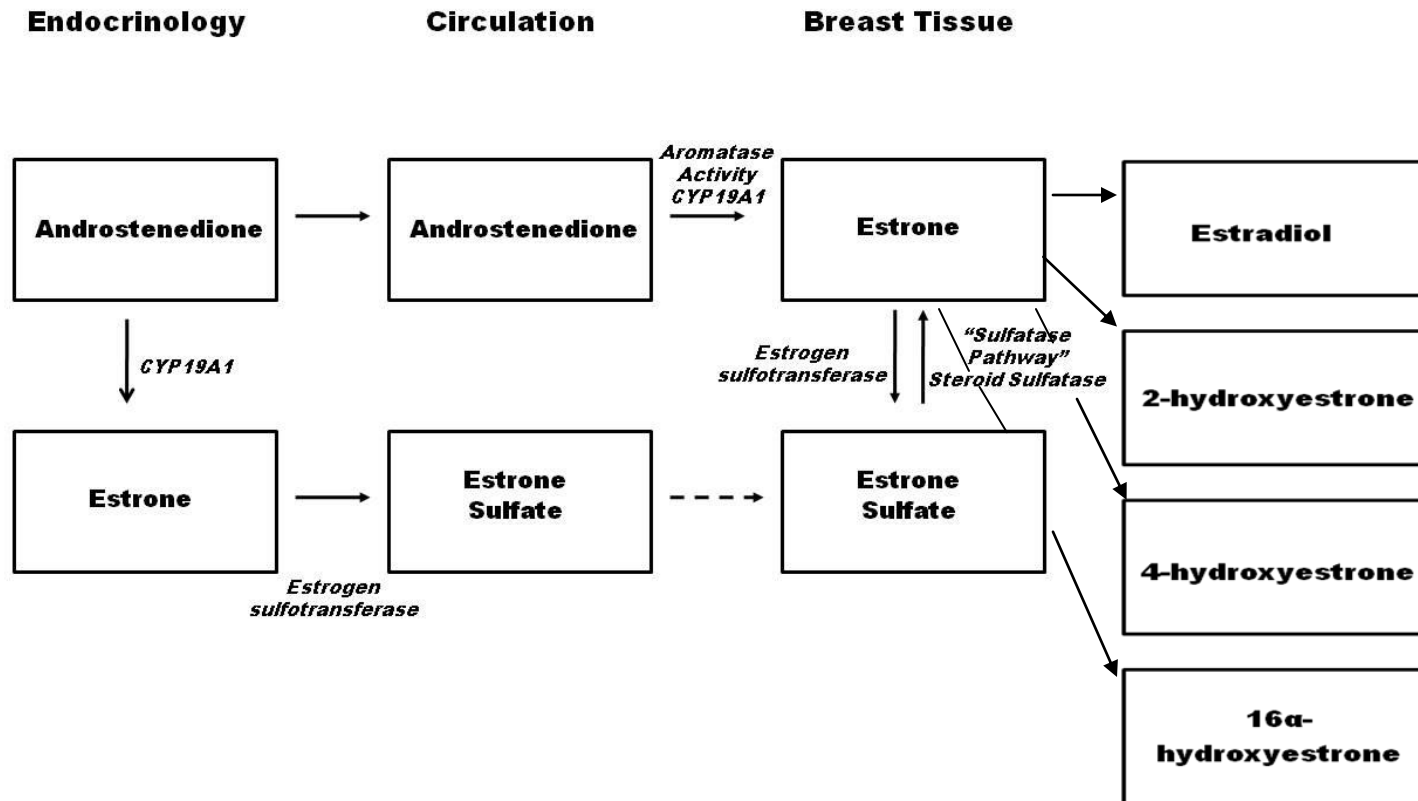


Figure 3. Intracrinology⁵

⁵ Adapted from "New Developments in Intracrinology of Human Breast Cancer," 2009, *Steroid Enzymes and Cancer*, 1155, p. 76-79. Copyright 2009 by New York Academy of Sciences.

Adapted from "Intracrine Mechanism of Estrogen Synthesis in Breast Cancer," 2003, *Biomedicine & Pharmacotherapy*, 57, p. 460-462. Copyright 2003 by Éditions Scientifiques et Médicales Elsevier SAS.

Adapted from "Intracrinology of Estrogens and Androgens in Breast Carcinoma," 2008, *Journal of Steroid Biochemistry & Molecular Biology*, 108, p. 181-185. Copyright 2007 Elsevier Ltd.

Endocrinology and Intracrinology systems operate using two separate mechanisms of action. In classical endocrinology, hormones are released into the blood to act on target tissues, therefore increasing plasma concentrations, and influencing the biological effects of hormone-dependent tissues (Foster, 2008). However, only a small portion of the hormones produced are actually utilized in the target tissues. The large majority of hormones produced in this fashion, are metabolized to biologically inactive forms. On the other hand, the intracrine system requires only modest amounts of hormones to elicit a maximum effect. Therefore, this system of hormone action is much more efficient in the development of hormone-dependent malignancies (Foster, 2008; Sasano et al., 2008).

1.5 CYTOCHROME P450 1B1

The *Cytochrome P450* is a superfamily of membrane-proteins that consists of 57 genes. These genes encode enzymes that function in the metabolism of drugs, foreign chemicals, arachidonic acid and eicosanoids, cholesterol metabolism, bile acid biosynthesis, steroid synthesis and metabolism, Vitamin D₃ synthesis and metabolism, and retinoic acid hydroxylation (Nebert & Russell, 2002). The proteins of the *Cytochrome P450* (*CYP*) family are arranged into families and subfamilies, based on the percentage of comparable amino acid sequence characteristics. Cytochromes that have greater than or equal to 40% of the same amino acid characteristics are assigned to one group that is denoted by an Arabic numeral. Then, those that share greater than or equal to 55% of the same amino acid characteristics are further arranged into subfamilies that are denoted by a letter. For example, sterol 27-hydroxylase and Vitamin D₃24-hydroxylase both belong to the *CYP27* family because they share greater than or equal to 40% of the same amino

acid characteristics. But, sterol 27-hydroxylase is further classified into the *CYP27A* subfamily, while Vitamin D₃24-hydroxylase belongs to the *CYP27B* subfamily, as these two enzymes do not share greater than or equal to 55% of the same amino acid characteristics. Moreover, if another enzyme(s) was found to have greater than or equal to 55% of the same amino acid characteristics as sterol 27-hydroxylase and Vitamin D₃24-hydroxylase, these enzymes would be further classified as *CYP27A2* and *CYP27B2*, and so on (Nebert & Russell, 2002). There are currently more than 270 *CYP* gene families, yet for this review, we will only focus on *CYP1B1*.

Cytochrome P450 1B1 (CYP1B1) maps to chromosome 2p22-p21, and consists of three exons and two introns. This gene is expressed in monocytes, macrophages, and in other extrahepatic organs, such as the kidney, prostate, uterus, ovary, and breast. *CYP1B1* is regulated by the aryl hydrocarbon receptor, a cytosolic transcription factor that is activated by polycyclic aromatic hydrocarbons, and dioxin-like compounds. *CYP1B1* can activate several carcinogens, such as arylenes, nitrosamines, and arylamines, all of which can cause DNA damage (Beuten et al., 2008; Ingelman-Sundberg, 2004; Singh et al., 2008; Wenzlaff et al., 2005).

There are several variants of this gene, but the most common is a C→G transversion at position 1666 in exon 3, the heme-binding domain of the gene, which is critical to its catalytic function. This change results in an amino acid substitution of leucine (Leu) to valine (Val) at codon 432 (Paracchini et al., 2007). According to Hapmap data, the frequency of the C allele is 0.867 among Han Chinese, 0.909 among Japanese, 0.15 among West African, and 0.558 among Caucasian populations (International HapMap Project, 2009).

CYP1B1 is highly involved in estradiol metabolism. This gene has been reported as being responsible for the hydroxylation of estrone at both C-2 and C-16 positions, leading to the formation of 2OHE₁ and 16OHE₁ (Badawi et al., 2001; Cribb et al., 2006; Lord et al., 2002;

Tsuchiya et al., 2005). As mentioned previously, 2OHE₁ is anti-estrogenic, while 16OHE₁ is a potent estrogenic molecule, and it is believed that women who metabolize estrogen principally through the C-16 pathway, experience an increased risk for developing breast cancer. This evidence suggests that alterations in the *CYP1B1* genotype could influence breast cancer risk. In fact, the Leu432Val polymorphism has been demonstrated to be associated with breast cancer risk.

In a meta and pooled analysis by Paracchini, Raimondi, Gram, et al. (2007), the authors observed racial, as well as age differences in the association between the *CYP1B1* Leu432Val polymorphism and breast cancer risk. A possible association between the *CYP1B1* Leu432Val polymorphism and breast cancer risk was also found, but only among Caucasians. The results of this subpopulation also revealed a significant age effect, with an increased risk of breast cancer occurring between the ages of 45 and 59 years. Yet, the observed risk was much lower for both younger and older women. However, this effect was not seen among Asians, while a possible negative association was found among African American or mixed populations (Paracchini et al., 2007).

In a rather large population study consisting of 1,521 breast cancer cases and 1,498 controls, a significant association was found among the *CYP1B1* Leu432Val polymorphism and breast cancer risk among postmenopausal women using HRT. The authors hypothesized that the effect of the *CYP1B1* genotype would be more pronounced among long-term users (≥ 4 years) of hormones. In fact, participants who expressed the homozygous variant genotype and who reported using HRT for ≥ 4 years, were at a greater risk of developing breast cancer, when compared to other postmenopausal women who reported using hormone therapy for ≥ 4 years, but did not carry this genotype (OR 2.0; 95% CI 1.1 – 3.5) (Rylander-Rudqvist et al., 2003).

A case-control study conducted in Turkey by Kocabas et al. (2002) assessed the association of the *CYP1B1* Leu432Val polymorphism and breast cancer risk. Utilizing 84 breast cancer patients and 103 controls matched for age, time of blood donation, and menopausal status, the authors found that the presence of the valine allele was associated with a significantly increased susceptibility of breast cancer. The adjusted odds ratio for age, age at menopause, age at first full term pregnancy, BMI, and smoking status was 2.32 (95% CI 1.26 - 4.25; $p = 0.007$). The authors also observed that the *CYP1B1* genotype was related to breast cancer among women with a BMI greater than 24 kg/m² (adjusted OR = 2.78; 95% CI 1.36 - 5.62; $p = 0.005$) (Kocabas, Sardas, Cholerton, Daly, & Karakaya, 2002).

To date, only one study has been identified that examined the association between the *CYP1B1* Leu432Val polymorphism and the 2OHE₁:16OHE₁ EMR. Paracchini et al (2005) assessed this relationship in 150 healthy men and premenopausal women, and observed that the presence of the valine allele was associated with an increased urinary 2OHE₁:16OHE₁ EMR among Caucasian women (*CYP1B1* Val/Val = 1.95 ± 1.06; *CYP1B1* Val/Leu = 2.08 ± 1.06; *CYP1B1* Leu/Leu = 2.55 ± 1.37) (Paracchini et al., 2005). While these results have elucidated significant genotype-phenotype correlations, they also emphasize the need for more definitive studies verifying the association between the 2OHE₁:16OHE₁ ratio and the *CYP1B1* Leu432Val, and their relationship to breast cancer risk.

1.6 SUMMARY AND CONCLUSIONS

The role of estrogens and the 2OHE₁:16OHE₁ EMR in the development and progression of breast cancer has long been established. However, this association has rarely been investigated

in relation to the *CYP1B1* Leu432Val polymorphism, mammographic density, and race. Nonetheless, recent evidence has found an association between the *CYP1B1* Leu432Val genotype and breast cancer, thereby suggesting that presence of the variant allele may mediate this risk. While the relationship between this genotype and the 2OHE₁:16OHE₁ EMR has not been readily examined, a significant association has been observed, and implies that the *CYP1B1* Leu432Val genotype may alter breast cancer risk through variations in the 2OHE₁:16OHE₁ EMR.

Thus far, the literature presents very little evidence regarding the 2OHE₁:16OHE₁ EMR by race, although some studies have investigated variations in mammographic density and, to our knowledge, only one study has examined differences in endogenous estrogen levels by race. However, the results of these studies are inconsistent, and need to be confirmed. Given the significant disparity in breast cancer morbidity and mortality between African American and Caucasian women, further research is warranted to identify a possible biological explanation for this difference.

Since evidence now suggests that the estrogens produced locally are capable of exerting maximum effects, with only modest quantities, it is of particular interest to examine concentrations of the 2OHE₁:16OHE₁ EMR *in vitro*. With an incomplete understanding of the etiology of disease, but an established relationship between cumulative estrogen exposure and breast cancer risk, it is feasible to conclude that 2OHE₁:16OHE₁ EMR concentrations in breast tissue may directly affect breast cancer risk. Furthermore, when taking race into account, investigations of this level may reveal more absolute evidence of differences in estrogen exposure and metabolism by race.

Breast cancer is a complex disease that affects all women. Although the overall mortality rate has declined in recent years, more research is needed to significantly improve current treatment and prevention methods that are sufficient for all women. Therefore, this study has significant implications for public health, as the overall goal of this research is to identify a possible biological explanation for the disparity in breast cancer between African American and Caucasian women. Furthermore, this study seeks to confirm the relationship between the *CYP1B1* Leu432Val polymorphism and the 2OHE₁:16OHE₁ EMR, which could lead to the identification of high risk populations. Additionally, the relationships between the 2OHE₁:16OHE₁ EMR, mammographic density, and race were also examined. As the biological mechanism by which mammographic density affects breast cancer risk is unknown, and the risk associated with mammographic density is significantly greater than the associated risk of all other clinical risk factors, this investigation serves to determine if the 2OHE₁:16OHE₁ EMR can be utilized to explain variations in mammographic density, and if this relationship differs by race. Lastly, this study seeks to determine if 2OHE₁:16OHE₁ EMR concentrations can be assayed *in vitro*, and if this ratio is altered by additional estrogen exposure and cell type. These results will provide a better understanding of the etiology of disease, as the 2OHE₁:16OHE₁ EMR was assayed directly from breast tissue.

2.0 SPECIFIC AIMS

Again, the objective of this study was to explore some of the relationships found between the 2OHE₁:16OHE₁ EMR, the *CYP1B1* Leu432Val polymorphism, mammographic density, race, and breast cancer risk. This goal was addressed in three manuscripts, each designed to satisfy the following specific aims.

The Effect of a CYP1B1 Polymorphism on the 2OHE₁:16OHE₁ Estrogen Metabolite Ratio and Breast Cancer Risk

Specific Aim I: To explore the relationship between the urinary 2OHE₁:16OHE₁ EMR and the role of the *CYP1B1* Leu432Val polymorphism in a case-control population of Caucasian women.

Recent evidence has found an association between the *CYP1B1* Leu432Val genotype and the urinary 2OHE₁:16OHE₁ EMR, suggesting that the presence of the variant allele may mediate breast cancer risk. We hypothesize a negative relationship will be observed when evaluating the 2OHE₁:16OHE₁ EMR and breast cancer risk, as the evidence suggest a low 2OHE₁:16OHE₁ EMR increases breast cancer risk. Moreover, we also hypothesize that a negative relationship will be observed when assessing the association of the *CYP1B1* Leu432Val and the 2OHE₁:16OHE₁ EMR. This polymorphism has been reported as being associated with the urinary 2OHE₁:16OHE₁ EMR and the presence of the variant allele has been reported to be associated with elevated breast cancer risk. Therefore, we believe that the Leu432Val polymorphism will be negatively associated with the 2OHE₁:16OHE₁ EMR.

The 2OHE₁:16OE₁ Estrogen Metabolite Ratio, Mammographic Density, and Race:

Examining the relationship between complex traits

Specific Aim II: To explore the relationship between serum 2OHE₁:16OHE₁ EMR and mammographic density, in a cohort of healthy, pre- and postmenopausal African American and Caucasian women.

Given the classical method of endocrinology and the use of postmenopausal women, the estrogen metabolites found in the blood will provide the best assessment of the estrogens being produced, as the ovaries in these women no longer produce sufficient quantities of estrogen. This investigation will provide insight to the disparity among African American women by revealing whether or not race influences the 2OHE₁:16OHE₁ EMR. We will also evaluate the relationship between this ratio and mammographic density, as well as other known breast cancer risk factors. We hypothesize a negative association will be observed with mammographic density and the 2OHE₁:16OHE₁ EMR, as increased breast density is associated with increased breast cancer risk. We also hypothesize that a negative relationship will be observed when evaluating the association between race and the 2OHE₁:16OHE₁ EMR. The risk of developing breast cancer for African American women is greatest under the age of 40, after this age, the risk becomes greater for Caucasian women, yet the mortality rate of African American women exceeds that of Caucasian women at every age. Therefore, we expect there to be a negative relationship between race/ethnicity and the 2OHE₁:16OHE₁ EMR.

An In Vitro Analysis of the 2OHE₁:16OHE₁ Estrogen Metabolite Ratio in Breast Epithelial Cells

Specific Aim III: To determine the 2OHE₁:16OHE₁ EMR in normal and tumor breast epithelial cell lines of African American and Caucasian women.

Evidence now suggests that the estrogens produced locally are capable of exerting a maximum effect, with only modest quantities. This novel *in vitro* assay will assess the 2OHE₁:16OHE₁ EMR in normal and tumor established breast epithelial cell lines derived from African American and Caucasian women. This evaluation has great implications, as to date; no other study has compared the effects of estradiol treatment in cell lines derived from these two racial/ethnic groups. We hypothesize that there will be a significant difference in the mean 2OHE₁:16OHE₁ EMR in tumor versus normal cells, in cell lines derived from African American versus Caucasian women, and in treated versus untreated cell lines. We also hypothesize that cell type, race, and estradiol treatment will all be significant predictors of the 2OHE₁:16OHE₁ EMR.

**3.0 THE EFFECT OF A CYP1B1 POLYMORPHISM ON THE 2OHE₁:16OHE₁
ESTROGEN METABOLITE RATIO AND BREAST CANCER RISK**

For Future Publication

Lloyd S¹, Taioli E², Jiangying Chen³, Gretchen Arhendt⁴, Feingold E¹, & Garte S⁵

Department of Human Genetics¹

Magee-Women's Hospital of UPMC⁴

Department of Environmental and Occupational Health⁵

University of Pittsburgh

Pittsburgh, PA

SUNY Downstate Medical Center²

Brooklyn, NY

Guangzhou Red Cross Hospital³

Guangzhou, Guangdong, China

3.1 ABSTRACT

Purpose: Urinary levels of the ratio of two estrogen metabolites, 2 – hydroxyestrone (2OHE₁) and 16 α – hydroxyestrone (16OHE₁) have been implicated as a marker of breast cancer risk. The Leu432Val polymorphism in the estrogen metabolism gene, *Cytochrome P450 1B1* (*CYP1B1*), has been found to be associated with urinary levels of the 2OHE₁:16OHE₁ estrogen metabolite ratio (EMR). The objective of this study was to determine if the Leu432Val polymorphism has any influence on breast cancer risk through variations in the urinary 2OHE₁:16OHE₁ EMR.

Methods: A case-control population of 137 Caucasian women was analyzed. Cases were identified through the Breast Cancer Surgical Registry at Magee-Women’s Hospital, Pittsburgh, PA, while controls were obtained from previous genetic association studies. Urinary estrogen metabolites were assayed using a basic Enzyme Linked Immunosorbent Assay (ELISA), and genotyping was done using a basic Restriction Fragment Length Polymorphism-PCR.

Results: A significant association between the 2OHE₁:16OHE₁ EMR and breast cancer was observed (OR = 0.289; 95% CI 0.137 – 0.613; p = 0.0012). Overall, the *CYP1B1* Leu432Val polymorphism was not associated with breast cancer risk, but was associated with the 2OHE₁:16OHE₁ EMR (p <0.0001). Associations with other known risk factors, such as age (p = 0.0064) and an age and BMI interaction (p = 0.0397), were also found.

Conclusion: The urinary 2OHE₁:16OHE₁ EMR may play a role in modifying breast cancer risk. The *CYP1B1* genotype does not appear to mediate this risk, but if it has any role in breast cancer risk, it may do so through variations in the 2OHE₁:16OHE₁ EMR.

3.2 INTRODUCTION

Since the report that estrogens are associated with breast cancer, epidemiological studies have focused on the effects that estrogen metabolites may have in modifying breast cancer risk (T. H. Lippert et al., 2000). The ratio of two metabolites, 2-hydroxyestrone (2OHE₁) and 16 α -hydroxyestrone (16OHE₁), has been implicated as a marker of breast cancer risk due to their contrasting biological properties (Lord et al., 2002). 2OHE₁ is capable of binding to the estrogen receptor (ER) with markedly reduced binding affinity, thus showing weakly estrogenic properties. Conversely, 16OHE₁ is a potent estrogenic molecule that functions by activating the ER (Zhu & Conney, 1998). Therefore, the ratio of 2OHE₁ and 16OHE₁ appears to regulate the proportion of anti-estrogenic molecules to estrogenic ones. It is hypothesized that women who metabolize estrogen primarily through the 2OHE₁ pathway have a lower risk of developing breast cancer, while those who metabolize estrogens principally through the 16OHE₁ pathway, are at an increased risk of developing breast cancer (C. Lippert, Seeger, & Mueck, 2003).

Estrogen biosynthesis and metabolism is catalyzed by the *Cytochrome P450 (CYP)* superfamily of genes, which encode a number of enzymes that are directly involved in drug and steroid metabolism (Nebert & Russell, 2002). The *CYP1B1* gene maps to chromosome 2p22-p21, and consist of three exons and two introns, which contain the entire coding sequence of the gene (Paracchini et al., 2005; Paracchini et al., 2007). An inducible enzyme capable of initiating a variety of carcinogens such as arylamines, nitrosamines, and arylenes, *CYP1B1* is expressed in a number of tissues including the kidney, prostate, uterus, ovary, and breast (Beuten et al., 2008; Ingelman-Sundberg, 2004; Paracchini et al., 2005). In the breast, this gene is believed to be involved in the metabolic regulation of estrogen homeostasis, and is highly expressed in breast tumors (Ingelman-Sundberg, 2004; Paracchini et al., 2005). Evidence also suggests that *CYP1B1*

is responsible for the hydroxylation of estradiol at the C-16 position, leading to the formation of 16OHE₁ (Badawi et al., 2001; Cribb et al., 2006; Lord et al., 2002; Tsuchiya et al., 2005). This implies that variation in this gene could modify estradiol catabolism, and may in turn alter breast cancer risk.

A single nucleotide polymorphism (SNP) located at position 1666 in exon 3, is a C→G transversion, which results in an amino acid substitution of leucine (Leu) to valine (Val) at codon 432 (Paracchini et al., 2005). Exon 3 codes for the heme-binding domain of the gene, a region that is critical to its catalytic function. While the mechanistic function of this SNP is unknown, it is believed to affect mRNA stability, and may be in linkage disequilibrium with transcriptional regulatory sequences such as response elements, or the regulating elements of RNA degradation of other *CYP1B1* variants (Paracchini et al., 2007). Previous literature suggests that the presence of the variant valine allele is associated with increasing breast cancer risk, yet these results are inconsistent (Greendale et al., 2005; Le Marchand, Donlon, Kolonel, Henderson, & Wilkens, 2005; Long et al., 2007; Rylander-Rudqvist et al., 2003; Sowers, Wilson, Karvonen-Gutierrez, & Kardia, 2006).

In a meta and pooled analysis by Paracchini, Raimondi, Gram, et al. (2007), the authors observed racial, as well as age differences in the association between the *CYP1B1* Leu432Val polymorphism and breast cancer risk. A possible association between the *CYP1B1* Leu432Val polymorphism and breast cancer was found, but only among Caucasians. Furthermore, this effect was not seen among Asians, and a possible negative association was found among African American or mixed populations. Additionally, among Caucasians, the results of their pooled analysis revealed a significant age effect, with an increase risk of breast cancer occurring

between the ages of 45 and 59 years, while this risk was much lower for both younger and older women (Paracchini et al., 2007).

The purpose of this study was to evaluate the effects of the *CYP1B1* genotype on the urinary 2OHE₁:16OHE₁ EMR and the association of the *CYP1B1* genotype and 2OHE₁:16OHE₁ EMR on breast cancer. The role of other known breast cancer risk factors, including age, BMI, and smoking status was also assessed.

3.3 MATERIALS AND METHODS

3.3.1 Study Population

A total of 137 Caucasian women were selected for this study. Participants' ages ranged from 20 to 78 years. Thirty-three cases were identified through the Breast Cancer Surgical Registry at Magee Women's Hospital, Pittsburgh, PA. This database, developed in 2005, consists of women undergoing breast cancer surgery at Magee Women's Hospital that consented to being added the registry (Im et al., 2009). A urine sample and matching breast epithelial tissue sample were obtained for each case. A total of 104 control participants were recruited to take part in several studies, and are described elsewhere (Paracchini et al., 2005). These investigations include analyses on the genetics of bone density, a study regarding diet and genetic factors, and a study on the effects of indole-3-carbinol on urinary estrogen metabolites. All control participants provided a urine sample and matching peripheral blood sample for genetic analysis.

Demographic variables such as age, BMI, and smoking status, were collected utilizing questionnaire data from controls, and the medical records of cases. BMI was calculated by

dividing the participants' weight in kilograms by their height in meters squared, while smoking status was a dichotomous variable, classifying subjects as "never" or "ever" smokers. Age and the 2OHE₁:16OHE₁ EMR are continuous variables, while the *CYP1B1* genotypes were categorized according to the subjects' respective genotype (i.e. reference genotype – Leu/Leu; heterozygous genotype – Leu/Val; homozygous variant – Val/Val).

3.3.2 DNA Extraction and Genotyping

Genomic DNA was extracted from the breast epithelial tissue and peripheral blood samples using the DNA extraction kit developed by Stratagene (Stratagene, LaJolla, CA) and Genra-System Tissue Kit (QIAGEN, Minneapolis, MN), respectively, according to the manufacturer's instructions. The *CYP1B1* genotype was determined using RFLP PCR in 50µl reactions (forward and reverse primers: 5'-CTGCCAACACCTCTGTCTTG-3' and 5'-CTGAAATCGCACTGGTGAGC-3'). PCR was performed at 95° C for 10 minutes, and 35 cycles at 95° C, 60° C, and 72° C for 1 minute each. A final annealing cycle was conducted at 72° C for 7 minutes and 12° C for 9 hours. The PCR products were then digested utilizing *Eco571* restriction digest in a 30µl reaction at 37° C for 12 hours. Digestion products were verified on a 2% agarose gel.

3.3.3 2OHE₁:16OHE₁ Estrogen Metabolite Assay

To quantify urinary concentrations of 2OHE₁ and 16OHE₁, an Enzyme Linked Immunosorbent Assay (ELISA) purchased from the Immuna Care Corporation (Immuna Care, Bethlehem, PA) was used. The estrogens were deconjugated by the addition of a β-glucuronidase and an

arylsulphatase enzyme. Seventy-five microliters of the enzyme digest were added to two (2) 96 well microtiter plates, one labeled “2OHE1” and the other “16OHE1.” These plates were then coated with anti-2OHE1 and anti-16OHE1 monoclonal antibodies. An alkaline phosphatase solution containing monoclonal antibodies to 2OHE₁ and 16OHE₁ was added directly to each well. After incubation, a basic solution of paranitrophenyl phosphate (pNPP) was added to detect the alkaline phosphatase solution (Klug, Bradlow, & Sepkovic, 1994). The microtiter plates were read using a Biotek ELx808 plate reader at 405 nm.

3.3.4 Statistical Analysis

All analyses were conducted using SAS version 9.2 (SAS Institute, Cary, North Carolina) software, and a 2-sided alpha level of 0.05. Logistic regression analysis was conducted to estimate odds ratios (OR) and 95% confidence intervals (CI) for the association between case-control status and the 2OHE₁:16OHE₁ EMR, the *CYP1B1* genotype, age, BMI, and smoking. Potential interactions, such as genotype and the 2OHE₁:16OHE₁ EMR; BMI and 2OHE₁:16OHE₁ EMR; age and BMI; age and 2OHE₁:16OHE₁ EMR; and the 2OHE₁:16OHE₁ EMR and smoking status were also examined.

Linear regression analyses of the 2OHE₁:16OHE₁ EMR on *CYP1B1* genotype, BMI, age, and case-control status were conducted to evaluate the association between the *CYP1B1* Leu432Val polymorphism and the 2OHE₁:16OHE₁ EMR separately in cases and controls. Potential interactions between age, BMI, and smoking were also investigated. For all regression analyses, the *CYP1B1* genotype was identified as 0 (reference genotype), 1 (heterozygous), 2 (homozygous variant).

3.4 RESULTS

Values for the 2OHE₁:16OHE₁ EMR ranged from 0.165611 to 5.20098, however one subject had a value of 19.30287, which was considered an extreme outlier. As a result, this observation was removed from all analyses to avoid extreme influential effects. Therefore, only 136 participants (104 controls and 32 cases) were included in the analysis.

Table 4 contains a detailed description of the study population. We observed that cases and controls differed significantly in age and BMI, ($p < 0.0001$) and ($p < 0.0001$), respectively. Additionally, a significantly lower 2OHE₁:16OHE₁ EMR was found among cases when compared to controls (1.42 versus 2.11; $p < 0.0003$). Similarly, there was also a statistical difference in the *CYP1B1* genotype frequency between cases and controls (Armitage trend test $p = 0.0129$). As there were so few smoking breast cancer cases in comparison to controls, this variable was removed from further analyses.

Table 4. Breast Cancer Case-Control Study Population Characteristics

	Cases	Controls	p – value
	N = 32	N = 104	
Age, years. (\bar{y})	56.0	35.1	<0.0001
BMI (\bar{y})	27.8	22.8	<0.0001
2OHE₁:16OHE₁ EMR (\bar{y})	1.42	2.11	0.0003
CYP1B1 Genotype *	Leu/Leu: 12	Leu/Leu: 18	0.0129**
	Leu/Val: 16	Leu/Val: 59	
	Val/Val: 4	Val/Val: 27	
Smoking Status	<i>Never</i> : 29	<i>Never</i> : 80	0.0147***
	<i>Ever</i> : 3	<i>Ever</i> : 24	

*Hardy Weinberg Equilibrium $\chi^2 = 0.2297$, ** Armitage trend test, *** Fisher's exact p- value

Logistic regression modeling was used to identify key variables that significantly predict the likelihood of being a case or a control (Table 5). The only statistically significant variables that contributed to the prediction of case status were age and the 2OHE₁:16OHE₁ EMR ($p < 0.0001$ and $p < 0.0012$, respectively). In fact, there was a positive relationship with age that increased the odds of being a case by 16% (OR = 1.161; 95% CI 1.094 – 1.233). On the other hand, a negative relationship was observed between the 2OHE₁:16OHE₁ EMR and case status, which decreased the risk of being a case by approximately 71% (OR = 0.289; 95% CI 0.137 – 0.613). However, the *CYP1B1* genotype was not a predictor of breast cancer status. Interactions between the 2OHE₁:16OHE₁ EMR and *CYP1B1* genotype; BMI and the 2OHE₁:16OHE₁ EMR; age and the 2OHE₁:16OHE₁ EMR; and age and BMI were also evaluated, yet, no significant relationships were observed.

Table 5. Predictors of Breast Cancer

Variable	Parameter Estimate	OR	95% CI	<i>p</i> value
Age*	0.1497	1.161	(1.094 - 1.233)	<0.0001
2OHE ₁ :16OHE ₁ EMR*	-1.2399	0.289	(0.137 – 0.613)	0.0012
CYP1B1*	-0.3725	0.689	(0.271 – 1.755)	0.4348
BMI*	0.0693	1.048	(0.915 – 1.200)	0.5007
EMR*CYP1B1 [€]	-0.1910			0.7570
BMI*2OHE ₁ :16OHE ₁ EMR [€]	-0.0412			0.6437
Age*2OHE ₁ :16OHE ₁ EMR [€]	0.0223			0.3425
Age*BMI [€]	-0.00625			0.1450

* Logistic Regression model 1; € Logistic Regression model 2

Multiple linear regression analyses were conducted to evaluate the association between the 2OHE₁:16OHE₁ EMR and the *CYP1B1* genotype (Table 6). Among all participants, significant positive associations between age, the *CYP1B1* genotype, and the 2OHE₁:16OHE₁ EMR were observed (age: $p = 0.0014$; *CYP1B1*: $p = 0.0294$). A significant inverse association between case-control status and the 2OHE₁:16OHE₁ EMR was also observed ($p < 0.0001$). Since age and BMI are two highly correlated variables, the interaction between these two and the 2OHE₁:16OHE₁ EMR: was assessed, however, this did not reach statistical significance.

Table 6. Association between the 2OHE₁:16OHE₁ EMR and the *CYP1B1* genotype

Variable	Parameter Estimate	<i>p</i> - value
Age	0.08432	0.0014
<i>CYP1B1</i> Genotype	0.28558	0.0294
BMI	0.02391	0.6555
Case/Control Status	-1.1317	<0.0001
Age*BMI	-0.00168	0.1001

To determine if the prediction of the 2OHE₁:16OHE₁ EMR varied by case-control status, additional linear regression analyses stratified by case-control status were conducted (Table 7). Among cases, age was positively associated with the 2OHE₁:16OHE₁ EMR, while the age and BMI interaction was negatively associated with the 2OHE₁:16OHE₁ EMR (age: $p = 0.0064$; age*BMI: $p = 0.0397$). The control population also exhibited a positive relationship between 2OHE₁:16OHE₁ EMR and age, as well as a positive relationship with the *CYP1B1* genotype (age: $p = 0.0081$; *CYP1B1*: $p = 0.0249$). The interaction between age and BMI was not statistically significant ($p = 0.0599$) (Table 7b).

Table 7. Association between the 2OHE₁:16OHE₁ EMR and the *CYP1B1* genotype Stratified by Case-Control Status

Cases Only

Variable	Parameter Estimate	<i>p</i> - value
Age	0.18125	0.0064
<i>CYP1B1</i> Genotype	0.26883	0.2815
BMI	0.21430	0.1114
Age * BMI	-0.00462	0.0397

Controls Only

Variable	Parameter Estimate	<i>p</i> - value
Age	0.13208	0.0081
<i>CYP1B1</i> Genotype	0.34863	0.0249
BMI	0.08449	0.2884
Age * BMI	-0.00380	0.0599

3.5 DISCUSSION

The results of this study demonstrate an association between the 2OHE₁:16OHE₁ EMR and breast cancer. Additionally, the analysis suggests that having a high 2OHE₁:16OHE₁ EMR is associated with a lower probability of developing breast cancer, and vice versa. These findings are in line with several studies. A small case-control study reported that postmenopausal women who developed breast cancer at least 6-months after baseline, had 2OHE₁:16OHE₁ EMR levels approximately 15% lower than matched controls. The authors also observed that participants with 2OHE₁:16OHE₁ EMR levels in the highest tertile experienced an approximately 30% lower

risk of developing breast cancer, than those in the lowest tertile (OR = 0.71; 95% CI 0.29 – 1.75; $p = 0.46$); however, the study lacked sufficient power to detect this difference (Meilahn et al., 1998). Nonetheless, similar results were observed in another case-control study, in which postmenopausal cases had significantly lower 2OHE₁:16OHE₁ EMR than did controls (cases: 1.41 ± 0.73 vs. controls: 1.81 ± 0.71 ; $p - \text{value} = 0.05$) (Kabat, Chang, & Sparano, 1997). An additional study reported that greater hydroxylation at the C-2 position was associated with a reduction in invasive breast cancer risk among premenopausal women, and those whose with a 2OHE₁:16OHE₁ EMR in the highest quintile reduced their risk of invasive breast cancer approximately 20% compared to the relative risk of those in the lowest quintile (lowest quintile $[\leq 1.80] = 0.76$; highest quintile $[\geq 3.29] = 0.55$) (Muti et al., 2000). These results were also duplicated in another study by Kabat et al. in 2006, but only in premenopausal women (Kabat et al., 2006).

Conversely, an analysis investigating urinary levels of the 2OHE₁:16OHE₁ EMR in a population of only postmenopausal women, did not observe any evidence to suggest that a high 2OHE₁:16OHE₁ EMR was associated with a decrease in breast cancer risk (mid-tertile $[1.17 - 1.73]$ OR = 0.34; 95% CI 0.12 – 0.98; highest tertile $[>1.73]$ OR = 1.13; 95% CI 0.46-2.78; p for trend = 0.96). This result remained constant even after adjustment for dietary intake (mid-tertile $[1.17 - 1.73]$ OR = 0.32; highest tertile $[>1.73]$ OR = 1.14) (Ursin et al., 1999). Likewise, a prospective study reported that increased concentrations of 2OHE₁ were associated with an increased risk of estrogen-receptor positive breast cancer, among postmenopausal, current HRT users, while 16OHE₁ levels were not found to be significantly associated with breast cancer (Wellejus et al., 2005). Another study assessed urinary levels of the 2OHE₁:16OHE₁ EMR before and after breast cancer treatment, and found that the association between the

2OHE₁:16OHE₁ EMR and breast cancer is modified by the urine collection protocol. The authors noted that in samples taken prior to surgery or other forms of breast cancer treatment, that an increased 2OHE₁:16OHE₁ EMR was associated with lower breast cancer risk, while in post surgical/post-treatment samples, an increased 2OHE₁:16OHE₁ EMR was associated with increased breast cancer risk (Fowke et al., 2003).

While no significant relationships between the *CYP1B1* genotype and breast cancer were found, we did observe a significant association between the *CYP1B1* genotype and the 2OHE₁:16OHE₁ EMR. From this finding, one can imply that if this genotype modifies breast cancer risk, it may do so by altering the 2OHE₁:16OHE₁ EMR. Paracchini et al (2005) studied the link between the 2OHE₁:16OHE₁ EMR and the *CYP1B1* Leu432Val polymorphism. This analysis consisted of 150 healthy men and premenopausal women. The healthy women present in our analysis were a subset of the women published by Paracchini et al. The authors observed that the presence of the valine allele was associated with an increased urinary 2OHE₁:16OHE₁ EMR among Caucasian women (*CYP1B1* Val/Val = 1.95 ± 1.06; *CYP1B1* Val/Leu = 2.08 ± 1.06; *CYP1B1* Leu/Leu = 2.55 ± 1.37) (Paracchini et al., 2005). Our data is consistent with this finding with respect to controls.

When evaluating the relationship between other known risk factors for breast cancer and the 2OHE₁:16OHE₁ EMR, we observed a significant, positive association between age and the 2OHE₁:16OHE₁ EMR. To date, we have identified only one study that has examined the association between the 2OHE₁:16OHE₁ EMR and age, and no significant relationship was found (Kabat et al., 1997). Obesity has long been a known risk factor for breast cancer, especially among postmenopausal women; given that the large majority of estrogens are produced by adipose tissue (Baglietto et al., 2008; Clemons & Goss, 2001; Dignam et al., 2006).

Furthermore, overweight, postmenopausal women have been reported to be at an increased risk of developing estrogen-positive tumors (Palmer, Adams-Campbell, Boggs, Wise, & Rosenberg, 2007). In the present study, we were able to demonstrate a significant, inverse relationship between the 2OHE₁:16OHE₁ EMR and the age*BMI interaction, two known risk factors for breast cancer risk that are highly correlated. In a case-control study by Modugno et al (2006), the authors found that among women not using hormone therapy, low serum levels of 2OHE₁ in combination with a high BMI, increased ones risk of developing breast cancer (adjusted OR = 3.67; 95% CI 1.26 – 10.95), while those with high concentrations of serum 16OHE₁ and a high BMI were also at an increased risk of developing breast cancer (adjusted OR = 3.51; 95% CI 1.34 – 9.16) (Modugno, Kip et al., 2006). While very few studies assess the relationship between other breast cancer risk factors and the 2OHE₁:16OHE₁ EMR, an early study evaluated the association between this ratio and having a family history of breast cancer in a population of premenopausal women; however, no difference was detected between the two groups (Ursin et al., 2002).

Breast cancer is a complex disease, with both genetic and environmental factors mediating ones risk of developing this disease. The objective of this study was to evaluate whether or not the Leu432Val polymorphism in the estrogen metabolism gene, *CYP1B1*, has any influence on the urinary 2OHE₁:16OHE₁ EMR, which has been implicated as a marker of breast cancer risk. Given the potential of this gene variant to alter estrogen metabolism, this SNP has been extensively studied to determine its association with breast cancer and other known risk factors for breast cancer as well (Han et al., 2004; Hanna, Dawling, Roodi, Guengerich, & Parl, 2000; Rylander-Rudqvist et al., 2003; Van Emburgh et al., 2008). While we did not observe a direct association with the *CYP1B1* genotype and breast cancer, previous studies have supported

the presence of a relationship. A case-control study conducted in Turkey (Kocabas et al. (2002)) assessed the association of the *CYP1B1* Leu432Val polymorphism and breast cancer. The authors found that the presence of the valine allele was associated with a significantly increased susceptibility to breast cancer. The adjusted odds ratio for age, age at menopause, age at first full term pregnancy, BMI, and smoking status was 2.32 (95% CI 1.26 - 4.25; p = 0.007). The authors also observed that the *CYP1B1* genotype was related to breast cancer among women with a BMI greater than 24 kg/m² (adjusted OR = 2.78; 95% CI 1.36 - 5.62; p = 0.005) (Kocabas et al., 2002).

Although our study presents the findings of some novel research, it is also subject to several limitations. A case-control study is a useful first step when exploring a source of disease, and allowed us to investigate a variety of characteristics that may lead to an adverse health outcome, we were unable to obtain the absolute risk (incidence) of disease, nor could we determine the relative risk of disease in the study population (Gordis, 2004). Furthermore, the use of a study population that consisted only of Caucasian women prevented applicable comparisons to other racial/ethnic groups. We also observed some dissimilarities between our cases and controls (e.g., age and BMI) that make drawing solid conclusions difficult.

Selection bias may also have been introduced into our research, as our controls were obtained from other studies. Therefore, the selection, as well as exclusion criteria may have differed for each group. Other limitations include reporting and recall bias, due to the use of self-reported questionnaires, and small sample sizes, which limited our power of detection.

Another limitation of this study was the inability to more accurately assess the role of the *CYP1B1* Leu432Val polymorphism in breast cancer risk. We have, however, shown an association between this polymorphism and the 2OHE₁:16OHE₁ EMR and a relationship

between 2OHE₁:16OHE₁ EMR and breast cancer risk. Additionally, the large majority of examinations assessing the 2OHE₁:16OHE₁ EMR and breast cancer risk, are case – control studies, that do not investigate the contributing factors to the 2OHE₁:16OHE₁ EMR. For this reason alone, our research is a great contribution to the literature.

In conclusion, our data suggests that the 2OHE₁:16OHE₁ EMR is associated with breast cancer risk. While we were unable to detect a direct association between the *CYP1B1* Leu432Val polymorphism and breast cancer risk, we have demonstrated that the *CYP1B1* genotype is associated with the 2OHE₁:16OHE₁ EMR, thereby suggesting that the *CYP1B1* Leu432Val polymorphism may alter breast cancer risk by modifying this ratio.

4.0 THE 2OHE₁:16OHE₁ ESTROGEN METABOLITE RATIO, MAMMOGRAPHIC DENSITY, AND RACE: EXAMINING THE RELATIONSHIP BETWEEN COMPLEX TRAITS

For Future Publication

Lloyd S¹, Taioli E², Feingold E¹, & Garte S⁴

Department of Human Genetics¹

Department of Environmental and Occupational Health⁴

University of Pittsburgh

Pittsburgh, PA

SUNY Downstate Medical Center²

Brooklyn, NY

4.1 ABSTRACT

Purpose: Mammographic density is one of the strongest predictors of breast cancer risk. While the role of estrogens in breast cancer risk has long been established, the association between estrogen metabolites and mammographic density has not been fully elucidated. This study assesses the relationship between mammographic density and serum concentrations of the 2OHE₁:16OHE₁ EMR according to race. **Methods:** The study population consisted of 120, cancer-free participants from the Mammograms and Masses Study. Serum estrogen metabolites were assayed by the Immuna Care Corp, utilizing a competitive, solid-phase enzyme immunoassay. Mammographic density was measured using the classification by Wolfe and a compensating polar planimeter. **Results:** Percent mammographic density was significantly lower in black than in white women, but no significant associations with the 2OHE₁:16OHE₁ EMR were observed. BMI and smoking were significantly inversely associated with percent mammographic density in white women, while no significant predictors of percent mammographic density were found among black women. **Conclusion:** Serum concentrations of the 2OHE₁:16OHE₁ EMR are not associated with percent mammographic density. Predictors of mammographic density are different between black and white women, suggesting different biological pathways to breast carcinogenesis.

4.2 INTRODUCTION

One of the strongest predictors of breast cancer risk is mammographic density (Baglietto et al., 2008; Bremnes, Ursin, Bjurstam, Rinaldi et al., 2007; Crandall et al., 2008; Greendale et al., 2005; Gruber et al., 2002; Johansson et al., 2008; Tamimi et al., 2007; Tamimi et al., 2005). In fact, the risk of developing breast cancer associated with mammographic density is greater than the risk of all other clinical risk factors (Greendale et al., 2005). Mammographic density is the proportion of fibrous, connective and epithelial tissue, to total breast area (Aiello et al., 2005; Martin & Boyd, 2008; Vachon et al., 2000). Women with mammographic breast density greater than or equal to 75%, are at a four to six-fold greater risk of developing breast cancer when compared to those with no measurable dense tissue (Aiello et al., 2005; N. F. Boyd et al., 2009; Greendale et al., 2005; Tamimi et al., 2007; Tamimi et al., 2005).

While the biological mechanism through which mammographic density predicts breast cancer risk is unknown, many of the established risk factors for breast cancer have also been shown to be associated with variations in breast density (i.e., age, menopausal status, parity, BMI, and HRT) (N. F. Boyd et al., 2009; Haiman et al., 2003; Vachon et al., 2000; Warren et al., 2006; Woolcott et al., 2009). As these risk factors all share a relationship to circulating hormone levels, investigators have sought to determine the role of endogenous estrogens on mammographic density. It has been hypothesized that breast density is related to cumulative estrogen exposure, and that more dense breasts represents increased cellular proliferation (Ziv et al., 2004). However, few studies have examined the association between estrogen metabolites and mammographic density.

The ratio of two particular estrogen metabolites, 2-hydroxyestrone (2OHE₁) and 16 α -hydroxyestrone (16OHE₁), has long been implicated as a marker of breast cancer risk. 2OHE₁ is

believed to be devoid of estrogenic property, while 16OHE₁ is believed to be an estrogenic compound, and as such has been shown to significantly increase cell proliferation. Therefore, it is hypothesized that women who metabolize estrogen primarily through the 2OHE₁ pathway have a lower risk of developing breast cancer, and vice versa for the 16OHE₁ pathway (Seeger et al., 2006; Vandewalle & Lefebvre, 1989; Zhu & Conney, 1998).

In this study, the association between mammographic density and the 2OHE₁:16OHE₁ EMR has been assessed in a population of healthy black and white women. Breast cancer is the most commonly diagnosed cancer among black women in the US. From 2001 to 2005, African American women experienced a 37% higher mortality rate than Caucasian women (American Cancer Society, 2008a, 2009). Furthermore, women of African descent are more likely to be diagnosed with breast cancer at a more advanced stage (which includes some stage III and stage IV tumors); are more likely to have larger, estrogen-negative, high grade tumors; and are more likely to have lymph node involvement, all of which are poor prognostic predictors of survival (Breastcancer.org, 2008; Chlebowski et al., 2005; Curtis et al., 2008). Current studies evaluating mammographic breast density in black women have been inconsistent. Some have reported that these women have significantly greater breast density than do white women (Chen et al., 2004; El-Bastawissi et al., 2001), but others have reported that black women have lower breast density when compared to white women (del Carmen et al., 2003; Ursin et al., 2003), or no difference in mammographic breast density, when comparing these two racial/ethnic groups (del Carmen et al., 2007). The present study explores the association between estrogen metabolites and breast density, while highlighting any potential differences that may occur by race.

4.3 MATERIALS AND METHODS

4.3.1 Study Population

This study population consisted of 120 participants from the Mammograms and Masses Study (MAMS), which is composed of pre- and postmenopausal women, age 18 and older, showing no sign of breast cancer after mammography or biopsy. Participants were excluded if they had a prior history of cancer (excluding cancer of the skin), drank more than five drinks per day, or weighed less than 110 pounds or greater than 300 pounds. Participants were enrolled from September 2001 until May 2005 from three hospital sources: 1) the Diagnostic Imaging Clinic at Magee-Women's Hospital, Pittsburgh, PA, 2) the Surgical Clinic at Magee-Women's Hospital, and 3) Magee-Women's Hospital or Magee Woman-care mammography clinics in the Pittsburgh area. Women receiving routine mammography screening exams were identified as „well controls,“ and were recruited through a study flyer that was attached to their negative screening results. Other participants with a suspicious lesion that was identified during mammography, and subsequently underwent breast biopsy were personally recruited by the research assistant during their visit to the Breast Biopsy Service if their biopsy showed no sign of cancer. The original MAMS study includes a total population of 1, 133 participants, in which personal medical history, HRT use, family history, and other demographics were obtained via self-report questionnaires, and non-fasting blood samples were taken. A subset of the population, 424 women (~30%) had serum estrogen metabolite measured.

This subpopulation included 374 Caucasians, 40 African Americans, and 10 participants that identified themselves as Asian/Pacific Islander, Hispanic/Latina, American Indian, or “other.” For the present study, all African American samples (40) matched on age and

menopausal status to 80 Caucasian women (N= 120) were selected. This study was approved by the Institutional Review Board of the University of Pittsburgh (Reeves, Gierach, & Modugno, 2007).

4.3.2 Measures of Mammographic Density

Consent was obtained from all MAMS participants for the research staff to receive copies of the participant's original screening mammogram that were taken immediately before the participant's breast biopsy or the corresponding mammogram to which the recruitment flyer was attached. Three different measures of mammographic density (dense breast area, total breast area, and percent breast density) were analyzed by M.S., an expert reader (Benichou et al., 2003; Haiman et al., 2003; Wolfe, Saftlas, & Salane, 1987). For all measures, the craniocaudal view was used, unless unavailable, wherein the mediolateral view was used instead. The reader was blinded to the identity of all mammograms. Participant's mammograms were analyzed with two different methods. The first approach involved visual inspection, and classification based on the system developed by Wolfe in 1976 (Wolfe, 1976). Each participant was placed into one of the four parenchymal pattern categories: N1 = little to no measurable dense tissue, where the risk of breast cancer is lowest; DY = mammographically dense breast, where the risk of breast cancer is highest; P1 and P2 = variable density levels, where the risk of breast cancer intermediate (Boyd et al., 2005). The second method involved a compensating polar planimeter (LASICO, Los Angeles, CA), a wax pencil, and a transparent overlay, to outline the entire breast, and areas of dense tissue, to quantify total breast area, and dense breast area. Breast density was calculated as the proportion of visibly dense breast to total breast area, expressed as a percentage.

To evaluate the reproducibility of the readings, a random sample of 28 mammograms were reassessed, and intraclass correlation coefficients (ICC) for intra-observer agreement were calculated for measure of breast density. The ICC for dense area measurements was $\rho=0.86$, $\rho=0.99$ for total area, and $\rho=0.89$ for percent breast density, indicating high reproducibility. The ICC derived for percent density is consistent with the reproducibility by the same reader in the Breast Cancer Detection Demonstration Project (Benichou et al., 2003).

4.3.3 ESTRAMET™ 2OHE₁ and 16OHE₁ Serum Assay

All serum estrogen metabolites were assayed using the *ESTRAMET™* 2OHE₁ and 16 α OHE₁ Serum Assay, in the laboratory of Dr. Thomas Klug (Immuna Care, Bethlehem, PA). This assay is a competitive, solid-phase enzyme immunoassay. First, each antigen, 2OHE₁ or 16OHE₁ was conjugated to an alkaline phosphatase solution. This enzyme-conjugate solution was then added to the specified 96-well microtiter plate, coated with anti-2OHE₁ or anti-16OHE₁ monoclonal antibodies, and incubated overnight. The microtiter plates were then washed, and a colored enzyme substrate solution was added, eliciting a chemical reaction with the antigen-enzyme conjugate bound to the plate. The plates were then read by a microtiter plate reader at 620 nm. This assay has a sensitivity of less than 20pg/ml for both 2OHE₁ and 16OHE₁. The coefficient of variation (CV) for 2OHE₁ ranged from 0.0 – 14.4, with only three samples having CVs outside of this range. CV values for 16OHE₁ ranged from 1.3 to 17.9, with only one observation having a value outside of this range.

4.3.4 Statistical Analysis

All analyses were conducted using SAS version 9.2 (SAS Institute, Cary, North Carolina) software, and a 2-sided alpha level of 0.05. Two sample t-tests were utilized to assess mean differences between continuous variables, and χ^2 tests were used to analyze discrete measures. Linear regression modeling was utilized to evaluate the relationship between mammographic density and the 2OHE₁:16OE₁ EMR. Demographic variables, such as age, BMI, race, family history of breast cancer, pregnancy status, number of live births, OC use, smoking status, HRT, and menopausal status, were included in all models, based on the results of previous literature that indicate the significant impact these factors have on mammographic density (N. F. Boyd et al., 2009; Haiman et al., 2003; Vachon et al., 2000; Warren, 2004; Woolcott et al., 2009). Age and BMI were added to the models as continuous variables, while the remaining variables (race, family history of breast cancer, pregnancy status, number of live births, OC use, smoking status, HRT, and menopausal status) were identified as categorical variables, and dummy variables were created as necessary. Subsequent analyses were also stratified by race, to evaluate if the relationship between mammographic density and the 2OHE₁:16OE₁ EMR varied by race.

An initial review of the data showed that the 2OHE₁:16OE₁ EMR and the percent density variable were not normally distributed; therefore, the 2OHE₁:16OE₁ EMR ratio were log transformed, and percent density was square root transformed, in order to normalize the data. However, the results are presented in the untransformed version for easy interpretation.

4.4 RESULTS

Of the total population, 42 participants were diagnosed with benign breast disease (BBD). Preliminary analyses were conducted to assess the relationship between BBD and mammographic density, but these results did not reveal any statistically significant difference in percent density between „well controls“ and those diagnosed with BBD. Similar results were obtained when stratified these analyses by race (data not shown). Therefore, all study participants were analyzed together.

The participants had a mean age of 55 years, were mostly postmenopausal, overweight, non-smoking women. Additionally, the majority of women did not have a family history of breast cancer and had given birth three or more times. Overall, the mean percent density of our study population was 30.9% (Table 1).

Table 8. MAMS Study Population Characteristics, overall and according to race

Variable	N = 120*	White women (n = 80)*	Black women (n = 40)*	p-value [‡]
Age, mean ± SD	55.6 ± 10	55.6 ± 10	55.5 ± 9.9	0.96
BMI, mean ± SD	28.8 ± 6.2	27.4	31.6	0.0004
% Density, mean ± SD	30.9 ± 2.1	36.4 ± 1.9	21.5 ± 2.2	0.0006
2OHE ₁ :16OHE ₁ EMR, mean ± SD	0.52 ± 0.6	0.54	0.48	0.35
Family History, n (%)	No: 97 (81) Yes: 19 (16)	No: 64 (80) Yes: 15 (19)	No: 33 (83) Yes: 4 (10)	0.42 [‡]
Ever Pregnant, n (%)	No: 23 (19) Yes: 91 (76)	No: 17 (21) Yes: 62 (78)	No: 6 (15) Yes: 29 (73)	0.59
Number of Live Births, n (%)	None: 30 (25) 1 Live Birth: 15 (13) 2 Live Births: 29 (24) 3+ Live Births: 40 (33)	None: 20 (25) 1 Live Birth: 10 (13) 2 Live Births: 23 (29) 3+ Live Births: 26 (33)	None: 10 (25) 1 Live Birth: 5 (13) 2 Live Births: 6 (15) 3+ Live Births: 14 (35)	0.60
OC Use, n (%)	No: 40 (33) Yes: 74 (62)	No: 27 (34) Yes: 52 (65)	No: 13 (33) Yes: 22 (55)	0.76
Ever Smoke, n (%)	No: 63 (53) Yes: 51 (43)	No: 48 (60) Yes: 31 (39)	No: 15 (38) Yes: 20 (50)	0.08
HRT, n (%)	Never: 48 (40) Former: 32 (27) Current: 36 (30)	Never: 32 (40) Former: 18 (23) Current: 30 (38)	Never: 16 (40) Former: 14 (35) Current: 6 (15)	0.05
Menopausal Status, n (%)	Premenopausal: 33 (27.5) Postmenopausal: 87 (72.5)	Premenopausal: 22 (28) Postmenopausal: 58 (73)	Premenopausal: 11 (28) Postmenopausal: 29 (73)	1.00

* Numbers for some characteristics may not add up due to missing variables; [‡] p-value: difference between blacks and whites; [‡] Fisher's Exact between white and black women

When the study population was stratified by race, black women had significantly higher BMI values than did whites, (31.6 vs. 27.3; $p = 0.0004$). A statistically significant difference in percent density was also observed between black and white women, with whites having breast density 41% higher than that of African Americans ($p = 0.0006$). Among white women, there was an equal distribution of current and never HRT users, while black women were more likely to have never undergone HRT. No statistically significant differences by race were observed for the 2OHE₁:16OHE₁ EMR, family history of breast cancer, pregnancy status, number of live births, OC use, as well as smoking and menopausal status (Table 2).

Among the predictors of mammographic density, the 2OHE₁:16OHE₁ EMR was not a significantly contributing factor. The only statistically significant relationships were between percent mammographic density and BMI ($p = 0.008$), and percent mammographic density and smoking status ($p = 0.002$) (Table 3).

Table 9. Association between Mammographic Density and demographic variables

Variable	Reference Group	Parameter Estimate	95% CI	p-value
2OHE ₁ :16OHE ₁ EMR	Continuous variable	-0.11	(-0.74 - 0.52)	0.74
Age	Continuous variable	0.002	(-0.05 - 0.06)	0.95
BMI	Continuous variable	-0.09	(-0.15 - -0.02)	0.008
African American Race	White	-0.61	(-1.46 - 0.24)	0.16
1+ 1 st Degree Relative	No family history	0.71	(-0.33 - 1.74)	0.18
Ever Pregnant	Never Pregnant	-0.17	(-1.87 - 1.52)	0.84
1 Live Birth	No Live Births	-0.30	(-2.02 - 1.41)	0.73
2 Live Births		-0.23	(-1.87 - 1.40)	0.78
3+ Live Births		-0.52	(-2.10 - 1.07)	0.52
OC Use	Never Use	0.58	(-0.34 - 1.51)	0.21
Ever Smoker	Never Smoke	-1.22	(-1.97 - -0.47)	0.002
Former HRT Use	Never HRT Use	-0.48	(1.54 - 0.58)	0.37
Current HRT Use	Never HRT Use	0.45	(-0.79 - 1.69)	0.47
Postmenopausal	Premenopausal	-0.27	(-1.81 - 1.28)	0.73

Stratified analyses did not show a statistically significant association between the 2OHE₁:16OHE₁ EMR and mammographic density among white women. Yet, both BMI and smoking were both significantly inversely associated with percent mammographic density (p = 0.02 and p = 0.007, respectively). However, none of the variables associated with percent breast density in white women resulted in significant relationships with breast density in black women; in fact, only BMI showed a weak, non significant inverse association with percent breast density (Table 4).

Table 10. Association between Mammographic Density and demographic variables Stratified by Race

Variable	<i>White Women</i>			<i>Black Women</i>		
	Parameter Estimate	p-value	95% CI	Parameter Estimate	p-value	95% CI
2OHE ₁ :16OHE ₁ EMR	0.13	0.71	(-0.57 – 0.83)	-1.18	0.26	(-3.28 – 0.92)
Age	0.005	0.89	(-0.06 – 0.07)	0.05	0.45	(-0.13 – 0.24)
BMI	-0.09	0.02	(-0.16 - -0.01)	-0.13	0.09	(-0.28 - -0.02)
1+ 1 st Degree Relative	0.62	0.30	(-0.56 – 1.80)	1.56	0.30	(-1.47 – 4.59)
Ever Pregnant	-0.65	0.58	(-2.95 – 1.66)	2.87	0.17	(-1.29 – 7.04)
1 Live Birth	-0.03	0.98	(-2.38 – 2.31)	-2.15	0.29	(-6.24 – 1.94)
2 Live Births	-0.38	0.77	(-2.55 – 1.89)	-1.40	0.47	(-5.36 – 2.56)
3+ Live Births	-0.80	0.48	(-3.02 – 1.43)	-1.31	0.47	(-5.01 – 2.39)
OC Use	0.63	0.21	(-0.37 – 1.64)	0.19	0.90	(-2.82 – 3.20)
Ever Smoker	-1.23	0.007	(-2.10 - -0.35)	-0.61	0.54	(-2.66 – 1.44)
Former HRT Use	-1.56	0.82	(-1.54 – 1.23)	-0.67	0.57	(-3.11 – 1.76)
Current HRT Use	0.45	0.54	(-1.0 – 1.89)	1.35	0.48	(-2.53 – 5.22)
Postmenopausal	-0.38	0.68	(-2.22 – 1.46)	-1.04	0.61	(-5.24 – 3.15)

4.5 DISCUSSION

In this study, no significant association with percent mammographic density and serum concentrations of the 2OHE₁:16OHE₁ EMR were observed in the total study population. To our knowledge, the current literature only presents two studies that have analyzed the connection between mammographic density and urinary levels of the 2OHE₁:16OHE₁ EMR. In 2001, Riza, dos Santos Silva, Stavola, et al conducted a large cross-sectional study of healthy, postmenopausal women from Halkidiki, Northern Greece. Utilizing the mammographic density classification system developed by Wolf (1976), the authors observed that women with P2/DY mammographic patterns were associated with higher urinary 2OHE₁:16OHE₁EMRs (Riza et al., 2001). Another study by Yong, Atkinson, Newton, et al. analyzed a group of healthy premenopausal women who were not currently using exogenous hormones. Urinary 16OHE₁ was found to have a borderline association with percent breast density while 2OHE₁ and the 2OHE₁:16OHE₁ EMR did not reach statistical significance (Yong et al., 2009). Differences between our results and the two published studies could be methodological in nature, as serum estrogen metabolites were measured in the current study, as opposed to urinary levels, however, these differences could also be due to sample size issues.

In our study, the stratified analyses by race did not show any association between the 2OHE₁:16OHE₁ EMR and mammographic density in either white or black women. However, among black women there was a non-significant negative relationship between the 2OHE₁:16OHE₁ EMR and percent mammographic density, compared to a positive relationship observed among white women. This suggests that estrogens and its metabolites may affect mammographic density, and indirectly breast cancer, differently between these two subpopulations.

Previous studies (Riza and Yong) did not evaluate the association mammographic density and the 2OHE₁:16OHE₁ EMR in black women, therefore to our knowledge, this is the first attempt to examine this association. While the relationship between mammographic density and breast cancer risk has been confirmed among white women, there is a lack of evidence supporting the association between mammographic density and breast cancer in black women. An early case-control study reported no difference in percent mammographic density between black cases and controls. Furthermore, the effect modification by race did not reach the level of statistical significance (Ursin et al., 2003). Another study demonstrated no difference in percent mammographic density between black and white women, but did report that black women had much higher absolute mammographic density than did white women between the ages of 35-64 years. However, these findings do not reflect the difference in breast cancer risk that is present between black and white women around 40-45 years of age (Chen et al., 2004). Similar results were observed in another more recent study (Habel et al., 2007). These findings support the necessity for future research to continue to unravel the biologic mechanism of mammographic density and to evaluate the role of race to determine whether the effects of mammographic density are the same in all women. Additionally, factors that significantly contribute to mammographic density should be analyzed by race, in order to develop an accurate risk profile.

Overall, the results of the current study demonstrated a significant association between BMI and percent mammographic density. Percent mammographic density has been consistently demonstrated to be inversely associated with body weight (N. F. Boyd et al., 2009; Boyd et al., 2005; Martin & Boyd, 2008; Vachon et al., 2000). One possible explanation is that as BMI increases the amount of fat (non-dense tissue) increases, thereby increasing total breast area while the area of dense tissue remains the same or progresses more slowly (Vachon et al., 2000).

This hypothesis was supported by a study of premenopausal women, in which weight was found to be positively associated with areas of non-dense and total breast area, and negatively associated with areas of dense tissue (Boyd et al., 1998). Similar to our results, in a study evaluating the relationship between mammographic density and breast cancer risk among both pre and postmenopausal women, an inverse relationship between breast density and BMI was also reported (Vacek & Geller, 2004). This finding was duplicated in a similar study of postmenopausal women by Tamimi in 2005 (Tamimi et al., 2005).

As obesity is a significant risk factor for breast cancer risk among postmenopausal women, some evidence suggests that this relationship may be due, in part, to the increase in endogenous estrogen exposure that occurs with increasing body weight (Baglietto et al., 2008). It is therefore feasible to hypothesize that obesity may alter breast cancer risk through mammographic density; however, the relationship between estrogen exposure and mammographic density remains unclear. Some studies have indicated significant inverse associations between estrogens and mammographic density, while others report significant positive associations (Aiello et al., 2005; Greendale et al., 2005; Johansson et al., 2008). In a study designed to evaluate the relationship between mammographic breast density, circulating hormone levels, and clinical breast cancer risk factors, a subgroup of women whose BMI fell below the study population median had significantly lower estradiol levels and significantly high breast density, suggesting that the inverse association between BMI and breast density is mediated by estrogens (Johansson et al., 2008). Conversely, other studies did not confirm the association between endogenous hormone concentrations and mammographic density, thereby implying that the effect of obesity on breast cancer risk is independent of mammographic density (Boyd et al., 2002; Tamimi et al., 2007; Verheus et al., 2007; Warren et al., 2006). With an

incomplete understanding of the biological relationship between mammographic density and breast cancer risk, it is plausible that both obesity and mammographic density are associated with breast cancer via two separate pathways.

The current study also supports a significant association between smoking and percent mammographic density. This finding is consistent with reports that identify some components of tobacco smoke as anti-estrogenic, which may function by inhibiting gonadotrophin releasing hormone and aromatase activity, as well reducing cell proliferation, and increasing estrogen metabolism in a manner that produces metabolites with anti-estrogenic properties (Bremnes, Ursin, Bjurstam, & Gram, 2007; Butler et al., ; Jeffreys, Warren, Gunnell, McCarron, & Smith, 2004; Modugno, Ngo et al., 2006).

When taking race into account, among white women, both smoking and BMI were significantly associated with percent mammographic density. Yet, these results were not significant predictors among black women. To our knowledge, the relationship between smoking and mammographic density in black women has yet to be explored.

While this study presents novel relationships in a rarely studied subpopulation, this study is subject to several limitations. Self-selection bias may be present as participants of MAMS were recruited from Magee Women's hospital, and its other satellite locations, and were subsequently selected on a voluntary basis. Hence, it is likely that these women may have only represented those who take an active approach to health, which limits the generalizability of these results to the general population. Other limitations include reporting and recall bias, due to the use of self-reported questionnaires. Additionally, due to small sample sizes, the power of detection was limited, as well as our ability to accurately investigate the role of race.

In conclusion, the current study presents for the first time, an analysis of mammographic density and the 2OHE₁:16OHE₁ EMR, in white and black women, and examines predictors of breast density separately for the two races. Our data suggests that serum concentrations of the 2OHE₁:16OHE₁ EMR are not associated with percent mammographic density. However, among white women, both BMI and smoking significantly contributed to percent mammographic density, a finding that is not confirmed in black women. These findings provide support to further investigate the relationship between estrogen exposure, as well as the 2OHE₁:16OHE₁ EMR, and mammographic density, to gain a better understanding of their biological relationship, as well as how these factors vary by race, and ultimately how they affect breast cancer risk.

**5.0 AN *IN VITRO* ANALYSIS OF THE 2OHE₁:16OHE₁ ESTROGEN METABOLITE
RATIO**

For future publication

Lloyd S¹, Taioli E², Feingold E¹, & Garte S⁴

Department of Human Genetics¹

Department of Environmental and Occupational Health⁴

University of Pittsburgh

Pittsburgh, PA

SUNY Downstate Medical Center²

Brooklyn, NY

5.1 ABSTRACT

Purpose: Evidence suggests that the *in situ* production of estrogen plays a significant role in breast physiology. This local production of hormone termed, “intracrine,” exerts its effects within the cells in which it is produced, thereby requiring only modest amounts of hormones to elicit a maximum effect. The objective of this study was to determine the 2OHE₁:16OHE₁ estrogen metabolite ratio (EMR) in normal and tumor cell lines derived from African American and Caucasian women, and to assess the effects of estradiol treatment on this ratio. **Methods:** Five breast epithelial cell lines were purchased from American Type Culture Collection (ATCC), and two from Cell Applications. There were five breast cancer cell lines, two of which were derived from African American women (MDA-MB-468 and MDA-MB-157) and three from Caucasian women (CAMA-1, SK-BR-3, and ZR-75-1). The remaining two cell lines were normal breast epithelial from Caucasian women (184B5 and HMEpC). Estrogen metabolite levels were quantified using Liquid – Chromatography Mass Spectrometry. **Results:** The 2OHE₁:16OHE₁ EMR was relatively stable across all passages of each sample. In fact, passage number was the only significant contributor to the 2OHE₁:16OHE₁ EMR. We did not observe any significant differences in the 2OHE₁:16OHE₁ EMR between cell lines derived from African American and Caucasian women, normal and tumor breast epithelial cell lines, or estradiol treatment. **Conclusions:** The 2OHE₁:16OHE₁ EMR may not be influenced by external factors, such as estrogen exposure but by intrinsic factors that induce subcellular changes, such as cellular aging or alterations that differentiate tumor from normal cells, (i.e., genetic variation).

5.2 INTRODUCTION

“Intracrinology” describes a mechanism of action in which hormones are synthesized and act on the same cells in which they are produced, without release into the extracellular space or into the general circulation (Foster, 2008; Kuhn-Velten, 2000; Sasano et al., 2008). This alternative to classic endocrinology was discovered when dihydrotestosterone (DHT) was found in the prostates of men with prostate cancer who had recently undergone castration or androgen deprivation therapy. Scientists observed that while testosterone levels in the blood dropped some 90-95%, concentrations of active DHT found in the prostate were only reduced by about 50% (Labrie et al., 2000). Since this time, many have attempted to ascribe this type of hormone production to other hormone-dependent malignancies.

Breast cancer is the most common, noncutaneous, cancer among women in the United States, with a lifetime risk of 12% and a 5% risk of death (American Cancer Society, 2009; Foster, 2008; National Cancer Institute, 2008b). Nearly two-thirds of all breast cancer cases occur during the postmenopausal period, when the ovaries have stopped producing estrogen (Pasqualini et al., 1996). However, evidence suggests that estrogens continue to be produced in extragonadal sites, such as adipose tissue, breast, brain, skin, osteoblasts and chondrocytes of bone, vascular endothelium, and aortic smooth muscle cells (Foster, 2008; Sasano et al., 2006; Simpson, 2003). Many have reported that aromatase, the enzyme responsible for the conversion of androgens to estrogens in the ovary, is expressed in a number of cell types in the breast. Immunolocalization revealed aromatase activity in the epithelial cells of ducts in the terminal ductal lobular units (TDLU) of normal breast tissue, and in the stromal cells as well as adjacent adipose tissue of breast carcinoma (Brodie et al., 1997; Sasano, Nagura, Harada, Goukon, & Kimura, 1994; Suzuki, Miki, Ohuchi, & Sasano, 2008).

The literature also suggests that while postmenopausal women have low circulating levels of estrogen, these concentrations are much higher in normal and tumor breast tissue of these women (Brodie et al., 1997; Chetrite, Cortes-Prieto, Philippe, & Pasqualini, 2007; Foster, 2008; Miettinen et al., 2000). In fact, it has been reported that the quantity of estradiol acquired from the circulation does not significantly contribute to the estradiol concentrations in breast tumors, as this estrogen largely originates from de novo biosynthesis. Furthermore, it has been reported that estradiol levels in breast cancer epithelium do not differ by menopausal status, despite the fact that plasma estradiol levels decrease approximately 90% after the onset of menopause (Billich, Nussbaumer, & Lehr, 2000; Russo, Lareef, Balogh, Guo, & Russo, 2003b; Suzuki et al., 2008). Therefore, it appears that intracrine action may be a significant contributor to the development of breast cancer.

While most epidemiological studies assess the association between estrogens and breast cancer risk utilizing serum or plasma estrogen levels, in this study we have evaluated this relationship using established breast epithelial cell lines. In studies assaying serum and/or plasma estrogen levels, the ratio of two catecholestrogens, 2-hydroxyestrone (2OHE₁) and 16 α -hydroxyestrone (16OHE₁), has been implicated as a marker of breast cancer risk. 2OHE₁ is reported to possess anti-estrogenic properties, while 16OHE₁ is said to be highly estrogenic (Lewis, Thomas, Klinge, Gallo, & Thomas, 2001). The 2OHE₁:16OHE₁ estrogen metabolite ratio (EMR) therefore regulates the proportion of anti-estrogenic molecules to estrogenic ones, leading to the hypothesis that women who metabolize estrogen primarily through the 2OHE₁ pathway have a lower risk of developing breast cancer, while those who metabolize estrogens primarily through the 16OHE₁ pathway, are at an increased risk of developing breast cancer. Therefore, the purpose of this study was to assess the association between the 2OHE₁:16OHE₁

EMR and breast cancer risk, in breast epithelial cells. This novel *in vitro* assay was conducted in seven breast epithelial cell lines, utilizing both tumor and normal cells, derived from African American and Caucasian women, to determine if differences in the 2OHE₁:16OHE₁ EMR exists by race, cell type (tumor or normal), estradiol treatment, or passage number.

5.3 MATERIALS AND METHODS

5.3.1 Cell Lines and Chemicals

Seven human breast epithelial cell lines were purchased to conduct this experiment; five (MDA-MB-468, MDA-MB-157, CAMA-1, SK-BR-3, and ZR-75-1) from ATCC (ATCC, Manassas, Virginia), and two (184B5 and HMEpC) from Cell Applications (Cell Applications, San Diego, California). Five of the seven cell lines, were breast tumor tissue, two from African American women (MDA-MB-468 and MDA-MB-157) and three from Caucasian women (CAMA-1, SK-BR-3, and ZR-75-1). The remaining two cell lines were normal breast epithelial, originating from Caucasian women that underwent breast reduction mammoplasty (184B5 and HMEpC). Each cell line was cultured according to the manufacturer's specified instructions and subcultured in T-75 flasks. MDA-MB-157 and MDA-MB-468 cell lines were propagated utilizing Leibovitz's L-15 Media, supplemented with 1.5mM L-glutamine and 2200 mg/L sodium bicarbonate, 10% fetal bovine serum (FBS), and maintained at 37°C with 100% air. The CAMA-1 cell line was cultured in Eagle's Minimal Essential Media, supplemented with non-essential amino acids, 2mM L-glutamine, 1mM sodium pyruvate, 1500mg/L sodium bicarbonate, 10% FBS, and maintained at 37°C with 5% CO₂. SK-BR-3 was propagated in McCoy's 5A

Medium, supplemented with 1.5mM L-glutamine and 2200mg/L sodium bicarbonate, 10% FBS, and kept at 37°C with 5% CO₂. ZR-75-1 was cultured using RPMI – 1640 media, supplemented with non-essential amino acids, 2mM L-glutamine, 1mM sodium pyruvate, 1500 mg/L sodium bicarbonate, 10% FBS, and maintained at 37°C with 5% CO₂. The 184B5 and HMEpC cell lines were cultured in serum free Mammary Epithelial Cell Basal Medium, purchased from Cell Applications, Inc (Cell Applications, San Diego, CA).

17β-estradiol was purchased from Sigma Aldrich (Sigma Aldrich, Saint Louis, MA), and was reconstituted in distilled water for a final concentration of 5nM. All cell lines were subcultured approximately every 2 to 3 days, and grown to about 40 to 45% confluence in an effort to keep the passage numbers of all cell lines in unison with one another. After the appropriate confluence was attained for each cell line, half the number of flasks accumulated for each cell line were treated with 0.75μl of reconstituted estradiol along with fresh media, while the other half received only fresh media. Seventy-two (72) hours after the administration of estradiol treatment, the media was aspirated from the cells and washed with 5ml of Phosphate Buffered Saline (PBS). The cells were then trypsinized, harvested, and centrifuged at 1600 rpm for 5 minutes at 4°C. Media from the cell pellet was aspirated, and the pellet was rinsed with 5 ml of PBS, and centrifuged again at 1600rpm for 5 min at 4°C. The PBS was aspirated, and the pellet was stored at -20°C.

5.3.2 Liquid Chromatography-Mass Spectrometry

Catecholestrogens were quantified utilizing Liquid Chromatography–Mass Spectrometry (LC–MS) in the laboratory of Dr. Timothy Veenstra, at the National Institutes of Health (Bethesda, MD). Each cell pellet was re-suspended in 0.5 ml of 25 mM sodium acetate buffer (pH 4.6), and

5 μ L dEM (8 μ g/ μ L) internal standard solution. The re-suspended pellet was then vortexed for 10 seconds, and placed in a sonicating water bath for 30 minutes. For an additional 30 seconds, tip sonication was used in 10 second intervals. Then, 0.5mL of 150mM sodium acetate buffer (pH 4.6), containing 1mg L-ascorbic acid, 5 μ L β -glucuronidase, and sulfatase enzyme mixture was added. The sample was then incubated at 37°C for approximately 20 hours. Following incubation, the sample was transferred to a screw-capped glass test tube, to undergo extraction with 6 mL dichloromethane. The dichloromethane was dried with N₂ gas and dansylated. Lastly, tandem mass spectrometry with selected reaction monitoring, coupled with baseline liquid chromatography was conducted, thereby significantly increasing the specificity of the measurement.

This methodology has a lower limit of quantitation (LLOQ) of 25 fg on column (in 1/10 of the cell pellet) for each estrogen. The LLOQ is the lowest level of estrogen that can reliably be measured with excellent accuracy and precision. The limit of detection (LOD) for this study was 1 fg on column (in 1/10 of the cell pellet) for each estrogen. The LOD represents the target estrogen peak that has a signal-to-noise ratio greater than three.

5.3.3 Statistical Analysis

All analyses were conducted utilizing SAS version 9.2 (SAS Institute, Cary, North Carolina) software, with an alpha level of 0.05. Due to the various growth patterns and properties of each cell line, numerous passages were attained while subculturing. A total of 138 observations were collected and analyzed, representing between 14 and 22 for each sample. Exploratory analysis showed that the 2OHE₁:16OHE₁ EMR was relatively stable across passage numbers (Figure 4). Considering this, actual passage numbers were utilized as covariates in the models.

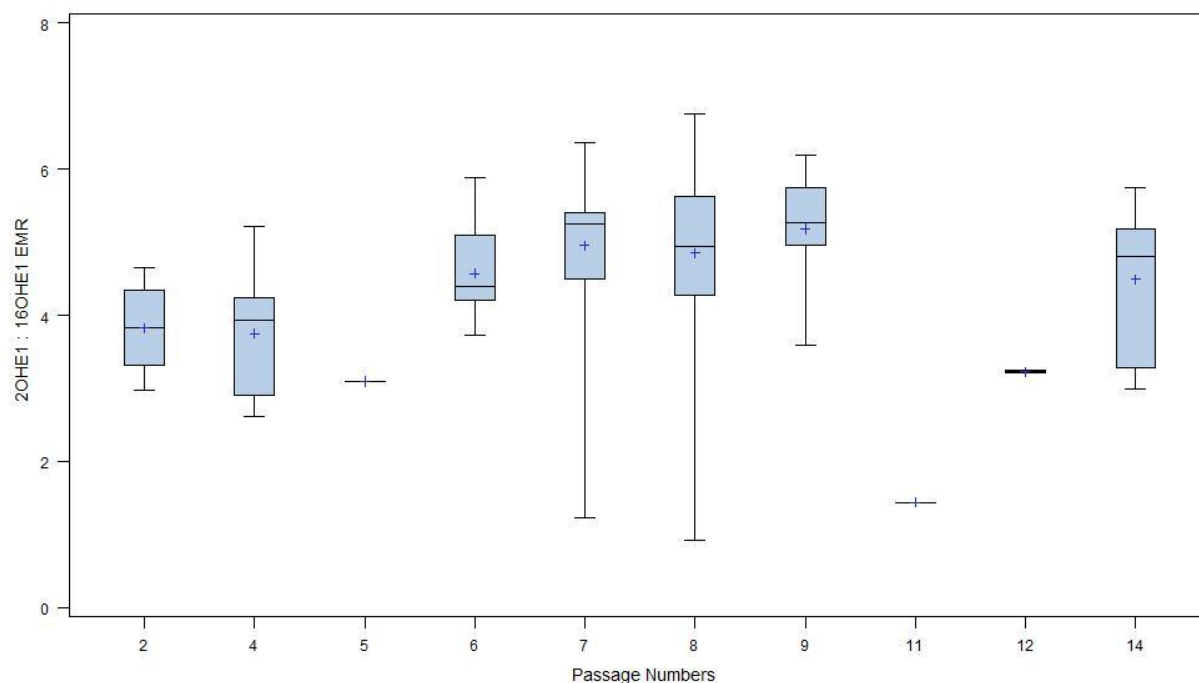
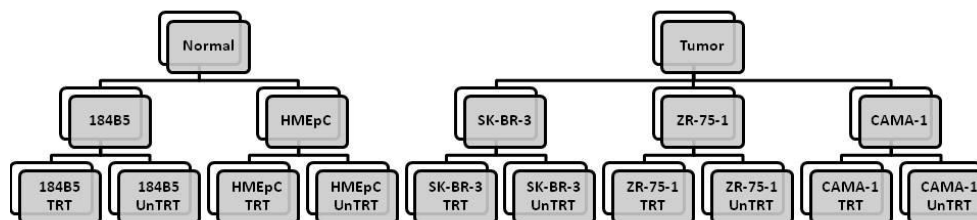


Figure 4. 2OHE₁:16OHE₁ EMR Distribution by Passage Number

Quantification of the catecholestrogens by LC-MS provided individual data for each estrogen metabolite, for each culture flask examined. The 2OHE₁:16OHE₁ EMR was calculated using the individual data for 2OHE₁ and 16OHE₁, respectively, and revealed a highly skewed distribution. Therefore, a log transformation of 2OHE₁:16OHE₁ EMR was conducted to normalize the data. Parametric statistical testing was utilized for all univariate analyses.

To assess the effects of cell type, estradiol treatment, passage number, and race on the 2OHE₁:16OHE₁ EMR, mixed linear modeling was utilized to account for both fixed effects (treatment, passage number, and cell type) and repeated measures (cell lines). The first model consisted of all cell lines derived from Caucasian women to assess the effects of cell type, estradiol treatment, and passage number on the 2OHE₁:16OHE₁ EMR, while the second was comprised of all breast carcinoma cell lines, to evaluate the effects of race, treatment, and passage number on the 2OHE₁:16OHE₁ EMR (Figure 5).

Mixed Linear Model I



Mixed Linear Model II

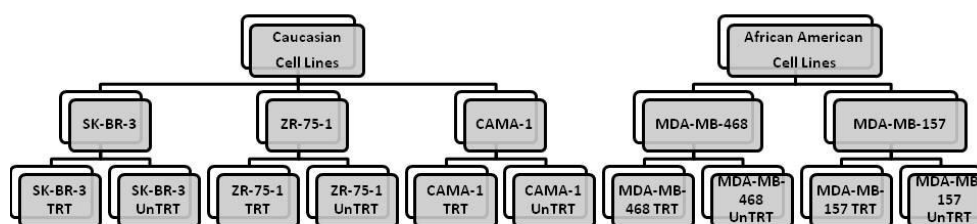


Figure 5. Mixed Linear Model Designs

5.4 RESULTS

Table 13 contains a detailed description of all cell lines analyzed. To assess mean differences between the logged $2\text{OHE}_1:16\text{OHE}_1$ EMR of treated and untreated cells for each cell line, two-sample t-tests were conducted. Our results demonstrated that there were no significant differences in the logged mean $2\text{OHE}_1:16\text{OHE}_1$ EMR of treated and untreated cells within the same cell lines.

Table 11. Cell Line Characteristics

						<i>Untreated</i>	<i>Treated</i>	
Cell Line	Race[‡]	Age	Cell Type[‡]	Tumor Type	ER Status	Mean EMR[*]	Mean EMR[*]	p value[*]
<i>HMEpC</i>	C	21	N	n/a	n/a	43.38	61.56	0.53
<i>184B5</i>	C	21	N	n/a	n/a	262.43	204.38	0.39
<i>CAMA-1</i>	C	51	T	Adenocarcinoma	+	104.58	81.45	0.61
<i>SK-BR-3</i>	C	43	T	Adenocarcinoma	-	139.77	100.48	0.29
<i>ZR-75-1</i>	C	63	T	Ductal carcinoma	+	101.49	121.51	0.62
<i>MDA-MB-157</i>	A	44	T	Medullary carcinoma	-	111.05	107.77	0.93
<i>MDA-MB-468</i>	A	51	T	Adenocarcinoma	-	127.74	129.02	0.99

[‡] C: Caucasian derived cell lines; A: African American derived cell lines, [‡] N: Normal cell lines; T: Tumor cell lines, ^{*} Mean has been untransformed for easy interpretation, ^{*} Difference in mean EMR of untreated and treated cell lines

Univariate analyses were also conducted to identify potential differences that may exist within each independent categorical variable (Table 14). These variables were then stratified to identify other possible differences in the logged mean 2OHE₁:16OHE₁ EMR. Neither the initial univariate nor stratified analyses revealed any statistically significant differences in the logged mean 2OHE₁:16OHE₁ EMR by race, cell type, or estradiol treatment.

Table 12. Univariate Analyses

Variable	2OHE₁:16OHE₁ EMR*		p - value
Race	<i>African American: 120.30</i>	<i>Caucasian: 111.05</i>	0.67
Cell Type	<i>Tumor: 111.05</i>	<i>Normal: 117.92</i>	0.78
Treatment Level	<i>Treated: 111.05</i>	<i>Untreated: 116.75</i>	0.76
Normal Only	<i>Treated: 117.92</i>	<i>Untreated: 117.92</i>	1.0
Tumor Only	<i>African American: 120.30</i>	<i>Caucasian: 106.70</i>	0.57
	<i>Treated: 107.77</i>	<i>Untreated: 115.58</i>	0.69
Treated Cells Only	<i>African American: 119.10</i>	<i>Caucasian: 106.70</i>	0.69
	<i>Tumor: 107.77</i>	<i>Normal: 117.92</i>	0.73
Untreated Cells Only	<i>African American: 122.73</i>	<i>Caucasian: 114.43</i>	0.86
	<i>Tumor: 115.58</i>	<i>Normal: 117.92</i>	0.97

* Mean has been untransformed for easy interpretation

The results of our mixed linear modeling can be found in Table 15. In both unadjusted models, significant positive associations between passage number and the 2OHE₁:16OHE₁ EMR were observed. This finding remained once all models were adjusted for all covariates (cell type, estradiol treatment, passage number, and race). Additionally, both unadjusted models revealed negative relationships between estradiol treatment and the 2OHE₁:16OHE₁ EMR. However, this effect was not seen in adjusted model 2; in fact, in this model, estradiol treatment and the 2OHE₁:16OHE₁ EMR were positively associated. The results of the first unadjusted model, demonstrated a negative association between cell type and the 2OHE₁:16OHE₁ EMR, yet in the adjusted model there was positive association was observed. On the other hand, there was a positive relationship between race and the 2OHE₁:16OHE₁ EMR, an effect that remained in the adjusted models as well. However, the findings of cell type, treatment, and race did not reach statistical significance.

Table 13. Mixed Linear Model Results

<i>Mixed Linear Design Model 1</i>		
(unadjusted)		
Variable	Parameter Estimate	p-value
Cell Type	-0.03359	0.96
Treatment	-0.06840	0.71
Passage	0.07327	0.05
<i>Mixed Linear Design Model 2</i>		
(unadjusted)		
Treatment	-0.06643	0.75
Race	0.1482	0.26
Passage	0.2146	0.004
<i>Mixed Linear Design Model 1</i>		
(adjusted)		
Variable	Parameter Estimate	p-value
Cell Type	0.06041	0.93
Treatment	-0.1008	0.58
Passage	0.07775	0.04
<i>Mixed Linear Design Model 2</i>		
(adjusted)		
Treatment	0.05800	0.7783
Race	0.02654	0.9055
Passage	0.02365	0.004

Additional exploratory analysis was also conducted to assess the effect of estrogen receptor (ER) status on the 2OHE₁:16OHE₁ EMR in the five breast cancer cell lines. A two-sample t-test was conducted to evaluate differences in the logged mean 2OHE₁:16OHE₁ EMR between ER positive (ER+) and ER negative (ER-) cell lines. However, no significant difference

was observed (Table 16). When this variable was added to our mixed linear model, it still did not reach statistical significance in either unadjusted or adjusted models (Table 17).

Table 14. Univariate Analysis of ER Status

Variable	2OHE₁ : 16OHE₁ EMR		p – value^{**}
ER Status	ER+ : 101.49	ER- : 120.30	0.38

Table 15. Mixed Linear Model Results

<i>Mixed Linear Design Model 2</i>		
(unadjusted)		
Variable	Parameter Estimate	p - value
ER Status	-0.01756	0.15
<i>Mixed Linear Design Model 2</i>		
(adjusted)		
Treatment	0.06819	0.74
Race	-0.02580	0.34
Passage	0.2520	0.002
ER Status	0.3791	0.19

5.5 DISCUSSION

While many studies have assessed total estrogen concentrations in cell culture, to our knowledge, no other study has quantified the 2OHE₁:16OHE₁ EMR, nor has any study evaluated potential racial differences in culture. Evidence suggests that the concentration of estrogen,

specifically estradiol, in tumor tissues is much higher than that of normal breast tissue (Chetrite, Cortes-Prieto, Philippe, Wright, & Pasqualini, 2000; Miettinen et al., 2000; Miller, Hawkins, & Forrest, 1982; Suzuki et al., 2008). While we were unable to replicate this effect, we did observe significant positive associations between passage number and the 2OHE₁:16OHE₁ EMR.

Passage number generally refers to the number of times a cell line has been subcultured into a new flask (American Type Culture Collection, 2007). It has been reported that over-subculturing cell lines, resulting in high passage numbers, can elicit adverse property changes that range from alterations in antigenicity, pathogenicity, cell morphology, growth rates, protein expression, signaling, transfection, and response to stimuli (American Type Culture Collection, 2007; Peterson, Tachiki, & Yamaguchi, 2004; Scott, Connor, Creelan, McNulty, & Todd, 1999). However, terms such as „high“ and „low“ passage are very subjective, as they vary by cell line, since there is no established methodology of determining the actual passage number (American Type Culture Collection, 2007). However, we have no reason to believe that we have over-subcultured our samples.

It is reasonable to examine the effects of passage number, as they may be equated with the aging process. A study by Peterson, Tachiki, and Yamaguchi (2004), examined MC3T3-E1 cells to study the effects of senescence on proliferation during osteogenesis *in vitro* which may be reflective of this process *in vivo*. The authors observed a 2-fold decrease in cell proliferation in cells at passage 42 after 11 days in culture, and also reported an overall decrease in population doubling after passage number 36 (Peterson et al., 2004). As aging decreases bone formation, studies such as these are very informative.

To date, only two studies have evaluated the relationship between age and the 2OHE₁:16OHE₁ EMR, one of which did not observe a significant relationship (Kabat et al.,

1997). However, we have previously reported a significant positive relationship with age and the 2OHE₁:16OHE₁ EMR (Lloyd, Taioli, Chen, Arhendt, Feingold, and Garte, 2010). Increasing age has previously been identified as a significant risk factor for breast cancer, as; in general, aging has been reported to increase genomic instability, induce global and promoter-specific epigenetic changes, and to alter the expression of genes involved in cell division and extracellular matrix remodeling (Yau et al., 2007). While we did not explicitly evaluate the effects of increasing passage numbers on the 2OHE₁:16OHE₁ EMR in these cell lines, our study design has permitted us to observe this association, and warrants further investigation.

While the objective of this study was to evaluate differences in the 2OHE₁:16OHE₁ EMR in normal and tumor breast epithelial cell lines, the majority of *in vitro* studies focus primarily on the biosynthesis of estrogen in culture. The formation of estrogen occurs through two major pathways; the aromatase pathway, which is responsible for the conversion of androgens (androstenedione) to estrogens (estrone), and the sulfatase pathway, which is responsible for the conversion of estrone sulfate to estrone, the parent substance of 2OHE₁ and 16OHE₁ (Chetrite et al., 2000; Nelson & Bulun, 2001; Sasano, Nagasaki, Miki, & Suzuki, 2009; Sasano et al., 2006). Aromatase activity has been detected in both normal and breast tumors, whereas sulfatase activity is reported to have activity levels 50 times greater in breast tumors compared to normal breast tissue, and has been detected in 90% of breast tumors (Foster, 2008; Miettinen et al., 2000; Pasqualini & Chetrite, 1999; Sasano et al., 2006; Suzuki et al., 2003). A study by Chetrite, Cortes-Prieto, et al. (2007) observed that the conversion of estrone sulfate to estrone was 7-10 times higher in the tumors of postmenopausal women with breast cancer than in morphologically normal tissue of the same patient. Furthermore, this study also found that the presence of estradiol exhibited an inhibitory effect on the enzymatic activity of estrone sulfate. The

conversion of estrone sulfate to estradiol was not observed in either tissue sample, however, the conversion of estrone sulfate to estrone could still be detected in both, normal and tumor tissue. Yet, the production of estrone was much greater in tumor than in normal tissue (Chetrite et al., 2007).

Given that 33-50% of all breast cancers respond to estrogen treatment, we evaluated the effect of estrogen receptor status on tissue concentrations of the 2OHE₁:16OHE₁ EMR (Pasqualini et al., 1996). However, we did not find any significant results. This finding is consistent with a study that assessed the correlation between ER status, and tissue concentrations of estrone, estradiol, estrone sulfate, estradiol sulfate, as well as aromatase and sulfatase enzymatic activities in postmenopausal women. The authors reported not observing any correlations between ER status and tissue concentrations of estrogens or the enzymatic activities of aromatase and sulfatase (Chetrite et al., 2000). This, as well as our findings suggests that ER status may not play a significant role in the local production of estrogen.

Intracrinology is a fairly new area of research, yet the theory on which this mechanism of action is based, has benefited numerous postmenopausal women with breast cancer. For the past 25 years, aromatase inhibitors have been used as one treatment method for metastatic breast cancer (Howell & Buzdar, 2005). It has been reported that the concentration of estradiol in the tumors of postmenopausal women is 20-fold greater than what is found in the plasma. However, administration of aromatase inhibitors produce a significant decline in intratumoral estradiol, estrone, and aromatase activities (Simpson, 2003). These results not only support the Intracrinology hypothesis, but also emphasize the importance of the local production of estrogen to the growth and progression of breast cancer.

While most studies focus primarily on the concentrations of estradiol and estrone, it is important to note that 2OHE₁ and 16OHE₁ are the most significant metabolites of estradiol. They are believed to function as signaling molecules; have variable binding affinities for the ER; are capable of covalently binding DNA and forming DNA adducts; and appear to be more mutagenic than their parent compound (Jefcoate et al., 2000; Liehr, 2001; Roy & Liehr, 1999). The ratio of these two catecholestrogens, has been implicated as marker of breast cancer risk in studies assaying plasma and serum, therefore the purpose of this study was to evaluate tissue concentrations of the 2OHE₁: 16OHE₁ EMR in cell culture, to determine if differences in race, cell type, and estradiol treatment could be detected. An analysis of this sort is very interesting, as it allows us to examine the disparity among African American women at the cellular level, as any differences found here would indicate that these women may metabolize estrogens differently from Caucasian women.

One limitation of this study was the use of FBS as part of the growth medium in the breast cancer cell lines. While the exact estrogen concentration in the FBS is unknown, we are satisfied with our findings, as levels of 2OHE₁ and 16OHE₁ were assayed directly from the cells themselves. Additionally, all cells were washed thoroughly with PBS before trypsinization, and were washed again after centrifugation. Therefore, we have no reason to believe that the administration of FBS would in any way, bias our results. Another limitation of this study was the inability to obtain normal breast epithelial cell lines from African American women, which would have allowed for better comparisons by race, and better overall analysis of differences by cell type.

In conclusion, these findings suggest that the process of cellular aging, rather than estrogen exposure, may be associated with alterations in the 2OHE₁:16OHE₁ EMR in breast

epithelial cells. As this was a novel investigation, and no significant associations between cell type, estradiol treatment, or race were observed, some of our findings were supported by the literature, warranting further investigation.

6.0 AN ANALYSIS OF ESTROGEN METABOLISM AND BREAST CANCER RISK

DISCUSSION

Again, the overall objective of this study was to investigate the association between the 2OHE₁:16OHE₁ EMR, the *CYP1B1* Leu432Val polymorphism, mammographic density, race, and breast cancer risk, to illustrate whether or not variation in estrogen metabolism was associated with differences in breast cancer risk. The results of this study were also designed to highlight a potential biological explanation for the disparity in breast cancer morbidity and mortality between African American and Caucasian women. We therefore, hypothesized that women who metabolized estrogen primarily through the 16OHE₁ pathway would have an increased risk of developing breast cancer.

Our first analysis evaluated the effects of the *CYP1B1* Leu432Val polymorphism on the urinary 2OHE₁:16OHE₁ EMR and breast cancer risk. As the *CYP1B1* gene catalyzes the formation of both 2OHE₁ and 16OHE₁, we hypothesized that the presence of the variant, valine, allele of this polymorphism may negatively alter the effect of the 2OHE₁:16OHE₁ EMR, which in turn, may negatively alter breast cancer risk. The results of this study demonstrated a significant association between the 2OHE₁:16OHE₁ EMR and breast cancer, but did not reveal any associations among the *CYP1B1* genotype and breast cancer risk. Conversely, the relationship between the 2OHE₁:16OHE₁ EMR and the *CYP1B1* genotype did reach statistical significance. Once these results were stratified by case-control status, we observed that this

association was only significant among our control population. Based on the results of this study, as well as evidence in the literature that supports the hypothesis that the *CYP1B1* genotype is associated with breast cancer risk, we can tentatively conclude that the *CYP1B1* Leu432Val polymorphism may indirectly influence breast cancer risk, by altering the 2OHE₁:16OHE₁ EMR (Kocabas et al., 2002; Paracchini et al., 2005; Paracchini et al., 2007; Rylander-Rudqvist et al., 2003).

When evaluating the role of race in the relationship between the 2OHE₁:16OHE₁ EMR and breast cancer risk, we examined the association between this ratio and mammographic density in a population of cancer-free pre and postmenopausal women. As the risk of developing breast cancer associated with mammographic density, is greater than the combined risk of all other clinical risk factors, this study served as a suitable proxy for assessing the association of the 2OHE₁:16OHE₁ EMR and breast cancer risk (Greendale et al., 2005). Given the association of mammographic density and endogenous, as well as exogenous, estrogen exposure, we hypothesized that mammographic density would have a negative association with the 2OHE₁:16OHE₁ EMR. Likewise, as the risk profiles for developing breast cancer varies between African American and Caucasian women, we also hypothesized that race would have a negative effect on the 2OHE₁:16OHE₁ EMR. However, we found that Caucasian and African American women in this study population differed only by BMI, HRT use, and mammographic density, but not the 2OHE₁:16OHE₁ EMR. Furthermore, we did not observe an association between mammographic density and the 2OHE₁:16OHE₁ EMR. Across the entire study population, the only significant predictors of mammographic density were BMI and smoking status, a finding that was also duplicated among Caucasian women, but not among African Americans. Although no significant relationships were observed between the 2OHE₁:16OHE₁ EMR and

mammographic density in either race, or in the total population, the results of this study did demonstrate differences in factors that contributed to mammographic density. These findings are consistent with reports that denote differences in risk profiles for African American and Caucasian women. For example, Caucasian women are more likely to developed breast cancer after the age of 50, while the risk of developing breast cancer for African American women is greater under the age of 40 (American Cancer Society, 2008a). Additionally, African American women are more likely to have larger, estrogen negative, high grade tumors (Breastcancer.org, 2008; Chlebowski et al., 2005; Curtis et al., 2008; Key et al., 2001). Therefore, we can conclude that the 2OHE₁:16OHE₁ EMR does not appear to affect mammographic density and the factors that are associated with mammographic density may vary by race.

The literature reports that the conversion of androgens to estrogens also occurs in breast tissue (Brodie et al., 1997). This local production of estrogen is termed Intracrine, as that which is produced is not released into the bloodstream. Therefore, this system of hormone production is much more efficient in the development of hormone-dependent malignancies, as only modest quantities of hormones are needed to elicit a maximum effect (Foster, 2008; Sasano et al., 2008). To date, no other study has sought to assess concentrations of the 2OHE₁:16OHE₁ EMR in breast tissue, hence, we conducted an *in vitro* assay in both normal and tumor breast epithelial cell lines to determine 2OHE₁:16OHE₁ EMR concentrations. The effects of race and estradiol treatment on tissue levels of the 2OHE₁:16OHE₁ EMR were also examined. We hypothesized that there would be significant differences in the mean 2OHE₁:16OHE₁ EMR between normal and tumor cell lines, as well as significant differences in the mean 2OHE₁:16OHE₁ EMR in cell lines derived from African American versus Caucasian women, and in treated versus untreated cell lines. We also hypothesized that cell type, race, and estradiol treatment would all be significant

predictors of the 2OHE₁:16OHE₁ EMR. As 2OHE₁:16OHE₁ EMR levels were fairly stable across all passage numbers, these numbers were added as an additional covariate. The results of this study revealed that there were no differences in the mean 2OHE₁:16OHE₁ EMR of normal and tumor cell lines, nor between treated and untreated cells, or in cell lines derived from African American and Caucasian women. In fact, the only contributor to the 2OHE₁:16OHE₁ EMR was passage number. This finding suggests that estrogen exposure alone may not be the underlying causative agent in the development of breast cancer, nor does it significantly alter estrogen metabolism. The literature often equates passage number to aging, as a number of sub-cellular changes occur with increasing passage number, thereby implying that other intrinsic alterations (i.e., genetic variation) may affect the 2OHE₁:16OHE₁ EMR (American Type Culture Collection, 2007; Peterson et al., 2004; Scott et al., 1999).

The results of this study highlight the complexity involved in assessing the relationship between the 2OHE₁:16OHE₁ EMR and breast cancer risk, as well as determining those risk factors that significantly contribute to it. However, we have successfully demonstrated an association between the 2OHE₁:16OHE₁ EMR and breast cancer risk, with the findings of specific aim one. While these findings should be confirmed, our logistic regression analysis in aim one revealed that a high 2OHE₁:16OHE₁ EMR was associated with a 72% decrease in the risk of being a case ($p < 0.0012$). Currently, the literature presents a few studies that have investigated the association of the urinary 2OHE₁:16OHE₁ EMR and breast cancer risk as we have hypothesized, and have found similar results. One such study was conducted by Muti, Bradlow, Micheli, et al. (2000), in which the authors conducted a nested case-control study of both pre- and postmenopausal women, and concluded that among premenopausal women, a high

2OHE₁:16OHE₁ EMR decreases breast cancer risk (Muti et al., 2000). This result was duplicated in 2006 by Kabat, O’Leary, Gammon et al. (Kabat et al., 1997).

Upon examination of the relationship between known breast cancer risk factors and the 2OHE₁:16OHE₁ EMR, we observed significant associations between age; and the age and BMI interaction. We observed a positive relationship between age and the 2OHE₁:16OHE₁ EMR in our first analysis, as well as a negative association with the age and BMI interaction. To date, we have only identified one other study that examined the relationship between age and the 2OHE₁:16OHE₁ EMR, but this study did not observe a consistent relationship (Kabat et al., 1997). Nonetheless, these findings can also be extended to our third aim, as passage number was the only significant contributor to the 2OHE₁:16OHE₁ EMR.

Age is an established risk factor for breast cancer, as, in general, risk for developing breast cancer increases with increasing age. Part of this association is due to the increasing life expectancy of Americans, but also includes factors such as long-term postmenopausal hormone therapy use due to changes in reproductive patterns (American Cancer Society, 2007). The effects of aging include a myriad of biological changes, from obesity, elevated blood pressure, and increasing lipid and lipoprotein levels (Owens, Matthews, Wing, & Kuller, 1992). Ageing has been defined by Kirkwood and Austad (2000) as a, “Progressive loss of function accompanied by decreasing fertility and increasing mortality” (p. 233). However, aging is also a complex process that is regulated by several signaling pathways and transcriptions factors, and is the subject of several evolutionary debates (Kenyon, ; Kirkwood & Austad, 2000). One such theory, is termed the „accumulation theory“ which describes the accumulation of late-effect deleterious alleles that are unselected for, and thereby exhibit heterogeneous distribution throughout the population (Kirkwood & Austad, 2000). This collection of genetic and epigenetic

changes results in macromolecular changes such as decreasing telomere length and dysfunctional DNA damage repair processes, which lead to senescence, apoptosis, and loss of replication, all of which are similar mechanisms involved in carcinogenesis (Bassi & Sacco, 2009; Neumeister, Albanese, Balent, Grealley, & Pestell, 2002). This intricate relationship between aging and cancer, may account for the lack of the association observed between the 2OHE₁:16OHE₁ EMR and breast cancer risk *in vitro* and mammographic density.

In the current study, we were unable to demonstrate an association between the 2OHE₁:16OHE₁ EMR and mammographic density, the most significant risk factor in breast cancer etiology. At the present time, there is no well defined biological mechanism to explain the influence of mammographic density on breast cancer risk, however, the results of specific aim 2 revealed that mammographic density was associated with factors such as smoking and BMI, findings that are supported in the literature (N. F. Boyd et al., 2009; Bremnes, Ursin, Bjurstam, & Gram, 2007; Butler et al., ; Jeffreys et al., 2004; Martin & Boyd, 2008; Vachon et al., 2000). Although age was not a significant predictor of mammographic density in this study, the literature presents consistent evidence to show that mammographic density does in fact decrease with increasing age (N. F. Boyd et al., 2009; Haiman et al., 2003; Vachon et al., 2000; Warren, 2004; Woolcott et al., 2009). As the mean age of this study population was 55.6 and most participants were postmenopausal, it is feasible to conclude that the effects of the aging may also influence the relationship between mammographic density and breast cancer risk.

As previously mentioned, there is a positive association between age and obesity, but there also exists, in general, a negative association between age and estrogen levels, given the onset of menopause significantly decreases circulating estrogen levels (Billich et al., 2000; Brodie et al., 1997; Russo, Lareef, Balogh, Guo, & Russo, 2003a). Obesity has long been

considered a risk factor for breast cancer among postmenopausal women, given the increasing amounts of estrogen produced by adipose tissue (Baglietto et al., 2008; Clemons & Goss, 2001; Dignam et al., 2006). However, the findings of specific aim 1 demonstrated a non-significant positive association between BMI and the 2OHE₁:16OHE₁ EMR for both cases and controls, and yet, a significant negative association was observed between the 2OHE₁:16OHE₁ EMR and an age and BMI interaction among cases, implying that increasing age in conjunction with increasing body weight is associated with lower estrogen metabolite levels. While both age and BMI are two highly correlated variables, the literature has reported similar findings between BMI and 2OHE₁:16OHE₁ EMR concentrations. In a study by Falk, Fears, Xu, et al. (2005), the authors observed that among Asian American premenopausal women, a low 2OHE₁:16OHE₁ EMR was found among those with a high BMI, >24.6 kg/m², and an even lower ratio was observed among obese women, BMI > 29 kg/m². This result was not repeated in postmenopausal women (Falk et al., 2005). However, there are very few studies that investigate other known breast cancer risk factors in relation to the 2OHE₁:16OHE₁ EMR.

Although our study presents the findings of some novel research, it is also subject to several limitations. A case control study is a useful first step when exploring a source of disease (Gordis, 2004). This study design allowed us to investigate a variety of characteristics that may lead to an adverse health outcome. However, one limitation to utilizing this design is that we were unable to obtain the absolute risk (incidence) of disease, nor could we determine the relative risk of disease in the study population. Furthermore, the use of a case-control study design that consisted only of Caucasian women prevented applicable comparisons to other racial/ethnic groups. We also observed some dissimilarities between our cases and controls (e.g., age and BMI) that make drawing solid conclusions difficult.

Selection bias may also have been introduced into our research, as the controls in our case-control study were obtained from other studies. Therefore, the selection, as well as exclusion criteria may have differed for each group. This bias may have also been present in specific aim two, as these participants were recruited from Magee Women's hospital, and its other satellite locations, and were subsequently selected on a voluntary basis. Hence, it is likely that these women may have only represented those who take an active approach to health, which limits the generalizability of these results to the general population. Other limitations include reporting and recall bias, due to the use of self-reported questionnaires in specific aim two, and small sample sizes, which limited our power of detection.

Overall, further research is warranted to confirm the results of this study. However, the large majority of examinations assessing the 2OHE₁:16OHE₁ EMR and breast cancer risk, are case – control studies, that do not investigate the contributing factors to the 2OHE₁:16OHE₁ EMR. For this reason alone, our research is a significant contribution to the literature. Additionally, few studies have addressed the serum 2OHE₁:16OHE₁ EMR in premenopausal women, and even fewer in African American women. Specific aim three is a completely novel experiment, with significant implications. Findings from studies such as these may one day allow investigators to evaluate the disparity facing African American women on a cellular level, to determine if the breasts of these women metabolize estrogens differently from Caucasian women. This project will also enhance the literature regarding the breasts' ability to synthesize estrogen, by expounding on the area of intracrinology. Although definitive conclusions cannot be made on the basis of this investigation, the uniqueness of our study designs and new results will enhance the body of evidence regarding the role of estrogens in breast cancer, and the genetics associated with this relationship.

Additionally, the current study also provides an indirect assessment of variation in concentrations of the 2OHE₁:16OHE₁ EMR that can be found throughout the body. This study evaluated 2OHE₁:16OHE₁ EMR concentrations from three different sources: urine, serum, and breast epithelial. Urine is the most common method utilized to assess 2OHE₁:16OHE₁ EMR levels and concentrations in our study ranged from 0.2-5, with an overall mean of 1.95. On the other hand, serum levels of the 2OHE₁:16OHE₁ EMR was considerably lower, ranging from 0.1-3, with an overall mean of 0.52, while concentrations assayed directly from breast tissue were highest, presenting values from 2.5-865, with an overall mean of 166.15. To our knowledge, this is first study ever to document such differences.

One goal of this research project was to explore the role of race in our analyses, in hopes of highlighting potential differences between African American and Caucasian women, given the incidence and mortality disparities between them. Many suggest that socioeconomic factors are largely to blame, citing that these women lack proper access to healthcare and receive inadequate treatment after diagnosis. Yet, studies such as those by Wojcik, Spinks, & Optenberg (1998) and Jatoi, Becher, & Leake, (2003) demonstrate that even within an equal access medical system, this disparity persists. Nevertheless, few studies have addressed the association of estrogen and breast cancer risk in African American women, but we attempted to do so. Our study sought to determine if there were racial differences in the 2OHE₁:16OHE₁ EMR. While we only assessed this relationship in two of our aims, we did not observe significant differences in the 2OHE₁:16OHE₁ EMR between Caucasian and African American women. However, we did observe evidence to suggest that the predictors of breast cancer risk may differ between African American and Caucasian women.

Unfortunately, one limitation of this study is that we were unable to explore the role of race in all three aims. This leaves to question whether or not there are differences in the 2OHE₁:16OHE₁ EMR among African American and Caucasian populations, and whether this ratio alters breast cancer risk in African American women. Differences found in this type of analysis, would further confirm that breast cancer risk factors may have opposing effects in racial/ethnic populations. Furthermore, given the inaccessibility of a variety of tumor and normal cell lines from African American women, we were unable to draw any conclusions as to whether estrogen metabolism in the breast of these women differs from that of Caucasian women. We suspect that one reason for this lack of association is the large differences in sample size.

Another limitation of this study was the inability to more accurately assess the role of the *CYP1B1* Leu432Val polymorphism in breast cancer risk, and the role of race in this relationship. In the literature we have seen a significant association between breast cancer risk and the *CYP1B1* Leu432Val polymorphism, but we were unable to confirm these findings across our entire study population. Furthermore, very few studies have assessed the *CYP1B1* Leu432Val polymorphism in African American women. To date, we have identified only one study that indicates the possibility of an increase in breast cancer risk among African American women carrying at least one copy of the valine allele, but these findings did not reach statistical significance (Van Emburgh et al., 2008). However, these results have not been duplicated, nor have they assessed this association with the 2OHE₁:16OHE₁ EMR. We have, however, shown an association between this polymorphism and the 2OHE₁:16OHE₁ EMR, a relationship between 2OHE₁:16OHE₁ EMR and breast cancer risk, and evidence to suggest that the predictors of the breast cancer risk may differ by race. While we believe that the *CYP1B1* Leu432Val

polymorphism may indirectly alter breast cancer risk through variations in the 2OHE₁:16OHE₁ EMR, we could also imply that the *CYP1B1* Leu432Val polymorphism may have an effect on other variables by race, therefore establishing a foundation for future research to examine whether the 2OHE₁:16OHE₁ EMR differs by race as well.

While we observed several associations between known breast cancer risk factors (i.e., age and BMI) and the 2OHE₁:16OHE₁ EMR, we are left to wonder what affects the 2OHE₁:16OHE₁ EMR may have on other known risk factors for breast cancer. Very few studies have examined these associations, and the results of such investigations would provide a more comprehensive understanding of the role of estrogen in breast cancer risk.

In conclusion, the objective of this study was to assess the association of the 2OHE₁:16OHE₁ EMR and breast cancer risk; as well as to explore the relationships between this ratio and the *CYP1B1* Leu432Val polymorphism, mammographic density, and race. The results of our study have been consistent with the findings in the literature, and provide some answers to the questions regarding the role of estrogens in breast cancer risk. Our study has also addressed the racial/ethnicity differences that exists in the incidence and mortality of breast cancer, and has provided a foundation for future studies to examine the relationship between the 2OHE₁:16OHE₁ EMR and the *CYP1B1* Leu432Val polymorphism in breast cancer risk among African American women. This research has established a basis for future studies to build upon, to provide a greater understanding of the risk factors and etiology of breast cancer, to develop better prevention and treatment methods for all women.

APPENDIX A

TABLE 1. HIGHLY PENETRANT CANCER SYNDROMES AND BREAST CANCER RISK CITATIONS

¹From “Li-Fraumeni Syndrome,” 2004, GeneReviews. Copyright 1993-2009 by the University of Washington, Seattle.

From “*PTEN* Hamartoma Tumor Syndrome (PHTS),” 2006, GeneReviews. Copyright 1993-2009 by the University of Washington, Seattle.

From “Puetz-Jeeghers Syndrome,” 2007, GeneReviews. Copyright 1993-2009 by the University of Washington, Seattle.

From “Ataxia-Telangiectasia,” 2005, GeneReviews. Copyright 1993-2009 by the University of Washington, Seattle.

From “Hereditary Diffuse Gastric Cancer,” 2006, GeneReviews. Copyright 1993-2009 by the University of Washington, Seattle.

From “Bloom’s Syndrome,” 2006, GeneReviews. Copyright 1993-2009 by the University of Washington, Seattle.

From “Werner Syndrome,” 2007, GeneReviews. Copyright 1993-2009 by the University of Washington, Seattle.

From “Xeroderma Pigmentosum,” 2008, GeneReviews. Copyright 1993-2009 by the University of Washington, Seattle.

From “Nijmegen Breakage Syndrome,” 2005, GeneReviews. Copyright 1993-2009 by the University of Washington, Seattle.

From “Fanconi Anemia,” 2008, GeneReviews. Copyright 1993-2009 by the University of Washington, Seattle.

From “Genetic Insights into Familial Cancers-update and Recent Discoveries,” 2002, *Cancer Letters*, 181, p. 125-164. Copyright 2002 by Elsevier Science Ireland Ltd.

From “Recognition and Management of Hereditary Breast Cancer Syndromes,” 2004, *The Oncologist*, 9, 13-24. Copyright 2004 by AlphaMed Press.

From “Highly Penetrant Hereditary Cancer Syndromes,” 2004, *Oncogene*, 23, p. 6445-6470. Copyright 2004 by Nature Publishing Group.

From “Hereditary Breast Cancer: From Molecular Pathology to Tailored Therapies,” 2008, *Journal of Clinical Pathology*, 61, p. 1073-1082. Copyright 2008 by David S. Tan, Caterina Marchio, and Jorge S. Reis Filho. From “Germ Line Mutations Associated with Breast Cancer Susceptibility,” 2001, *European Journal of Cancer*, 37, p. 300-321. Copyright 2001 Elsevier Science Ltd.

APPENDIX B

TABLE 2. LOW PENETRANCE BREAST CANCER SUSCEPTIBILITY VARIANTS

CITATIONS

From “A systematic Review of Genetics Polymorphisms and Breast Cancer Risk,” 1999, Alison M. Dunning, Catherine S. Healey, Paul D. P. Pharoah, M. Dawn Teare, Bruce A. J. Ponder, and Douglas F. Easton.

From “Susceptibility to Breast Cancer: Hereditary Syndromes and Low Penetrance Genes,” 2006, 2007, *Breast Disease*, 27, p. 21-50. Copyright 2006, 2007 by IOS Press and authors.

From “Genetic Predisposition to Breast Cancer: Past, Present, and Future,” 2008, *Annual Review of Genomics and Human Genetics*, 9, p. 321-345. Copyright 2008 by Annual Reviews.

From “Polygenes, Risk Prediction, and Targeted Prevention of Breast Cancer,” 2008, *New England Journal of Medicine*, 358, p. 2796-2803. Copyright 2008 Massachusetts Medical Society.

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