2:16α-HYDROXYESTRONE METABOLITE RATIO AND BREAST CANCER: A COMBINED ANALYSIS

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Estrogen metabolites may play an important role in breast carcinogenesis. Animal and in vitro studies suggest differing biological effects of the 2-hydroxyestrone ($2\text{-}OHE_1$) and 16α-hydroxyestrone ($16\alpha\text{-}OHE_1$) metabolites, lending support to the use of $2{:}16\alpha\text{-}OHE_1$ as a measure of estrogen balance. Although previous studies have evaluated the association between these specific metabolites and breast cancer among pre- and postmenopausal women, the results have been inconclusive. The sample size of individual studies is often small and lacks the statistical power to draw conclusions or to adequately assess the relationships within subgroups. Furthermore, the relationship between various lifestyle factors and personal characteristics and estrogen metabolites remains unclear. We evaluated the association between the $2\text{-}OHE_1$, $16\alpha\text{-}OHE_1$ and $2{:}16\alpha\text{-}OHE_1$ metabolites and breast cancer among premenopausal (183 cases/548 controls) and postmenopausal (319 cases/647 controls) women using a combined analysis of individual level data from previously published research studies. In separate study adjusted conditional logistic regression models matched on 5-year age groups, higher levels of $2{:}16\alpha\text{-}OHE_1$ were not associated with breast cancer among pre- or postmenopausal women, although the premenopausal analyses suggested a reduction in risk [Premenopausal: $OR_{\geq 2.67 \text{ vs. } <1.76}=0.81$ (95% CI: 0.49, 1.32); Postmenopausal: $OR_{\geq 2.46 \text{ vs. } <1.53}=0.87$ (95% CI: 0.58, 1.29)]. Using multivariable regression analyses adjusted for study, we evaluated various predictors of estrogen

metabolites among the control populations of the participating studies (544 premenopausal/720 postmenopausal). Among premenopausal women, BMI was negatively associated with 2-OHE_1 and 2:16α-OHE_1 ($p < 0.05$). Analyses among postmenopausal women revealed significant associations ($p<0.05$) between age, age at menopausal status, and history of benign breast disease. In summary, this combined analysis does not support an association between urinary estrogen metabolites and breast cancer among pre- or post menopausal women. However, our results do suggest potential differences in factors related to estrogen metabolite levels among pre- and postmenopausal women. Enhancing our knowledge of estrogen metabolites among breast cancer patients and among healthy populations of women is a significant contribution to public health. By improving our understanding of estrogen metabolites we may be able to identify women at higher risk of breast cancer as well as increase our understanding of breast cancer etiology.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

I would like to take this opportunity to thank my mentors, family and colleagues who have helped me achieve my doctoral degree. The members of my committee have been invaluable, and I am fortunate to have had the opportunity to work with and learn from experts in the field of breast cancer research and women's health. I thank my committee members, Dr. Emanuela Taioli, Dr. Jane Cauley, Dr. Roberta Ness, Dr. Roslyn Stone and Dr. Victor Vogel for their mentorship, guidance, and encouragement throughout my doctoral training, all of which have helped me successfully complete this dissertation.

I would like to thank the collaborators of this combined analysis project for contributing their data and supporting this work. I am also appreciative for the opportunity to have been part of the National Cancer Institute Predoctoral Cancer Epidemiology Training Program at the University of Pittsburgh; it truly has been a remarkable learning experience. I thank Dr. Weissfeld for his mentorship as part of the R25 training program and I also express my gratitude to my fellow doctoral students and R25 trainees for their encouragement. I have enjoyed working and learning with each of you and I am thankful for your support.

I thank my parents, my brothers and my friends for their enduring patience and support. Without their love and encouragement, this would not have been possible. This dissertation is dedicated to my family. I also dedicate this work to the women in my family who fought and continue to fight against breast cancer.

1.0 INTRODUCTION

Estrogen exposure has been established as a hormonal risk factor for breast cancer yet the mechanisms by which estrogen increases breast cancer risk remains unclear. One hypothesis implicates estrogens in the proliferation of human breast epithelial cells. Estrogens may indirectly influence carcinogenesis by stimulating cell division and thereby increasing the probability a mutation will occur. Many of the known breast cancer risk factors relate to a woman's cumulative lifetime exposure to estrogen; however, the underlying mechanisms are also unknown. Furthermore, many of the identified risk factors are not potentially modifiable, posing challenges for the implementation of prevention efforts. Estrogen metabolites may provide a measure of energy balance and may also have the potential to be modified. In view of these factors and the role of lifetime estrogen exposure in breast cancer development, understanding the role of estrogen metabolites in relation to breast cancer risk has become increasingly important in recent years. Two specific metabolites, 2-hydroxyestrone (2-OHE_1) and 16α-hydroxyestrone ($16\alpha\text{-OHE}_1$) have been the focus of previous investigations due to their opposing estrogenic properties. Moreover, these metabolites are mutually exclusive, rendering their ratio a useful measure of estrogen balance.

Previous studies have evaluated the relationship between these estrogen metabolites and breast cancer among pre- and postmenopausal women yet the results have been inconclusive. Furthermore, the sample size of individual studies is often too small and lacks the statistical

power to draw conclusions or to adequately assess the relationships within subgroups. The intent of this present study is to enhance our understanding of the potential relationship between these urinary estrogen metabolites (2-OHE_1, 16α-OHE_1, and 2:1616α-OHE_1) and breast cancer among a larger combined sample of pre- and postmenopausal women.

In this combined analysis of primary data from previously published studies, individual level data from eligible and participating studies were used in order to address the following specific aims: 1) to assess the relationship between the 2-OHE_1, 16α-OHE_1 and 2:16α-OHE_1 urinary estrogen metabolites and breast cancer among premenopausal women, 2) to evaluate the association between 2-OHE_1, 16α-OHE_1, and the 2:16α-OHE_1 and breast cancer among postmenopausal women and 3) to evaluate the association between known breast cancer risk factors and each of these estrogen metabolites. In the background and significance section that follows, the literature review includes an overview of breast cancer epidemiology and known breast cancer risk factors, a description of estrogen and estrogen metabolites, and the potential link between estrogen metabolites and breast cancer.

2.0 BACKGROUND AND SIGNIFICANCE

2.1 EPIDEMIOLOGY OF BREAST CANCER

Breast cancer is the most common cancer diagnosed among women and the second leading cause of cancer death in the United States, with approximately 182,460 new cases and 40,480 breast cancer deaths expected in 2008 (American Cancer Society 2008). Worldwide, breast cancer is the second most common cancer overall with an estimated 1.15 million incident cases in 2002 (Parkin 2005). An elevated incidence is reported in both developed and developing countries (Figure 1); however, the incidence rates are slightly higher in developed countries with the highest age-standardized incidence rates reported in North America, Western Europe, Australia/New Zealand, and Northern Europe (Parkin 2005). This high incidence of breast cancer as well as the reasonable prognosis due to screening programs and early detection explains, in part, the worldwide prevalence of breast cancer (Parkin 2005). Although the incidence of breast cancer in the U.S. slightly increased during the period of 1980–2001, the trend has shifted with a reported decrease in rates by 3.5% per year during the period of 2001-2004, according to data from the American Cancer Society (ACS) (ACS 2007). Possible explanations for this observed decrease may include changes in mammography patterns and the decreased use of hormone replacement therapy (Ravdin 2007).

Breast cancer incidence and mortality rates differ by race, with the highest age-adjusted incidence rates reported among white women (130.8 per 100,000) and African American women (111.5 per 100,000). Although the incidence rate of breast cancer among African American women is slightly lower than white women, African American women are more likely than white women to die from this disease, with a death rate of 34.4 per 100,000 as compared to 25.4 per 100,000 (Jemal 2007). This racial difference in mortality rate is observed even after adjustment

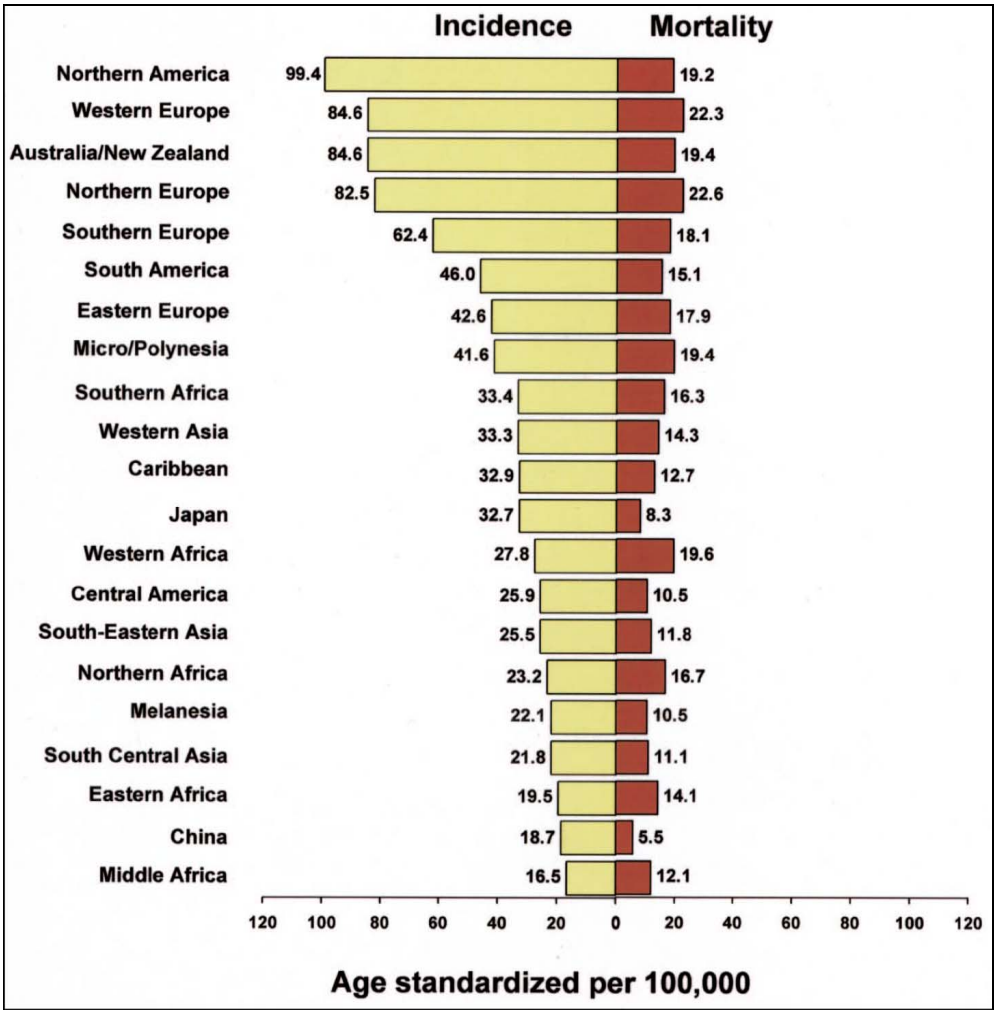

Figure 1 Age Standardized Incidence and Mortality Breast Cancer Rates (Parkin et al. 2005)

for differences in stage of diagnosis (Chlebowski 2005). In addition to racial differences, the incidence of breast cancer varies by age and menopausal status, with breast cancer rates highest among postmenopausal women. The incidence of breast cancer increases with age (until age 80), with 95% of breast cancer cases diagnosed in women over the age of 40, according to the 2000-2004 data from the American Cancer Society. The median age at diagnosis is reported as 61 years of age, with the highest age-specific incidence rate of 464.8 cases per 100,000 occurring among women ages 75-79 (ACS 2007).

2.1.1 Breast Cancer Risk Factors

Numerous factors have been implicated with an increase in risk of developing breast cancer, such as older age, family history of breast cancer, increased breast density, later age at first pregnancy, use of postmenopausal hormones (ACS 2007), and most notably, higher cumulative estrogen exposure during a woman's lifetime (Henderson 1998). Age, reproductive factors, family history and mammographic density are among the strongest risk factors for breast cancer. Family history in a first degree relative is associated with increased breast cancer risk, with risk increasing with younger age at onset as well as increasing numbers of affected first degree relatives (CGFBC 2001). Furthermore, two inherited breast cancer susceptibility genes have been identified (BRCA1 and BRCA2), although these genes only account for approximately 7% of breast cancer cases (reviewed in Hulka 2001).

Mammographic density is one of the strongest predictors of breast cancer risk, even after adjustment for other known breast cancer risk factors (Boyd 2005). Increased breast density has been associated with an increase in breast cancer risk, in both case-control and cohort studies (Boyd 2005). Women with high levels of breast density appear to have a 2-6 fold increased risk

of breast cancer compared to those with lower breast density (increase in fatty tissue) (Boyd 1998, Byrne 2001, Ursin 2003, Byrne 1995, McCormack 2006).

Lifestyle factors such as alcohol and physical activity may also affect breast cancer risk. Alcohol consumption of two drinks or more per day has consistently been associated with increase breast cancer risk by approximately 30% (Smith-Warner 1998, Hamajima 2002, Horn-Ross 2002, Ellison 2001). Physical activity has been shown to have a protective effect on breast cancer risk. Studies have reported modest decreases in breast cancer risk with regular physical activity with estimates ranging on average from 15-40% depending on the study population and the type of physical activity (Vainio 2002). Additionally, reproductive factors such as breastfeeding and increasing parity are associated with decreased breast cancer risk whereas early age at menarche and late age at menopause are associated with increased breast cancer risk.

Exogenous postmenopausal hormone therapy is associated with increased breast cancer risk, with levels of risk varying by the duration of use and type of treatment (estrogen versus combined estrogen and progestin) (Rossouw 2002, Bernstein 2006). As reviewed by Bernstein, the use of combined estrogen and progestin regimens can increase the risk of breast cancer up to 10% per year of use (Bernstein 2006). While some of the known breast cancer risk factors may potentially be modifiable, such as postmenopausal obesity, hormone replacement therapy, alcohol use and physical inactivity, others are not (age, family history, age at menarche, age at menopause, and parity). Mammographic density may also be a potentially modifiable factor. Although limited studies have been conducted in this area, results from a dietary intervention reported a reduction in the area of breast density (Boyd 1997). However, whether these effects are sustainable over a long period of time remains unclear.

Table 1 Summary of Breast Cancer Risk Factors

Known Risk Factors	Direction of Association
Age (↑)	↑
Family History of Breast Cancer	↑
History of Benign Breast Disease	↑
Reproductive Factors	
Parity	↓
Younger age at first birth	↓
Older age at menarche	↓
Obesity	
Premenopausal	↓
Postmenopausal	↑
Endogenous Estrogens (↑ levels)	↑
Hormone Therapy	↑
Breast Density (↑ dense tissue)	↑

2.1.2 Obesity and Breast Cancer

The prevalence of overweight and obesity remains elevated in the United States (Ogden 2006). According to the National Health and Nutrition Examination Survey (NHANES), 30% of adults in the United States were classified as obese in 2003-2004 with a BMI of $\geq$ 30 kg/m^2. More specifically, the prevalence of obesity among women ages 40-59 in the US was 36.8% during this time period (Ogden 2006). This is of particular importance considering the multiple environmental and genetic factors that interact and contribute to the development of obesity (Froguel and Boutin, 2001), with more than 430 genes, markers and chromosomal regions linked to phenotypes of human obesity (Snyder 2004). Furthermore, obesity has been linked with an increased risk of multiple chronic diseases including cancers of the colon and endometrium (Calle 2004).

The association of obesity with breast cancer risk varies by menopausal status. Obesity is associated with an increased risk of postmenopausal breast cancer (EHBCCG 2003, van den Brandt 2000, Key 2003); however, among premenopausal women, obesity has been suggested to be protective (Ursin 1995, Van Den Brandt 2000). Furthermore, the association of obesity and postmenopausal breast cancer risk may be dependent on the use of hormone therapy. Studies have reported a significant association between obesity and postmenopausal breast cancer among those who had never taken hormone therapy, whereas no association was reported among those who had ever used hormone therapy (Morimoto 2002, Feigelson 2004). In addition to baseline measures of obesity or obesity levels pre-diagnosis, adult weight gain appears to be associated with increased postmenopausal breast cancer risk (Eliassen 2006, Morimoto 2002, Feigelson 2004).

Potential explanations for the differing associations between obesity and breast cancer by menopausal status have been proposed. Although it remains largely unclear why premenopausal obese women have a reduced breast cancer risk it may be, in part, due to the impaired ovulatory function and the decrease in gonadal steroidogenesis that is associated with abdominal obesity. Among postmenopausal women, one possible biological mechanism involves the higher estrogen levels associated with increased obesity, resulting from an increase in aromatase activity that occurs in adipose tissue (McTiernan 2003, Colditz 1993, Vainio 2002, Sitteri 1987). The aromatization of steroids to estrone is the main source of endogenous estrogen and estrogen metabolites among postmenopausal women. Additionally, lower levels of sex hormone-binding globulin (SHBG) in obese women may lead to higher levels of unbound estrogen. The increased bioavailable estrogen caused by excess body fat is not bound by SHBG but rather is available to interact with breast tissue (Rock 2002, Verkasalo 2001). Insulin and insulin growth factors may

also play a role in explaining the relationship between obesity and postmenopausal breast cancer (Rock 2002). During the menopausal transition, women experience a decrease in ovarian function and estradiol levels and an increase in weight, percent body fat, and percent intra-abdominal fat (Kuller 2000). The combination of these factors may influence a woman's risk of breast cancer. Although the increase in peripheral levels of estrogens is the primary proposed explanation for the observed increase in postmenopausal breast cancer risk amongst obese women, other hormones related to body fat may also play a role.

2.2 ENDOGENOUS ESTROGENS AND BREAST CANCER

Endogenous estrogen is an important component in the development of breast cancer. Cumulative estrogen exposure is affected by normal life events such as age at menopause, age at menarche, and reproductive factors, all of which are known to be associated with breast cancer risk. Many of the risk factors identified for breast cancer may directly or indirectly affect the levels of endogenous estrogen exposure. Higher levels of endogenous estrogen have been associated with an increased breast cancer risk among postmenopausal women (EHBCCG 2002, Eliasssen 2006, Cauley 1999, Zeleniuch-Jacquotte (1995), Missmer 2004, Helzlsouer 1994, Toniolo 1995, Berrino 1996, Dorgan 1996, Thomas 1997). In most studies, estrogen levels were measured using blood samples collected from healthy women who were then followed until their diagnosis of breast cancer. Although the endogenous estrogen levels were likely not affected by disease status (as blood samples were collected many years prior to diagnosis), one limitation to many of these studies is the use of a single measurement of hormone levels.

A reanalysis of nine prospective studies by the endogenous hormones and breast cancer collaborative group reported a strong association between endogenous estrogens and breast cancer risk (Endogenous Hormones and Breast Cancer Collaborative Group). The results of this study reported a significant increasing trend in breast cancer risk with increasing quintiles of estradiol concentrations ($p<.001$). Those in the highest quintile of estradiol concentrations had a relative risk two times that of those in the lowest quintile (95% CI: 1.47-2.71). A similar pattern was observed when comparing quintiles of free estradiol among those with the highest versus lowest levels (RR=2.58, 95% CI: 1.76-2.78). Elevated relative risks were also reported in a prospective case-cohort study of breast cancer among women 65 years and older (Cauley 1999). Women with the highest concentration of bioavailable estradiol were 3.6 times as likely to develop breast cancer as were those with the lowest concentration (95% CI: 1.3-10.0) (Cauley 1999).

Few studies have evaluated the association between endogenous estrogen levels and breast cancer risk according to estrogen and progesterone receptor status of the tumor (Missmer 2004, Zeleniuch-Jacquotte 1995). Missimer et al. a reported a strong association between estradiol and ER^+/PR^+ breast cancer tumors but observed weak or no associations for ER^+/PR^- and ER^-/PR^-. For those in the highest quartile compared to those in the lowest quartile the relative risk for ER^+/PR^+ tumors was 3.3 (95% CI: 2.0-5.4). Although these results suggest a stronger association with ER^+/PR^+ tumors, only one other study has examined this relationship and that study reported no difference in the association between endogenous estrogen levels and type of estrogen receptor tumor status (Zeleniuch-Jacquotte 1995).

The relationship between estradiol levels and postmenopausal breast cancer also has been evaluated by body mass index. In a reanalysis of individual data from eight prospective studies

conducted by the Endogenous Hormones and Breast Cancer Collaborative Group, the observed increase in breast cancer risk with increasing levels of BMI was reduced after adjustment for free estradiol and other estrogens. These results support the notion that the observed increase in postmenopausal breast cancer with increasing obesity may be related to an increase in bioavailable estradiol.

The potential mechanism by which estradiol could affect breast cancer risk may involve the process of mitosis in breast epithelial cells. Increased estradiol levels stimulate the mitotic process, and thereby increase the mitotic rate in breast epithelial cells. As the mitotic rate increases, the chances of a mutation replicating prior to repair also increases (Preston-Martin 1990, Anderson 1982, Fiegelson and Henderson 1996). An alternative mechanism also has been proposed in which estrogen, through the formation of depurinating estrogen adducts, acts as a cancer initiator (Cavalieri 2006). Therefore, endogenous estrogens as well as their metabolites are important components of the carcinogenic pathway.

2.3 ESTROGEN METABOLITES, ESTROGEN QUINONES, AND ADDUCTS

Estrogen metabolism most often occurs by the oxidation pathway (Lippert 1999). The biotransformation of estrogen includes multiple steps, the first of which involves the conversion of estradiol to estrone in the C17 position, a reversible process. The breakdown of estrone continues via two main pathways involving hydroxylation sites C2, C4 or C-16, leading to the formation of either A-ring or D-ring metabolites (Mueck 2002, Lippert 2000). Main metabolites of the A-ring pathway include 2-OHE_1 and 4-hydroxyestrone (4-OHE), while 16α-OHE_1 and estriol result from the D-ring pathway (Figure 2, from Mueck et al. 2002). 2-hydroxylation of

estradiol or estrone is a main metabolic pathway in the liver, while 4-hydroxylation represents a smaller portion of the metabolic products formed (Kerlan 1992). Although these metabolites are the main products from both the A-ring and D-ring pathways, additional metabolites are formed both pathways. Table 2 includes a list of estrogen metabolites formed from both the A-ring and D-ring pathways.

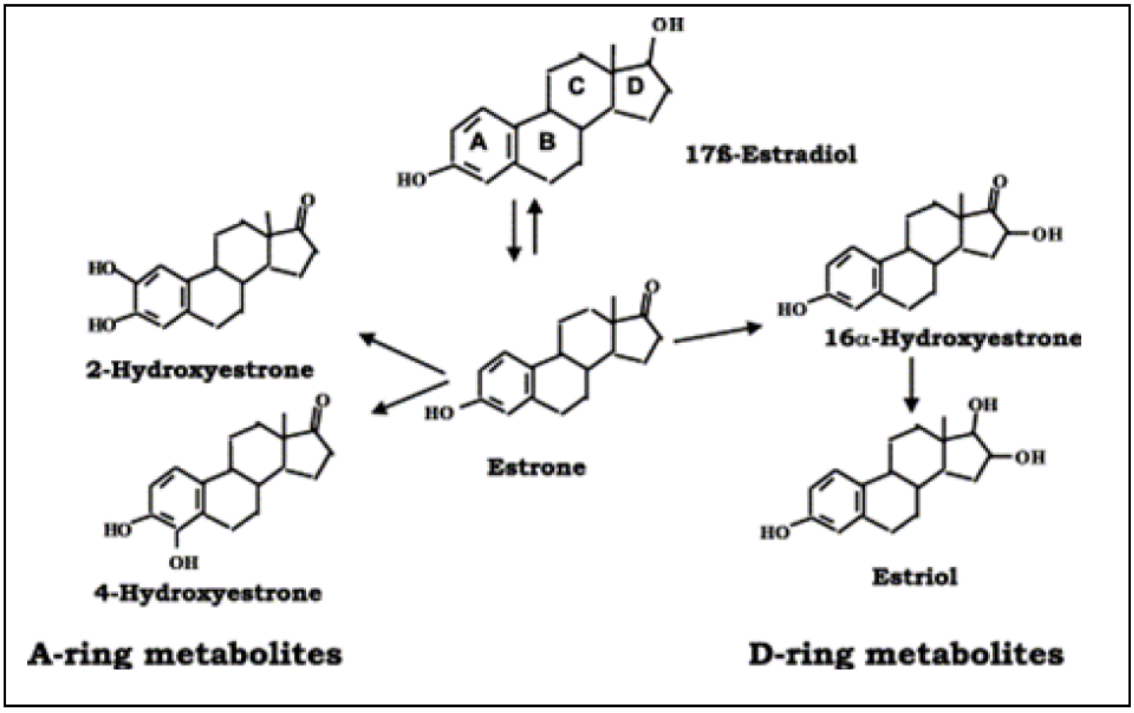

Figure 2 Structures of the Parent Hormone 17β estradiol and its main A- and D- ring metabolites.

[Figure 2 from Mueck et al. 2002. Maturitas. 43: 2]

Table 2 List of Main Metabolites of 17β -estradiol and Estrone*

Compound Name
2-Hydroxyestrone
2-Methoxyestrone
2-Hydroxyestradiol
2-Methoxyestradiol
2-Hydroxyestriol
2-Methoxyestriol
4-Hydroxyestrone
4-Methoxyestrone
4-Hydroxyestradiol
4-Methoxyestradiol
16-epiestriol
16-ketoesradiol
16α-Hydroxyestrone
17-epiestriol
2-hydroxyestrone-3-methyl ether
Estrone
Estriol
Estetrol

*Zhu 1998

All of the estrogen metabolite products are formed with the help of specific enzymes mainly within the cytochrome P450 family. The estrogen 2-hydroxylation which occurs in the human liver is mostly catalyzed by the cytochrome P450 1A2 and the 3A family of enzymes (Kerlan 1992, Yager 2006). Cytochrome P450 1B1 is involved in the 4-hydroxylation of estradiol in target tissues such as the breast (Hayes 1996), whereas the cytochrome P450 3A family is thought to be involved in the 4-hydroxylation in human liver microsomes (Kerlan 1992, Yager 2006). This process of estrone and estradiol metabolism is referred to as Phase I metabolism (Yager 2006).

Among women with high ovarian estradiol secretion, mainly premenopausal women, this metabolic process begins with estradiol. However, among postmenopausal women, estrogen formation occurs by aromatization of androgens to estrone, the main circulating estrogen which

subsequently is released from the body by metabolism into either the A-ring or D-ring metabolites. These metabolites are suggested to perform differently, with metabolite 16α-OHE_1 having higher estrogen properties based on its ability to bind to the estrogen receptor (Lippert 2000), and 2-OHE_1 having lower estrogen activity, in part due to the reduced affinity to estrogen receptor binding. Additionally, 16α-OHE_1 has been shown to have properties similar to those of estradiol, an estrogen agonist (Cavalieri 2006). Although both 2-OHE_1 and 16-OHE_1 have estrogenic properties, they vary in regards to their ability to bind to the estrogen receptor. Additionally, these metabolites are mutually exclusive, rendering the ratio 2:16α-OHE_1 a useful measure. An increase in one pathway will lead to a reduction in the product of the competing pathway. The 4-OHE metabolite also binds to the estrogen receptor and additionally it may undergo redox cycling leading to the formation of reactive oxygen species and potentially oxidative damage (Liehr 1990a, Liehr 1990b). Similar to the 4-OHE metabolite, 2-hydroxyestradiol and 2-OHE_1, also can undergo metabolic redox cycling to form reactive oxygen species (Liehr 1990a, Liehr 1990b). Further oxidation can then lead to the formation of reactive semi-quinone and quinone products, specifically, estradiol-3,4-semiquinone, estradiol-3,4-quinone, estradiol-2,3-semiquinone, and estradiol-2,3-quinone (Cavalieri 2006).

As reviewed by Cavalieri et al., the catechol quinones that are formed can react with DNA to form depurinating adducts, which are released from DNA to form apurinic sites. These DNA adducts can lead to altered forms of DNA which can either be repaired or misrepaired, resulting in mutations. An adduct generally forms when a chemical covalently binds to a molecule such as DNA, and in this case either a stable adduct or depurinating adduct is formed when the catechol quinones bind with DNA. The depurinating adducts that are formed include 2-OHE_2-6-N3Ade which results from the 2-OHE pathway while 4-$OHE_1(E_2)$-1-N3Ade and 4-

$OHE_1(E_2)$-1-N7Gua are formed when the catechol estrogen, $E_1(E_2)$-3,4-Q reacts with DNA. These depurinating adducts comprise more than 99% of the total DNA adducts formed (Zahid 2006, Cavalieri 2006). The estrogen metabolism pathway and DNA adduct formation, along with the genes involved in these processes are depicted in figure 3 (adapted from Cavalieri 2006). The activating enzymes and DNA adducts are shown in red while the protective enzymes are shown in green.

Cavalieri and colleagues discuss this model in relation to homeostatic balance (Cavalieri 2006). The notion behind the proposed role of depurinating adducts as cancer initiators involves an imbalance between the activating and deactivating pathways shown in Figure 3. The disruption of estrogen balance may lead to increased levels of estrogen quinones which would then be available to react with DNA. Potential causes of this imbalance may be the over expression of CYP1B1 or low levels of other substances in the pathway such as quinone reductase or glutathione (Cavalieri 2006).

2.3.1 Studies of estrogen metabolites in healthy populations

Since the early 1980's, approximately 45 studies have evaluated the individual estrogen metabolites (2-OHE_1, 16α-OHE_1) , or the 2:16α-OHE_1 ratio in relation to various factors including dietary and lifestyle factors, race, hormone therapy, genetic polymorphisms and family history. A summary list of studies that have evaluated the 2-OHE_1 and 16-OHE_1 metabolites among healthy populations is presented in Table 3. Studies have been conducted among populations of both pre- and postmenopausal healthy women, and the majority of studies measured estrogen metabolites in urine.

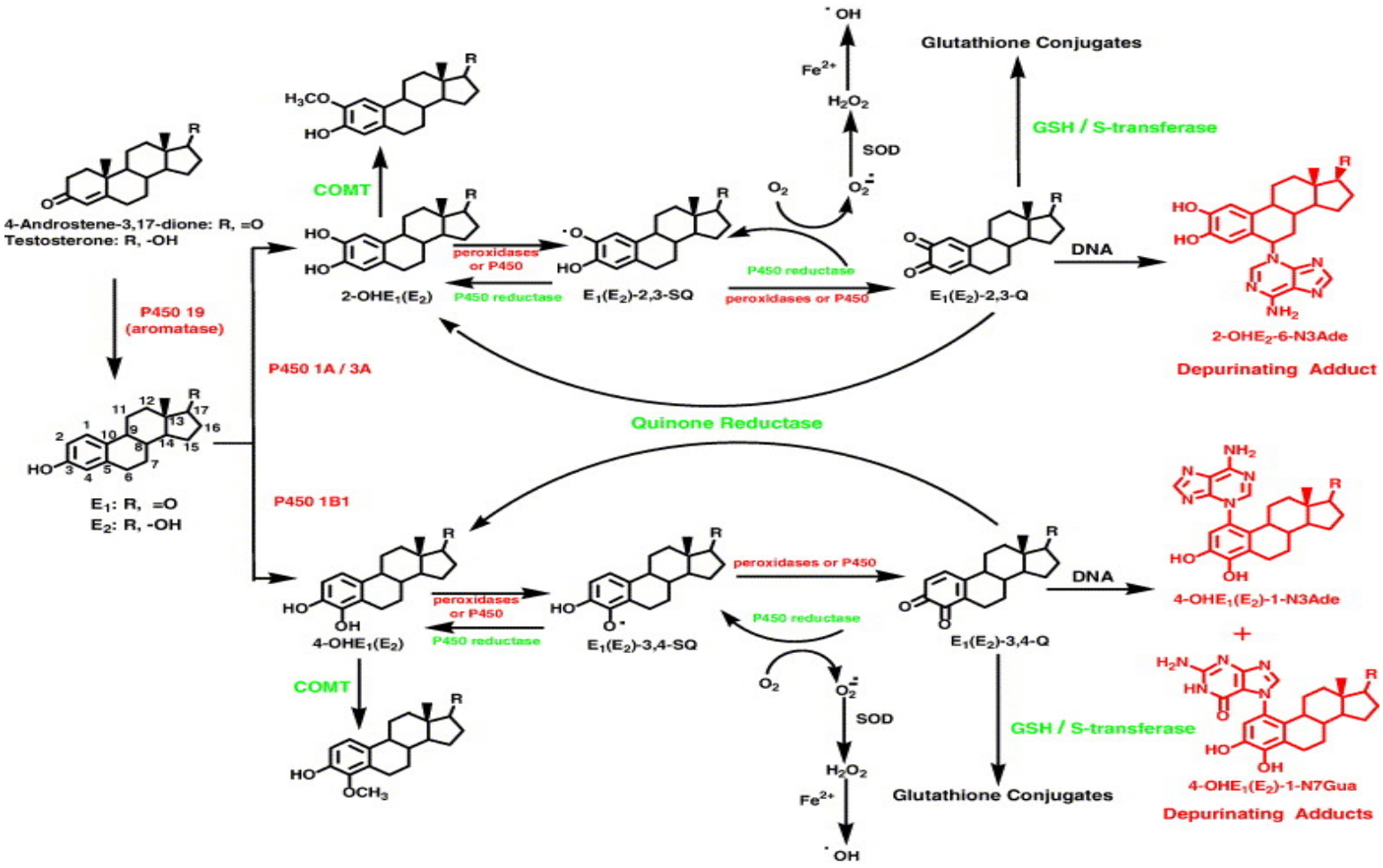

Figure 3 Estrogen Metabolism and DNA Adduct Formation

[Figure from Cavalieri et al. 2006. Biochim Biophys Acta. 1766(1): 66]

Some studies have evaluated potential racial differences in estrogen metabolites (Aldercruetz 1994, Ursin 2001, Jernstrom 2003, Falk 2005**,** Taioli 1996), while other studies have assessed the role of dietary factors on estrogen metabolite levels. These dietary factors include flaxseed consumption (Haggans 2000, McCann 2007, Sowers 2006), Brassica vegetable consumption (Fowke 2000), macronutrient intake (Fowke 2001), soya diet (Lu 2000), and indole-3-carbinol supplementation (Michnovicz 1997). Additional studies have evaluated the effects of lifestyle factors such as physical activity on estrogen metabolite levels (Pasagian-Macaulay 1996, Atkinson 2004, Bentz 2005, Matthews 2004) as well as family history of breast cancer (Ursin 2002, Greenlee 2006) and postmenopausal hormone use (Alvarez-Vasquez 2002, Mueck 2001). Factors suggested to increase 2-hydroxyestrone levels include vigorous exercise, intake of cruciferous vegetables, indole-3-carbinole, flax, soy, estrogen replacement therapy, thyroid hormone medication, and smoking. Cruciferous vegetables include foods such as cabbage, broccoli, kale, otherwise known as Brassica foods which release indole-3-carbinole, a possible anticarcinogenic compound suggested to increase levels of 2-hydroxylation (Lord 2002). Obesity has been suggested to decrease 2-OHE_1 levels, subsequently increasing 16α-OHE_1 levels (Sowers 2006).

Estrogen metabolite levels vary among populations of healthy women. The mean values reported across studies are presented by race, menopausal status or in relation to dietary or lifestyle factors; describing the overall mean levels of estrogen metabolites among healthy subjects in the population can be difficult. Additionally, some studies may report only the mean values of the individual 2-OHE_1 and 16α-OHE_1 levels and not the ratio. In a small study of 67 Singapore Chinese and 58 African American women, Ursin et al. reported no significant difference in the mean 2:16α ratio for Chinese women (1.63 95%CI: 1.40-1.89) versus African

American women (1.48 95% CI: 1.27-1.74). Taioli et al. reported a significant difference in mean 2:16α ratio levels for Caucasian (2.25 ± 0.89) and African American (1.42 ± 0.61) women among a study population of 33 healthy women ages 18-73. These findings suggest that estrogen metabolism may differ between some racial groups.

Table 3 Summary of Studies of Estrogen Metabolites in Healthy Populations of Women

Author	Year	Topic	N	Race	Age (years)	Menopausal Status	Assay	# measures	2:16	Mean or Median	2:16 Ratio Presented by
Aldercreutz	1986	dietary fiber	23	Finnish	31.7/34.6	Pre	GC-MS	2	yes	geometric	diet groups
Aldercreutz	1994	multiethnic	25	Asian/Finnish	23.3/31.9	Pre	GC-MS	1	no	mean	2 or 16-OHE only
Alvarez-Vasquez	2003	HRT	56	White^	?	Post	EIA	1	yes	mean	overall and by HRT
Armamento-Villareal	2004	HRT	310	White	avg. 60	Post	EIA	1	yes	mean	HRT status
Atkinson	2003	equol excretion	126	White/Asian/AA	25-59	Pre & Post?	EIA	1	yes	geometric	overall
Atkinson	2004	pa intervention	173	mostly White (86%)	50-75	Post	EIA	3	yes	geometric	intervention
Bentz	2005	physical activity	77	White	18-51	Pre	EIA	1	yes	mean	overall
Brooks	2004	flaxseed RCT	46	White	avg. 52-54	Post	EIA	2	yes	mean	treatment
Campbell	2007	aerobic fitness	30	White	20-42	Pre	EIA	2	yes	mean	fitness, menstrual
Chen	1999	methods	10	White	23-58	Pre and Post	EIA	8	yes	mean	No
Falk	2005	multiethnic	511	Asian women	25-55	Pre & Post	EIA	1	yes	geometric	Too specific
Fowke*	2000	brassica vegetables	34	White	>45	Post	EIA	2	yes	mean	intervention
Fowke*	2001	dietary factors	37	White^	>45	Post	EIA	2	yes	Mean	0verall
Frankenfeld	2004	daidzein	89	White^	50-75	Post	EIA	1	yes	geometric	equol groups
Greenlee	2007	family hx of br ca	64	Mostly white (91%)	avg 50	Pre & Post	EIA	1	yes	mean	menopause & age
Haggans	2000	Flaxseed	16	mostly white	20-38	Pre	EIA	3	yes	mean	diet group
Jernstrom	2003	Multiethnic	513	Asian/AA/White/Indian	17-35	Pre	EIA	1	yes	geometric	phase
Lim	1997	osteopenia, BMD	59	Korean	55-60	Post	GC-MS	1	no	mean	groups
Lu	2000	soy, isoflavones	85	White?	33	Pre	EIA	Daily	yes	mean	diet
Leelawattana	2000	bone density	71	**	47-59	Post	**	**	yes	**	**
Longcope	1987	low vs.high fat diet	64	White?	**	Pre	RIA	2	**	**	**
Martini	1999	soy intake	36	White?	18-40	Pre	EIA	2	yes	Mean	diet & OC use
Masi	2006	blood pressure	85	White/AA/Latino	50-67	Post	EIA	1	yes	mean	HRT status

Table 3 continued

Author	Year	Topic	N	Race	Age (years)	Menopausal Status	Assay	# measures	2:16	Mean or Median	2:16 Presented by
McCann	2007	Flax, genes	132	mostly white (97%)	45-75	Post	EIA	2	yes	mean	overall
Matthews	2004	physical activity	157	White/Chinese	61.7/ 47.1	Pre & Post	EIA	2	yes	mean	race
Michnovicz	1988	smoking	29	White?	21-44	Pre	RIA	1	no	mean	smoking
Michnovicz	1997	indole3-carbinole	10 women	White?	32.3	Pre	GC-MS	2	no	mean	No
Mueck	2001	OC & HRT	55, 63	White?	26/55	Pr& Post	EIA	2	yes	mean	pre & post HRT or OC
Mueck	2004	OC	**	**	**	Pre	EIA	2	yes	mean	pre & post OC
Napoli	2005	genes/ bone density	156	White	63.5	Post	EIA	1	yes	mean	genotype
Nettleton	2005	soy intake	40	White?	avg 56.2	Post	EIA	4	yes	geometric	overall (controls)
Pasagian-Macaulay	1996	lifestyle factors	174	White?	44-50	Pre	EIA	baseline & 6 months	yes	mean	treatment group
Riza	2001	breast density	140	Greek	40-65	Post	EIA	1	yes	Mean	breast density
Sowers*	2006	genes	1340	AA/white/Asian/Hispanic	45-52	Pre & Peri	EIA	1	no	mean	race
Sowers*	2006	diet &lifestyle	1881	AA/white/Asian/Hispanic	45-54	Pre &Pero	EIA	1	yes	mean	race
Taioli	1996	multiethnic	33	White/AA	18-73	Pre & Post	EIA	1	yes	mean	race
Ursin	2001	multiethnic	125	Chinese, US AA & White	45-75	Post	EIA	1	yes	geometric	race
Ursin	2002	family hx of br ca	97	White	20-50	Pre	EIA	1	yes	geometric	family history
Westerlind	2007	diet & exercise	24	White	avg 31.5	Pre	EIA	5	yes	mean	menstrual phase
Xu	1999	Menstrual cycle	6	White?	avg 27.8	Pre	GC-MS	Daily (during cycle)	yes	mean	menstrual phase
Xu	1998	soy isoflavones	12	white?	avg 26	Pre	GC-MS	3	yes	mean	diet period
Xu	2000	soy diets	18	white?	avg 56.9	Post	GC-MS	2	yes	mean	Overall & diet

Abbreviations: GC-MS=Gas chromatography mass spectrometry, EIA=Enzyme ImmunoAsssay, RIA=Radiometric ImmunoAssay, Geometric: refers to geometric mean, ^=requires confirmation,?=not indicated in publication, hx=history, men=menopause status, *Potentially same study populations, **=pending article

2.3.2 Measurement of estrogen metabolites

The 2-OHE_1 and16α-OHE_1 metabolites are present in low concentrations in the blood, and thus detection methods have focused on the measurement of these metabolites in urine. Earlier studies have utilized the gas chromatography-mass spectrometry technique to measure estrogen metabolites in urine (Aldercreutz 1976). However, this method can be labor intensive (Fotsis 1987) and more prone to errors. In 1994, Klug et al. developed an Enzyme ImmunoAssay (EIA) technique to measure the 2-OHE_1 and16α-OHE_1 metabolites in urine (Klug 1994). This assay kit has been validated against other methods of estrogen metabolite detection, such as the gas chromatography-mass spectrometry (Falk 2000).

In a methodological study by Ziegler et al., the original kit developed by Klug et al. (1994) was found to have difficulty with the limit of detection when measuring the metabolite levels in urine samples from postmenopausal women (Ziegler 1997). Concentrations of estrogen metabolite levels are lower among postmenopausal women versus premenopausal women due to overall lower levels of estrogen in the body. Subsequently, a modified version of the kit was developed with an increased sensitivity level of 0.625 ng/ml (Bradlow 1998). The components of the original kits are similar to those of the modified kit, with the exception of the modifications to the antibody concentrations, enzyme concentrations and standards.

2.3.3 Reliability, reproducibility and validity of estrogen metabolites

Limited research has been conducted in relation to the reliability, reproducibility, and validity of estrogen metabolites in urine as well as in other biological samples such as serum, plasma, and

tissue. Furthermore, the studies that have been conducted often are based on small sample sizes. Falk et al. conducted a study to evaluate the reproducibility, validity and assay performance of the modified ELISA kit compared with the gas chromatography-mass spectrometry method (GC-MS) (Falk 2000). The results of this comparison demonstrated that the absolute values of the 2-OHE_1 and 16α-OHE_1 metabolites were lower, and the 2:16α-OHE_1 ratio was higher when measured with the new ELISA kit compared to the GC-MS. Despite the differences in the absolute values, the correlations between both laboratory methods were high. For the 2-OHE_1 metabolite, the Spearman correlation coefficients were 1.00 for both pre and postmenopausal women. Similarly, for the 16α-OHE_1 metabolite, the Spearman correlation coefficient between both methods was 0.70 and 1.00 for pre- and postmenopausal, respectively (Falk 2000).

In addition to the overall performance of the assay, it is also important to consider potential differences in estrogen metabolite levels when measuring these compounds in various biological samples. The ELISA kit can be used to measure the 2-OHE_1 and 16α-OHE_1 metabolites in both plasma and urine, but few studies have compared the levels in both sample types (Bradlow 2006) or in relation to serum or tissue levels. In a study by Bradlow et al., plasma and urine samples were collected from 511 nulliparous, multiethnic women, aged 17-35. For the estrogen metabolite levels in plasma and urine, the reported correlation coefficient was 0.52 ($p<0.0001$) (Bradlow 2006). The correlation between the levels from both sample types also was evaluated in relation to various factors such as oral contraceptives, phase of the menstrual cycle and ethnicity. The correlation between plasma and urinary levels was higher among those not currently taking oral contraceptives ($r_s=0.58$; $p<0.0001$) compared to those who are currently taking oral contraceptives ($r_s=0.34$; $p<0.0001$) (Bradlow 2006). In addition to comparisons between plasma and urine, 2-OHE_1 and 16α-OHE_1 metabolites were measured in tissue using gas

chromatography-mass spectrometry. Tissue samples were obtained from mammary fat taken from multiple specimens during mammoplasty for breast reduction. Due to the variability and wide range of both the 2-OHE_1 and 16α-OHE_1 metabolite levels in the tissue samples, tissue levels could not be compared with urinary or plasma levels (Bradlow 2006). Additional studies have not compared estrogen metabolite levels in tissue versus plasma or serum. Thus, it remains unknown whether urinary levels of estrogen metabolites reflect tissue levels in the breast.

Limited research has been conducted in the area of within-person variability and the stability of estrogen metabolite levels over time. Chen et al. conducted a study to assess the within-person variability of the ratios of urinary 2-OHE_1 to 16α-OHE_1 (Chen 1999). Ten healthy Caucasian women aged 23-58 years provided an overnight fasting morning urine sample once a week for eight weeks. Over this two-month period, the 2-OHE_1 and 16α-OHE_1 metabolites measured at any one time point correlated with the average ratio over the eight week study period (mean correlation coefficient: 0.85) (Chen 1999). Although this study supports the stability of the estrogen metabolites over a two-week period, the results are based on a small sample size.

The temporal reliability of hormone levels and in particular, the 2-OHE_1 and 16α-OHE_1 metabolites was measured by Williams et al. in a longitudinal study in which five samples were collected over a year (n=34). Twelve analytes were measured and the investigators observed that only two analytes, sex hormone binding globulin and estrone sulfate, could be adequately estimated by a single measure with the potential to account for 64% of the true variance. This study suggests that a single measure of the estrogen metabolites may not adequately account for at least 50% of the true variance, and preferably, that 2-4 measures might be adequate (accounting for 81% of the true variance). In regards to the overall stability of the five measures

of 2-OHE_1 and 16α-OHE_1, the intraclass correlation coefficients were 0.46 (0.30-0.63) and 0.56 (0.41-0.71), respectively (Williams 2002). Despite the studies by Chen and Williams, questions still remain with regard to the within-person variation of estrogen metabolites over time and the validity of a single measure over multiple measures, as well as additional methodological issues concerning the measurement of estrogen metabolites.

2.4 ESTROGEN METABOLITES AND BREAST CANCER

Lifetime estrogen exposure is a known breast cancer risk factor. Estrogens are involved in the proliferation of human breast epithelial cells and may indirectly influence carcinogenesis by stimulating cell division (Preston-Martin 1990, Fiegelson 1996). As the rate of mitosis increases, the possibility that a mutation will occur also increases (Preston-Martin 1990, Fiegelson 1996). Estrogen exposure is affected by normal life events such as age at menarche, age at first pregnancy and age at menopause, all of which may impact breast cancer risk but are often not modifiable. Considering the role of lifetime estrogen exposure in breast cancer development, along with the possible variation of estrogen metabolism in women, studies have evaluated the relationship between estrogen metabolite levels and breast cancer risk (Cauley 2003, Fowke 2003, Ho 1998, Kabat 1997, Kabat 2006, Meilahn 1998, Modugno 2006, Muti 2000, Ursin 1999, Wellejus 2005), however, the results have varied across studies.

Earlier studies by Schneider et al. (1982) and Fishman et al. (1984) first measured the role of 16α-OHE_1 using a radiometric assay in a small sample of women with breast and endometrial cancer (n=10 controls, n=33 breast cancer patients and n=10 endometrial cancer patients). The results of these studies suggested elevated levels of 16α-OHE_1 among women with

breast and endometrial cancer as compared to the controls. Fishman et al. reported a statistically significant difference in the mean (± SE) 16α-hydroxylation values for breast cancer patients (14.9 ± 1.5) versus normal women (9.3 ± 0.8), p-value=0.01 (Fishman 1984). Since the publication of these initial studies, twelve studies have evaluated the association between estrogen metabolites and breast cancer, including seven case-control, four nested case-control and one case-cohort study. The tables below summarize the study characteristics and main findings of these 11 published nested case-control and case-cohort studies (Table 4), and case-control studies (Table 5) (summary of studies current as of July 15, 2008).

While the results of some studies support an overall association for a reduction in breast cancer risk with a higher 2:16α -OHE_1 ratio (Kabat 1997, Ho 1998), other studies suggest either a modest association or a lack of statistical significance (Meilahn 1998, Muti 2000, Wellejus 2005), or show no association at all (Ursin 1999, Cauley 2003). Additionally, the relationship between the 2:16α -OHE_1 ratio and breast cancer risk may depend on whether the metabolites were measured before or after treatment (Fowke 2003) and on factors such as obesity and hormone replacement therapy (Modugno 2006).

Possible explanations for the inconsistent findings across studies include differences in the timing of the specimen collection, the covariates included as potential confounders, whether subgroups were considered in the analyses, sample size, the type of biological sample (serum vs. urine) and potential biases. The few studies that reported significant findings likely detected a protective effect with the 2:16α-OHE_1 ratio because multiple confounding variables and important subgroup analyses also were considered (Modugno 2006, Fowke 2003, Wellejus 2005).

Both BMI and hormone therapy are important breast cancer risk factors that may affect endogenous hormone levels. Assessing the role of estrogen metabolites in relation to these potential effect modifiers as well as other factors such as menopausal status, stage of disease and estrogen receptor status may help elucidate the relationship between estrogen metabolites and breast cancer. However, individual study sample sizes are often too small to allow for such stratification. The sample sizes of the study populations ranged from approximately 40 to 426 cases and 60 to 426 controls. The largest study, conducted by Wellejus et al., included 426 cases and 426 controls while the study by Kabat et al. (1997) included 42 cases and 64 controls.

Inconsistent findings have been reported across the studies, regardless of the type of study design utilized. Muti et al. conducted a nested case-control study within the Guernsey study and reported a non-significant reduction in breast cancer risk among women in the highest versus lowest tertiles of 2:16α-OHE_1 (premenopausal: OR=0.75, 95% CI: 0.35-1.62; postmenopausal: OR=0.71, 95% CI: 0.29-1.75). Another nested case-control study was conducted among women ages 35-60 years of age who were participants in the ORDET Study (Hormones and Diet in the Etiology of Breast Cancer). Results suggested that a higher ratio of 2-OHE_1 to 16α-OHE_1 conferred a protective effect among pre-menopausal women in the highest quartile compared to those in the lowest quartile (OR=0.58, 95% CI: 0.25-1.34). However, among postmenopausal women, a non-significant increased risk was observed among those in the highest versus lowest quartile of 2:16α-OHE_1 (OR=1.29, 95% CI: 0.53-3.10) (Muti 2000). Both Modugno et al. and Wellujus et al. assessed the association between the estrogen metabolites and breast cancer by specific subgroups. Wellejus et al. reported a statistically significant difference in the mean 2:16α-OHE_1 values by hormone therapy status (p=0.0001) and specifically, higher 2:16α-OHE_1 values were observed among hormone therapy users. The case-

cohort study conducted by Cauley et al. did not detect an association between the estrogen metabolite ratio and breast cancer among women ≥ 65 years of age who were participants in the Study of Osteoporotic Fractures (SOF) (Cauley 2003).

Similar variation in study findings was observed among the published case-control studies. Ursin et al. reported no significant difference in mean 2:16α-OHE_1 values by case/control status (p=0.58). Significant findings were reported by both Ho et al. and Kabat et al.; however, the results should be interpreted carefully as these estimates are based on fairly small sample sizes. Fowke et al. stratified analyses by the timing of urine collection and observed a significant increase in breast cancer risk with an elevated 2:16α-OHE_1 among those with urine samples collected after treatment (Fowke 2003). Based on the results of published studies to date, the relationship between the 2-OHE_1 and 16-OHE_1 metabolites and breast cancer remains unclear. Conducting a combined analysis of individual data from published studies to evaluate this research question will help clarify whether the 2:16α-OHE_1 ratio is significantly associated with breast cancer among pre- and postmenopausal women. Figures 4 and 5 present a graphical summary of the individual published study estimates of the association between the 2:16α-OHE_1 ratio and breast cancer (Tables 6 and 7 indicate the corresponding study estimates).

Table 4 Nested Case-Control or Case-Cohort Studies of Estrogen Metabolites (2-OHE_1/16α -OHE_1) and Breast Cancer

Authors (year)	No. of cases/controls	Study Population	Age	Sample Source/ Assay	Major Findings OR/RR (95% CI)
Meilahn et al. (1998)	Postmenopausal: 42/139 Premenopausal: 60/184	Guernsey III cohort study 1977-1985 1:3 matching on age (± 2 years), date of baseline exam (± 1 year), menopausal status (if premenopausal, matched on phase on menstrual cycle)	≥ 35 years mean age (SD) (cases/controls): Premenopausal: 40.5 ± 4.3 / 40.5 ± 4.2 Postmenopausal: 59.1 ± 6.6 / 59.0 ± 6.2	spot urine/ enzyme immunoassay	highest tertile vs. lowest tertile of 2/16 ratio and breast cancer (unadjusted) Postmenopausal: OR=0.71 (95% CI: 0.29-1.75) Premenopausal: OR=0.75 (95% CI: 0.35-1.62) 2:16 median (cases/controls): Pre: 2.1 / 2.1 Post: 1.6 / 1.7
Muti et al. (2000)	Postmenopausal: 71/274 Premenopausal: 67/264 Italian women,	Residents of Varese province, northern Italy, enrolled as part of the 'Hormones and Diet Etiology of Breast Cancer Study' (ORDET) 1:4 matching on age, menopausal status, time of blood draw, centers	35-60 years	urine/ enzyme immunoassay	≥ 3.66 vs. ≤ 1.77 (adjusted) Postmenopausal: OR=1.31 (95% CI: 0.53-3.18) Premenopausal: OR=0.55 (95% CI: 0.23-1.32) *similar unadjusted OR values 2-OHE1& 16-OHE1 median: Pre: 40.3 ng/ml & 17.5 ng/ml Post: 9.7 ng/ml & 4 ng/ml
Cauley et al. (2003) Case-cohort	272/291 Postmenopausal	Participants from the Study of Osteoporotic Fractures (Portland, Minneapolis, Baltimore, Monongahela Valley)	≥ 65 years	serum/ Estramet 2/16 enzyme immunoassay	> 0.92 vs. ≤ 0.58 (age & bmi adjusted): HR:1.17 (0.73-1.87) Geometric mean (cases/controls): 0.74 (0.71-0.77) / 0.73 (0.70-0.77)
Wellejus et al. (2005)	426/426 Postmenopausal	Danish women from the Diet, Cancer and Health Cohort matched by age at diagnosis, baseline age and HRT use	50-64 years	Spot urine/ Estramet 2/16 enzyme immunoassay	Among HT users: 2:16 ratio per doubling age adjusted IRR =1.27 (95% CI: 1.00-1.60) 2:16 median by HRT status (cases/controls): HRT users: 2.2 (0.7-5.3) / 1.9 (0.7-5.8) HRT nonusers: 1.6 (0.7-32) / 1.6 (0.6-3.5) p-value (HRT vs. non) = 0.0001
Modugno et al. (2006)	200/200 Postmenopausal Majority white (92% of cases & controls)	Women's Health Initiative (women from 40 US clinical centers) matched by ethnicity, enrollment date, clinic site, type of HT and years since menopause	50-79 years	serum/ Estramet 2/16 enzyme immunoassay	↑ BMI & ↑ 16α-OHE_1 vs. ↓BMI & ↓16α-OHE_1 among Non HT users: OR=3.51 (1.34-9.16) Median 2:16 levels (cases/ controls): No HT 0.43/ 0.46 ERT 0.97/ 0.93 PERT 1.03/1.01 Any HT 1.01/ 0.99

Table 5 Case-Control Studies of Estrogen Metabolites (2-OHE_1/16α -OHE_1) and Breast Cancer

Authors (year)	No. of cases/controls	Source of Controls	Study Population	Age	Sample Source/ Assay	Major Findings OR (95% CI)
Kabat et al. (1997)	Overall: 42 invasive/64 Postmenopausal: 23 invasive/28	Women undergoing routine mammogram or screening	Women evaluated or treated at Montefiore Medical Center (Bronx, New York)	mean (SD) (cases/controls): 53.8 ± 15.1 / 54.2 ± 10.4	spot urine/ enzyme immunoassay	2:16 ratio < 1.38 vs. >1.91 OR=1.95 (0.64-5.97) Postmenopausal only: OR=32.74 (3.36-319.09) 2:16 mean (cases/controls) overall: 1.67 ± 0.80 / 1.72 ± 0.66 (p=0.7) post: 1.41 ± 0.73/ 1.81 ± 0.71 (p=0.05)
Zheng et al (1998)	20/20 Menopausal status not listed	Primarily from female employees in the Shanghai Cancer Institute	Two hospitals in Shanghai and Hangzhou, China	No provided	Urine/Estramet 2/16 enzyme immunoassay	Mean (cases/controls): 1.16/1.52, p=0.046 No other information provided
Ursin et al. (1997) Pilot study	25/23 Postmenopausal	Eligible community controls from previous case-control study	English speaking white women (including Hispanics) Los Angeles county	55 - 64 years	urine/ Estramet 2/16 enzyme immunoassay	Mean ± SE (cases/controls) 2:16 mean 1.39 ± 0.10 vs. 1.58 ± 0.20 (p=0.58)
Ursin et al. (1999)	66/76 Postmenopausal	Eligible community controls from previous case-control study	English speaking white women (including Hispanics) Los Angeles county Individual matched by age, ethnicity and area of residence	50 - 64 years	urine/ Estramet 2/16 enzyme immunoassay	≥ 1.91 vs. < 1.38 Adj OR=1.13 (0.46-2.78) 2:16 ratio mean (cases/controls) 1.78 (1.58-2.01) vs. 1.76 (1.60-1.93) (p=0.84)
Ho et al. (1998)	65/ 36 Pre & Postmenopausal	Randomly selected women who underwent breast biopsy (national breast screening project) confirmed to have benign breast disease	Cases did not receive chemotherapy or radiotherapy. Excluded insulin dependent diabetics, patients with hepatic dysfunction or loss of more than 5% premorbid weight	Mean Age± SE (cases/controls) 54.0 ± 1.2 54.8 ± 0.9	urine/ Estramet 2/16 enzyme immunoassay urine collected after overnight fast	2/16 ratio (continuous variable) and breast cancer, adjusted OR=0.10 (0.03-0.38) ≤ 0.9 vs. 0.9 adjusted OR=6.45 (2.05-20.3) 2/16 ratio Mean ± SE (cases/controls): All: 0.7 ± 0.1 / 2.0 ± 0.3 Post Menopausal: 0.7 ± 0.1 / 2.2 ± 0.5

Table 5 continued

Authors (year)	No. of cases/controls	Source of Controls	Study Population	Age	Sample Source/ Assay	Major Findings OR (95% CI)
Fowke et al. (2003)	110/110 pairs	random controls selected from a complete registry of the Shanghai population	Incident breast cancer cases identified from registries (hospital or tumor) in Shanghai, China Individually matched by menopausal status, age and pre or post treatment urine collection	25 - 65 years (median = 46 years)	urine/ Estramet 2/16 enzyme immunoassay	≥ 1.22 vs. ≤ 0.69 Urine collection: Pre-treatment: adj OR: 0.5 (0.2,1.1) Post: adj OR: 8.1 (1.6,47.1) *78 pretreatment pairs, 32 posttreatment 2:16 median (cases/controls): Pretreatment: 0.9/1.0 Posttreatment: 1.3/ 0.8
Kabat et al. (2006)	269 invasive, 158 in situ/326 Pre & postmenopausal	Random sample of Nassau and Suffolk county residents < 65 identified from RDD and > 65 from Health Care Financing Administration	The Long Island Breast Cancer Study Project, Nassau and Suffolk Counties, Long Island Primary invasive & insitu cases identified through rapid case ascertainment	range 20-98	spot urine/ Enzyme linked immunoassay	≥ 2.3 vs. ≤ 1.4 Postmenopausal: Invasive 0.78 (0.46-1.33) In situ 0.83 (0.44-1.57) Premenopausal: Invasive 0.50 (0.25-1.01) In situ 1.45 (0.66-3.19)

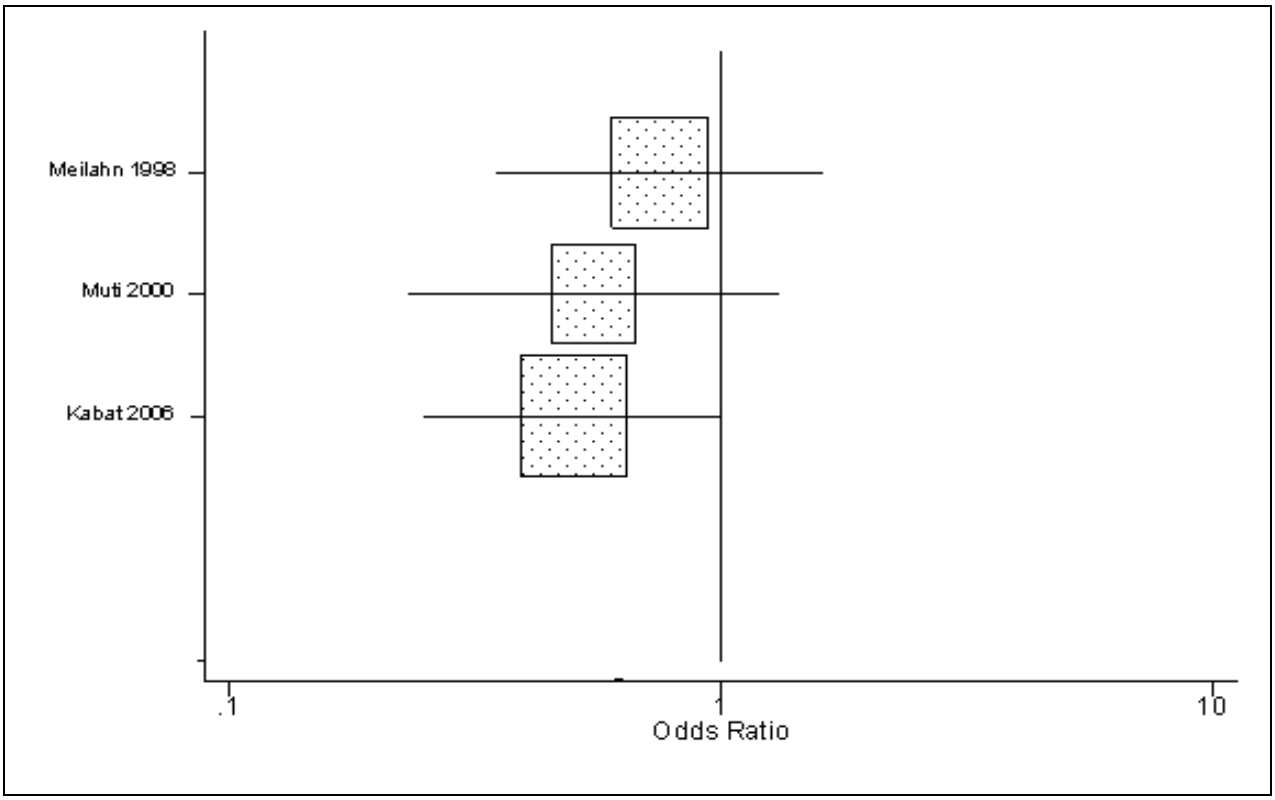

Figure 4 Premenopausal Studies: Association between 2:16α-OHE_1 and Breast Cancer*

*Estimates shown reflect those in the original publication.

Table 6 Premenopausal Study Specific Published Estimates Corresponding to Figure 3

Study*	2:16α-OHE_1 cutpoints	Estimate**	Confidence Limits
Meilahn 1998	<1.72,1.72-2.43, ≥2.44	OR=0.75	(0.35, 1.62)
Muti 2000	≤1.80,1.80-2.30, 2.31-2.72, 2.72-3.29, ≥ 3.29	OR=0.55	(0.2, 1.32)
Kabat 2006	≤ 1.4, 1.5-2.2, ≥ 2.3	OR=0.50	(0.25, 1.01)

*Studies by Fowke et al. (2003) and Ho et al (1998) which are listed in Table 5 also included premenopausal women; however, the published data were not presented stratified by menopausal status.
**Estimates presented in Figure 4 and Table 6 reflect the highest category vs. lowest category of 2:16α-OHE_1.

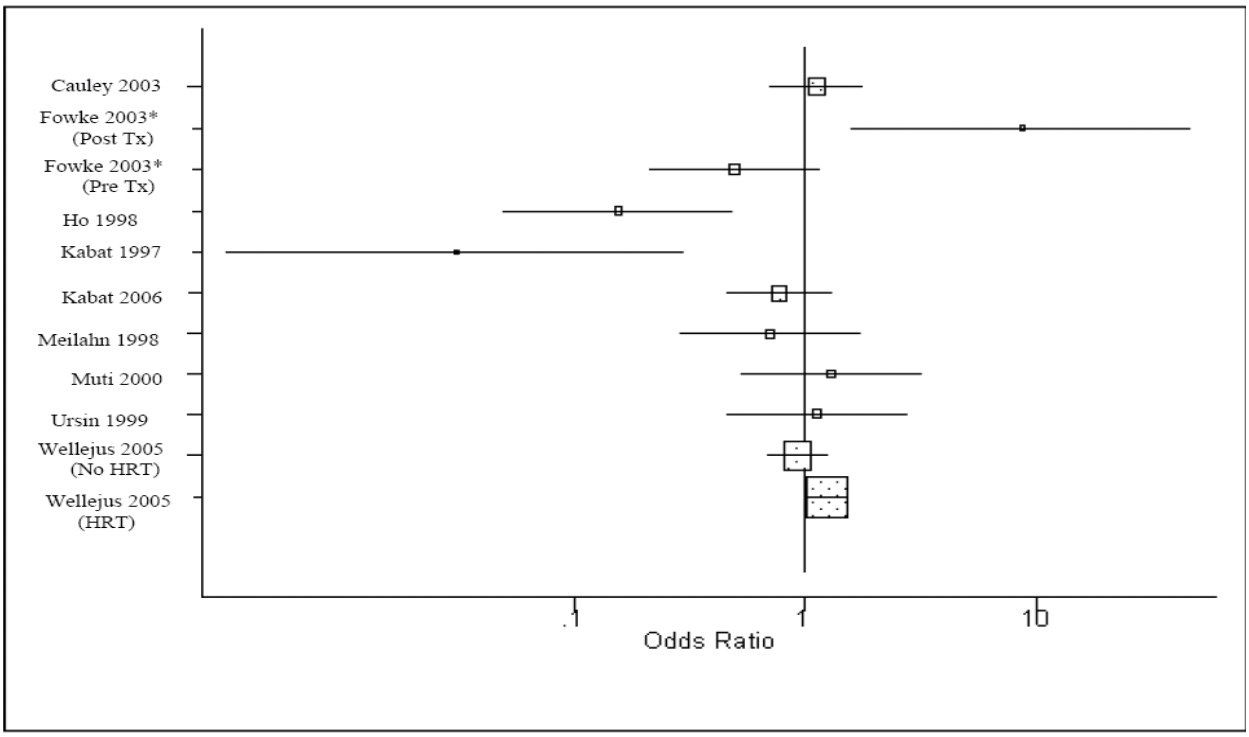

Figure 5 Postmenopausal Studies: Association between 2:16α-OHE$_1$ and Breast Cancer

*Published results in Fowke et al. (2003) were presented by timing of urine collection (pre and post treatment). Estimates presented for Fowke et al. and Ho et al. are for all women (including pre-menopausal) as the estimates in the publications were not stratified by menopausal status. All estimates presented reflect those in the original publications.

Table 7 Postmenopausal Study Specific Published Estimates Corresponding to Figure 4

Study*	2:16α-OHE cutpoints	Estimate	Confidence Limits
Cauley 2003	≤ 0.576, 0.577-0.749, 0.750-0.923, >0.923	1.17	(0.73, 1.87)
Fowke 2003 (Post Tx)	≤ 0.69, 0.70-1.22, ≥ 1.22	8.70	(1.6, 47.1)
Fowke2003 (Pre Tx)	≤ 0.69, 0.70-1.22, ≥ 1.22	0.50	(0.2, 1.1)
Ho 1998	≤ 0.9, > 0.9	0.16	(0.49, 0.49)
Kabat 1997	<1.38, 1.8-1.90, > 1.91	0.31	(0.003, 0.3)
Kabat 2006	≤1.5, 1.6-2.2, ≥ 2.3	0.78	(0.46, 1.33)
Meilahn 1998	<1.39, 1.39-2.08, >2.09	0.71	(0.29, 1.75)
Muti 2000	≤1.77, 1.77-2.26, 2.26-2.80, 2.80-3.66, ≥ 3.66	1.31	(0.5, 3.18)
Ursin 1999	≤ 1.16, 1.17-1.73, > 1.73	1.13	(0.46, 2.78)
Wellejus 2005 (No HRT)	per doubling of the ratio	0.94	(0.69, 1.26)
Wellejus 2005 (HRT)	per doubling of the ratio	1.25	(1.02, 1.53)

*Modugno et al. (2005) results are not included in this table as estimates for the ratio were not presented in the publication (only median values and estimates for the individual metabolites). Abbreviations: Tx=treatment, HRT=Hormone Replacement Therapy

2.4.1 Possible mechanisms underlying the association between estrogen metabolites and breast cancer

Estrogen metabolites are thought to influence the growth of estrogenic target cells (Lippert 2000). More specifically, the 16α-OHE_1 metabolite, due to its high affinity to bind to the estrogen receptor, has been associated with cell proliferation and metastasis, whereas the 2-OHE_1 may not have a role in these processes (Seeger 2006). Estrogens have mainly been considered breast cancer promoters due to their ability to stimulate cell proliferation. Estrogens are thought to increase the proliferation of breast epithelial cells lending cells susceptible to genetic errors which if not repaired, can lead to carcinogenesis (Fiegelson 1996). However, an additional carcinogenic model has been proposed suggesting that estrogen may act as a cancer initiator (Cavalieri 2006, Yager 2000). The estrogen depurinating adducts which are formed from the 2-OHE_1 and 4-OHE estrogen metabolites may directly lead to the initiation of cancer by inducing genetic mutations (Cavalieri 2006). The estrogen metabolites may also indirectly lead to genotoxic effects through oxidative damage, which occurs as a result of redox cycling (Yager 2000).

2.5 ESTROGEN DEPURINATING ADDUCTS AND BREAST CANCER

Limited studies have been conducted in the area of estrogen conjugates and depurinating adducts in relation to cancer, and specifically breast cancer (Rogan 2003, Markushin 2003). Rogan et al. evaluated breast biopsy tissues from 28 breast cancer cases and 49 women without breast cancer.

Tissue samples were tested for estrogen metabolite levels as well as catechol estrogen quinone conjugates. Higher levels of 4-OHE and catechol estrogen conjugates were detected in breast cancer tissue compared to healthy tissue, with levels of the 4-catechol estrogen approximately three times higher in breast cancer tissue compared to control tissue (Rogan 2003). The results from this study suggest that breast cancer cases have higher levels of catechol estrogen conjugates than non-cases. This may result in an increased possibility for the conjugates to react with DNA leading to the formation of DNA adducts.

A study by Markushin et al. (2003) aimed to assess whether the catechol estrogen quinone DNA adducts can be detected in human breast tissue extract using tissue samples from two women (one woman with breast cancer and one without). Although the focus of the study was on detection methods and was conducted on a sample size of n=2, the results of this study not only showed that the 4-OHE_1-1-N3Ade and 4-OHE_2-1-N3Ade adducts can be detected in breast tissue extract, but also that the adduct levels may differ by cancer status. The levels of the 4-OHE_1-1-N3Ade adduct in breast tissue extract from a breast cancer patient were 8.40 ± 0.05 pmol/g of tissue compared to 0.25 ± 0.05 pmol/g in the healthy tissue. Levels of the 4-OHE_2-1-N3Ade adduct were similar for both tissue samples. Although these results would need to be replicated in larger studies, they demonstrate the presence of the catechol estrogen quinone DNA adducts in breast tissue. In a recent study by Gaikwad et al., estrogen metabolite levels, estrogen conjugates and depurinating adducts were measured using urine samples from 46 healthy control women, 12 high-risk women and 17 women with breast cancer (Gaikwad 2007). Higher levels of the ratio of depurinating adducts to their relevant estrogen metabolites and conjugates were reported among high risk ($p < .0001$) and breast cancer patients ($p < .0001$) as compared to healthy controls (Gaikwad 2007).

In addition to studying the relationship between estrogen adducts and breast cancer, recent research has evaluated estrogen DNA adducts in relation to prostate cancer, also a hormonally driven cancer. Markushin et al. reported the detection of the depurinating adduct 4-OHE_1(E2)-1-N3Ade in urine samples of prostate cancer patients. Interestingly, this particular adduct was not detected in the urine of healthy men (Markushin 2006). The results from these studies warrant future investigations of estrogen DNA adducts in relation to breast cancer and in relation to breast density, an intermediate marker of breast cancer risk.

2.6 ESTROGEN METABOLITES AND BREAST DENSITY

Considering the potential association between estrogen metabolites and breast cancer, as well as the strong link between estrogen levels and breast cancer risk, it may be beneficial to evaluate the role of estrogen metabolites in relation to breast density, a strong predictor of breast cancer risk. To date, only one study has examined the relationship between breast density and estrogen metabolites. A cross-sectional study by Riza et al. reported a significant association between a higher 2:16α-OHE_1 ratio and a P2/DY parenchymal pattern among postmenopausal women (Riza 2001). The Wolfe's mammographic parenchymal patterns classification system was used to measure breast density in this study in which the P2/DY parenchymal pattern pertains to high breast density. Thus, the results of this study suggest that higher, rather than lower levels of the 2:16α-OHE1 ratio may be associated with parenchymal patterns known to increase breast cancer risk. The results of Riza et al. suggest a different direction of association than what is suggested in relation to breast cancer. Additional research is needed in this area in order to gain a better understanding of the relationship between estrogen metabolites and breast density, a strong predictor of breast cancer risk. Thus, it remains unclear whether estrogen metabolite levels may in part explain some of the observed variation in breast density.

2.7 EPIDEMIOLOGIC METHODS: COMBINED ANALYSIS

In epidemiology, existing literature is often reviewed and summarized, but these synopses can sometimes be subjective to the reviewer's opinions. Two methods currently used to systematically review literature and to provide a more objective summary are a meta-analysis of published results or a pooled analysis of individual level data from published studies. Meta-analyses combine summary measures whereas pooled analyses combine individual level data. Friedenreich discusses the methods and issues that should be considered when combining data from epidemiologic studies (Friedenreich 1993). Advantages to conducting a combined analysis of individual level data include the ability to better assess confounding and interaction in a large sample size of combined studies, as well as the ability to study rare exposures. As discussed by Friedenriech, a combined analysis of individual data is more advantageous than a meta-analysis due to the ability to re-analyze the raw data using uniform coding, definitions, and cutpoints as well as the adjustment for similar confounders.

Fredenreich discusses eight steps to consider when conducting a combined analysis of individual data. Those include (1) identifying and locating all relevant studies, (2) selecting eligible studies, (3) requesting and obtaining the primary data from the original investigators, and preparing the data, (4) estimating the study-specific effects, (5) assessing the potential heterogeneity of the study-specific effects, (6) estimating the combined effect, (7) assessing the potential heterogeneity between studies, and (8) performing a sensitivity analysis (Friedenreich 1993).

Although methodological approaches to a combined analysis of individual level data have been summarized (Friedenrich 1993, Bletnner 1999, Taioli 2002), few studies have addressed the statistical issue that arises when trying to combine individual data from studies of

different designs. Moreno et al. has discussed methods for the combined analysis of matched and unmatched case-control studies (Moreno 1996). Moreno et al. suggest the use of an interaction term between the individual risk factors and an indicator variable for study group. The interaction term coefficient would estimate the difference for a risk factor between the matched and unmatched studies. Despite the analytic challenges of conducting a combined analysis, an analysis of individual data has advantages, as noted above.

2.8 SUMMARY OF BACKGROUND AND SIGNIFICANCE

Estrogen exposure is an established hormonal risk factor for breast cancer but the mechanism by which increased estrogen exposure increases breast cancer risk is not clear. Potential mechanisms to explain this estrogen link have been proposed. One potential hypothesis includes the notion of estrogen's proliferative effect on breast cancer cells in which an increase in mitotic activity may increase the chances of a mutation occurring. A more recent hypothesis suggests that estrogen (and in particular estrogen metabolites) may act as cancer initiators, leading to DNA damage through the formation of DNA adducts. Despite these proposed hypotheses, the mechanism by which estrogen affects breast cancer risk, and the role of estrogen metabolites, remains to be elucidated.

Many of the known breast cancer risk factors impact a woman's cumulative lifetime exposure to estrogen. Understanding the potential role of estrogen metabolites in the carcinogenic process may contribute insight into the underlying mechanism of estrogen exposure. In addition, estrogen metabolites may serve as potential biomarkers of breast cancer,

and may help identify high-risk women. The intention of the combined analysis was to clarify the association between estrogen metabolites (2-OHE_1 and 16α-OHE_1) and breast cancer.

3.0 URINARY 2:16 ALPHA-HYDROXYESTRONE AND BREAST CANCER: OVERALL METHODS FOR THE COMBINED ANALYSIS

3.1 METHODS

3.1.1 Study design

We utilized a combined analysis approach, using individual level data from previously published studies in order to examine the relationship between urinary estrogen metabolites, specifically 2-OHE_1, 16α-OHE_1, and the 2:16α-OHE_1 ratio and breast cancer. Although individual studies have evaluated the potential association between these urinary estrogen metabolites and breast cancer, a combined analysis is advantageous in that it provides an increase in sample size and corresponding power which is necessary for the investigation of specific estrogen metabolites and breast cancer. This study design also allows for the assessment of potential effect modification by factors such as body mass index and tumor characteristics such as estrogen and progesterone receptor status.

3.1.2 Study Identification and Selection

Original research studies that evaluated the relationship between estrogen metabolites and breast cancer were identified by searching the National Library of Medicine and National Institutes of Health Pubmed database. The search strategy (Figure 6) involved the following keyword search terms: (1) estrogen metabolites (n=2901 articles) and (2) estrogen metabolites with additional limits to females and English language (n=974 articles), (3) estrogen metabolites AND breast cancer (n=376 articles). Each of the 376 citations and abstracts were reviewed and 12 articles were identified as having examined estrogen metabolites in relation to breast cancer. The remaining articles were considered ineligible for the following reasons: (1) review articles, (2) other metabolites such as tamoxifen metabolites, (3) in vitro studies, (4) other cancers, and (5) unrelated topics. Reference lists from retrieved articles were also reviewed in order to identify additional eligible articles; no additional studies were identified. This search is current as of July 15, 2008.

Articles were considered eligible for review if they met the following pre-determined inclusion criteria: (1) an original research study, (2) exposure measured as 16α -OHE_1, 2-OHE_1, and/or the ratio of these two main estrogen metabolites, (3) urine as the sample source, (4) breast cancer assessed as the main outcome and (5) sample size of at least 50 subjects. These criteria were determined *a priori* and were selected to maximize the comparability across studies.

3.1.3 Selection results

Of the eight eligible published studies, three are nested case-control studies (Meilahn 1998, Muti 2000, Wellejus 2005, and five are case-control studies (Kabat 1997, Ho 1998, Ursin 1999,

Fowke 2003, Kabat 2006). Two studies by Ursin et al. were identified, one of which is a pilot study (Ursin et al. 1997) with data on a subset of the study population published in Ursin et al. 1999. Therefore, the more inclusive study by Ursin et al., published in 1999 will be included in this analysis.

3.1.4 Data collection and extraction

Study investigators were asked to submit their study specific datafile along with a copy of the original questionnaire, a description of the variables and study methods, and a datafile which included the main exposures of interest, the 2-hydroxyestrone and 16α-hydroxyestrone metabolites, along with important breast cancer covariates. In addition, for each participating study, information on the type of study design, the study population, matching variables (when appropriate), case and control status, as well as important covariates were collected. Covariates requested include demographics (age, race, education, socioeconomic status), lifestyle factors (alcohol consumption and smoking), height, weight, menopausal status, reproductive factors (age at menarche, age at first birth, parity, number of live births), timing of urine collection, treatment, tumor characteristics, family history of breast cancer, benign breast diasease, oral contraceptive and hormone therapy use. Upon receipt of each datafile, the data was checked for consistency and uniformity with previously published results. Any identified discrepancies were resolved with the study investigators. Appendix A includes a checklist, which indicates the completeness of each datafile received. The coding for each covariate provided from the study

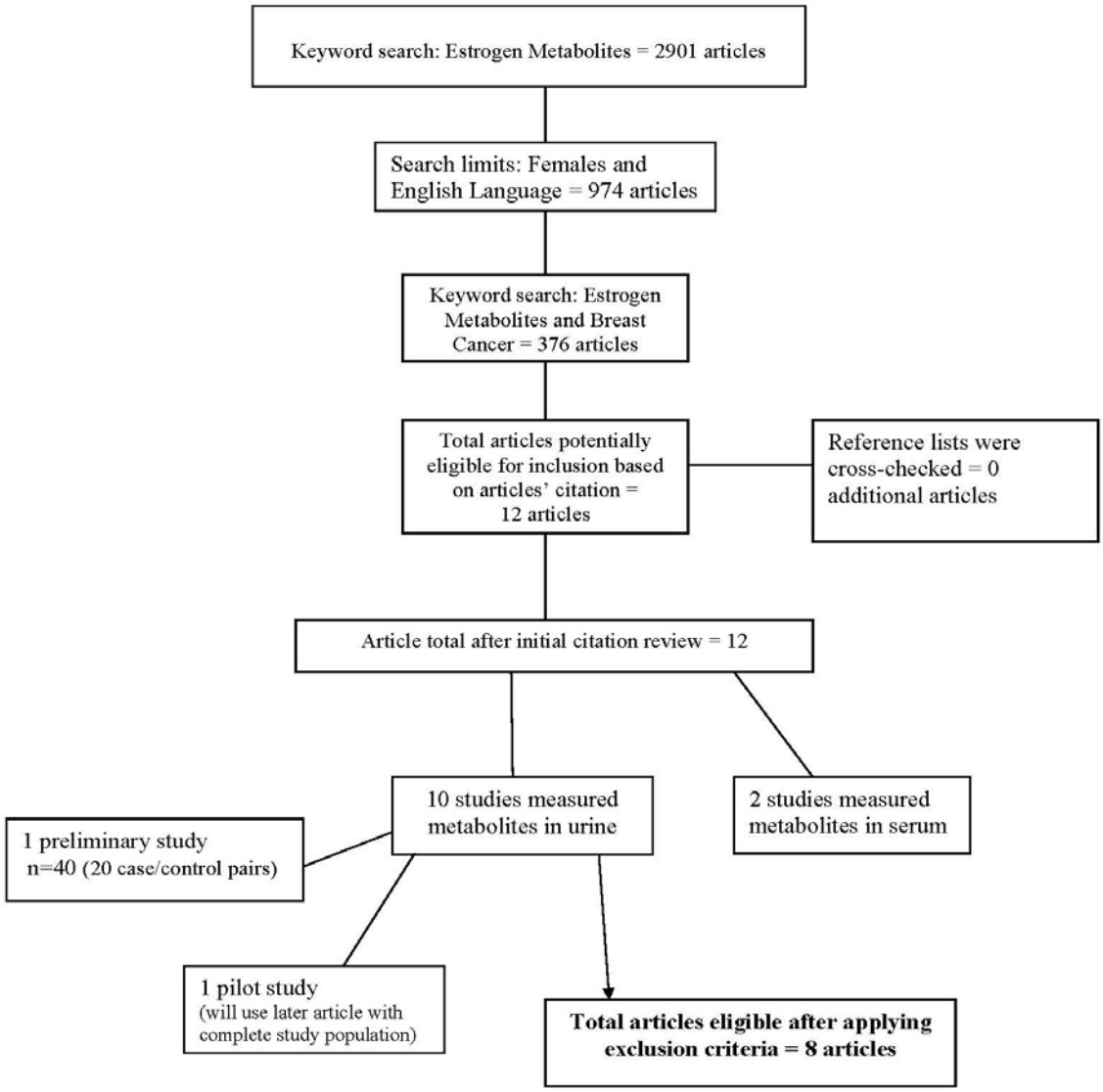

Figure 6 Pubmed Search for Articles Investigating the Relationship between Estrogen Metabolites and Breast Cancer

investigators was reviewed and a common definition was created for potential covariates. After review of each datafile, less than 5% of observations were missing values for the 2:16α-OHE_1 ratio. Details on the matching, case and control selection and exclusion criteria for each participating study are also presented in Appendix A.

Complete staging information was available for cases in the study by Muti et al. Stage categories were provided by Fowke et al.; however, details on the category definitions were not provided. Stage was defined as unknown for cases from Guernsey et al. since only information on grade and node was available. Due to the incomplete information on tumor staging, we were unable to assess metabolite levels by stage of disease.

3.1.5 Statistical Analysis

Listed below are the preliminary descriptive analyses performed for each of the specific aims. The additional details on the specific analyses utilized for each aim are included within each corresponding chapter.

Aim I and II: To explore the distribution of each metabolite and the assumption of linearity on the logit scale, Lowess smoother plots on the logit scale (and p scale) and normal histograms were generated for the combined population and for each study (Appendix B and C).

Aim III: The distribution of each metabolite among premenopausal and postmenopausal controls, by study, were assessed using normal histograms and by kernel density smoother plots. Study specific correlations between continuous variables (age, BMI, waist-to-hip ratio, age at menarche, and age at first pregnancy) and estrogen metabolites (2-OHE_1, 16α-OHE_1, and 2:16α-OHE_1) were estimated using the nonparametric Spearman rank correlation coefficient. Study specific differences in median levels of estrogen metabolites by categorical variables (ethnicity,

family history of breast cancer, history of benign breast disease, smoking, alcohol consumption, BMI, oral contraceptive use and parity) were assessed using the nonparametric Wilcoxon rank sum test. These graphs and analyses are presented in Appendix D.

4.0 URINARY ESTROGEN METABOLITES AND BREAST CANCER AMONG PREMENOPAUSAL WOMEN:

A COMBINED ANALYSIS OF INDIVIDUAL LEVEL DATA

To be submitted for publication

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4.1 ABSTRACT

Objective: Estrogen metabolism may play a role in breast carcinogenesis; however, only a few small studies have evaluated this association among premenopausal women. The objective of this analysis was to evaluate the role of the two urinary estrogen metabolites, 2-hydroxyestrone (2-OHE_1) and 16α-hydroxyestrone (16α-OHE_1), and their ratio (2:16α-OHE_1) in relation to breast cancer among premenopausal women by conducting a combined analysis of previously published studies.

Methods: Primary data from three previously published studies yielded a study sample of 731 premenopausal women, including 183 invasive breast cancer cases and 548 controls. Urinary estrogen metabolite levels were measured using an ELISA assay. Both study-specific and combined unadjusted and multivariable adjusted odds ratios (ORs) and 95% confidence intervals (CIs) were estimated using conditional logistic regression matching on 5-year age groups. Sensitivity analyses were performed combining only the Caucasian studies. All statistical tests were two-sided.

Results: Unadjusted median levels of the 2:16α-OHE_1 ratio were significantly lower among cases (1.8) than among controls (2.2) ($p < 0.001$). Unadjusted median 2-OHE_1 levels were also elevated among controls ($p<0.001$) whereas no difference in 16α-OHE_1 levels by case/control status was observed ($p=0.26$). The highest tertile of the 2:16α-OHE_1 ratio compared to the lowest was associated with reduced breast cancer risk (OR=0.51, 95% CI: 0.33, 0.78); although, this reduction was attenuated and no longer statistically significant after adjustment for study (OR= 0.81, 95% CI: 0.49, 1.32). The sensitivity analyses including only the Caucasian studies also suggested modest protective association; although the results were not statistically significant (study adjusted $OR_{\text{Tertile 3 vs. Tertile 1}}$ =. 0.72, 95% CI: 0.43, 1.20).

Conclusions: This combined analysis using primary data from three previous studies of premenopausal women does not support a significant association between the 2:16α-OHE_1 ratio and invasive breast cancer; however, the results are suggestive of a reduced risk of breast cancer with higher levels of 2:16α-OHE_1.

4.2 INTRODUCTION

Although breast cancer occurs more often in women after the menopausal transition, the occurrence in premenopausal women has a strong public health impact because of issues such as impaired reproductive function and long-term management and survival. However, few relevant risk factors have been identified for premenopausal breast cancer. One of the areas of potential significance among premenopausal women includes understanding the role of estrogen and estrogen metabolites on breast cancer development. Endogenous estrogen exposure throughout a woman's lifetime is one of the recognized factors implicated with breast cancer (Henderson 2000, Bernstein 2002, Key 2002). The association with circulating estrogens and post-menopausal breast cancer is well established (Key 2002, Eliassen 2008); however, among premenopausal women, measurement issues surrounding the timing of urine collection during the menstrual cycle render this association less clear (Bernstein 2002, Eliassen 2006, Sturgeon 2004).

Estrogens are involved in the proliferation of human breast epithelial cells and may influence carcinogenesis indirectly by stimulating cell division (Preston-Martin 1990, Fiegelson 1996) or by inducing genotoxic effects as a result of oxidative damage (Yager 2000). An alternative mechanism has also been proposed in which estrogen, through the formation of

depurinating estrogen adducts, acts as a cancer initiator (Cavalieri 2006). Estrogen metabolism occurs by the oxidative pathway, and among premenopausal women with high ovarian estradiol secretion, this metabolic process begins with the conversion of estradiol to estrone. The breakdown of estrone continues via two main pathways involving hydroxylation sites C2, C4 or C-16, leading to the formation of either A-ring or D-ring metabolites (Mueck 2002, Lippert 2000). Main metabolites of the A-ring pathway include 2-hydroxyestrone (2-OHE_1) and 4-hydroxyestrone (4-OHE), while 16-alpha-hydroxyestrone (16α-OHE_1) and estriol result from the D-ring pathway (Mueck 2002, Yager 2006).

Although both 2-OHE_1 and 16α-OHE_1 have estrogenic properties, their ability to bind to the estrogen receptor as well as the nature of their estrogenic properties varies. The 16α-OHE_1 metabolite has been shown to have higher estrogen properties based on its ability to covalently bind to the estrogen receptor (Swaneck 1988) while also sharing properties similar to those of estradiol (Lippert 2003, Seeger 2006), whereas the 2-OHE_1 metabolite exhibits lower estrogen activity, in part due to the reduced affinity to estrogen receptor binding as well as less cell proliferative activity (Schneider 1984). Furthermore, these metabolites result from mutually exclusive pathways (Zhu 1998), rendering the ratio of 2:16α-OHE_1 a useful measure of exposure to active estrogen metabolites.

Few studies have evaluated the relationship between premenopausal urinary estrogen metabolite levels and breast cancer risk (Fowke 2003, Kabat 1997, Kabat 2006, Ho 1998, Meilahn 1998, Muti 2000). Some studies suggest a reduction in premenopausal breast cancer risk with higher levels of the urinary 2:16α-OHE_1 ratio (Meilahn 1998, Muti 2000, Kabat 2006) while others include premenopausal cases but do not present the results separately according to menopausal status (Ho 1998, Kabat 1997). In general, the number of premenopausal breast

cancer cases included in each study is small, and the individual studies may lack the statistical power to detect a significant association.

The objective of this analysis was to evaluate both the individual levels of the 2-OHE_1 and 16α-OHE_1 metabolites, and their ratio, in relation to breast cancer among premenopausal women by performing a combined analysis of primary data from three previously published studies. We hypothesized that higher levels of the 2-OHE_1 metabolite would be associated with a reduction in breast cancer risk while higher levels of the 16α-OHE_1 metabolite would be associated with an increase in the risk of breast cancer among premenopausal women, and that a higher 2:16α-OHE_1 ratio would be associated with a reduction in premenopausal breast cancer risk.

4.3 METHODS

4.3.1 Study design

We conducted a combined analysis of individual level data from three previously published studies, including one case-control study conducted in Shanghai, China (Fowke et al.) and two nested case-control studies conducted in Northern Italy (Muti et al.) and Guernsey Island (Meilahn et al.). Primary data from the participating studies were obtained and potential heterogeneity in exposure distributions assessed prior to generating a summary estimate of the combined data. Table 8 provides a brief description of the participating studies with additional details provided in the original publications (Muti 2000, Meilahn 1998, Fowke 2003).

4.3.2 Study Identification and Selection

Study Identification and Selection Criteria

Original research studies that evaluated the relationship between estrogen metabolites and premenopausal breast cancer were identified by searching the National Library of Medicine and National Institutes of Health Pubmed database. The search strategy involved the following keyword search terms: (1) estrogen metabolites (n=2901 articles) and (2) estrogen metabolites with additional limits to females and English language (n=974 articles), (3) estrogen metabolites AND breast cancer (n=376 articles). Each of the 376 citations and abstracts were reviewed, and 12 articles were identified as having examined estrogen metabolites in relation to breast cancer, with six studies including premenopausal women. Reference lists from retrieved articles were also reviewed in search of additional eligible articles; no additional studies were identified. This search is current as of July 15, 2008.

Articles were considered eligible for review if they met the following pre-determined inclusion criteria: (1) an original research study, (2) inclusion of premenopausal women, (3) exposure measured as 16-alpa-hydroxyestrone (16α-OHE_1), 2-hydroxyestrone (2-OHE_1), and/or the ratio of these two main estrogen metabolites, (4) urine as the sample source, (5) breast cancer assessed as the main outcome and (6) total sample size of at least 50 subjects. These *a priori* criteria were selected to maximize the comparability across studies.

4.3.3 Selection results

Five of the six studies (Fowke 2003, Kabat 1997, Ho 1998, Kabat 2006, Meilahn 1998, Muti 2000) that included premenopausal cases in their original study fulfilled the above eligibility

criteria for inclusion in this combined analysis. Of these, two were nested case-control studies (Meilahn 1998, Muti 2000), and three were case-control studies (Fowke 1998, Kabat 1997, Kabat 2006), with a total potential sample size of 1,020 premenopausal women (307 cases, 713 controls). Investigators from the eligible studies were contacted and invited to participate in this combined analysis. Multiple invitations were extended in an attempt to enlist the participation of all eligible studies. Three studies contributed data to the present analysis, two nested case-control studies (Muti et al., Meilahn et al.) and one case-control study (Fowke et al.), with a combined analysis sample size of 731 premenopausal women (183 cases, 548 controls).

4.3.4 Data Collection and Extraction

Data Collection and Extraction

The three participating studies utilized structured questionnaires to ascertain information on demographics, lifestyle and anthropometric factors, hormone use, reproductive history, and other known breast cancer factors. Investigators were asked to submit their study-specific datafile along with a copy of the original questionnaire, a description of the variables and study methods, and an electronic datafile. Each datafile was checked for consistency and uniformity with previously published results. Identified discrepancies were resolved with the study investigators. Policies for data publication and authorship were reviewed and accepted by study investigators.

Completeness of Data

All variables requested were coded with a common format for this analysis with the exception of education and socioeconomic status, due to differences in classification systems. Information on waist-to-hip ratio and alcohol consumption was available in two studies (Fowke, Muti). Data on estrogen receptor (ER) and progesterone receptor (PR) status was available for

62.8% and 60.3% of cases from all three studies, respectively. In the Guernsey study, PR data was not available and ER status was unknown for the majority of cases. Due to incomplete information on tumor staging, we were unable to assess metabolite levels by stage of disease.

4.3.5 Sample Collection and Laboratory Analysis

Spot urine samples were collected in the studies by Meilahn et al. and Fowke et al; and an overnight spot urine sample was collected in the study by Muti et al. The use of a spot urine collection has been shown to be a reliable sample source for the measurement of urinary estrogen metabolites (Chen 1999) with no reported significant differences between spot urine samples or multiple urine collections over a 24 hour period (Westerlind 1999). Additionally, the potential affect of long term sample storage on metabolite levels was assessed in the original study by Meilahn et al.; no variations in metabolite levels were reported. All participating studies measured urinary 2-OHE_1 and 16α-OHE_1 using a commercially available competitive, solid-phase enzyme immunoassay kit (ESTRAMET) produced by Immunacare corp (Bethlehem, PA, USA). With this assay kit, the binding of the monoclonal antibodies to estrogen metabolites (antigen) is captured directly on the solid phase. The urinary forms of 2-OHE_1 and 16α-OHE_1 are found as glucuronide conjugates and require the removal of sugars before the monoclonal antibodies in the assay kit can detect the urinary metabolites. Studies by Fowke et al. and Muti et al. utilized the more recent ELISA kit (Bradlow 1998) whereas Meilahn et al. used the original assay developed by Klug (Klug 1994). The sensitivity of the modified assay is reported to be 0.625ng/ml for the 16α-OHE and 2-OHE metabolites (Bradlow 1998, Falk 2000). The reported mean within assay variability of this kit is approximately 4% while the mean between assay variability is about 10%. The main components of the recent kit are similar to the original assay,

with the exception of modifications that allow for an increased sensitivity level among postmenopausal women (Bradlow 1998, Falk 2000).

4.3.6 Statistical analysis

The 2-OHE_1 and 16α-OHE_1 metabolites (ng/ml) were divided by the urinary creatinine concentration (mg/dl) as a means of standardizing the metabolite values by total volume of urine. The 2:16α-OHE_1 variable in this analysis is based on the ratio of these two individual estrogen metabolites. 2-OHE_1 and 16α-OHE_1 metabolite data was available on 99.3% of this study sample. The estrogen metabolite exposures (2-OHE_1, 16α-OHE_1 and the 2:16α-OHE_1 ratio) were all non-normally distributed and were analyzed as categorical variables using common categories (tertiles) based on the distribution among the total combined control population.

The main outcome of this analysis is invasive breast cancer. *In situ* breast cancer cases were excluded from the present analysis due to the limited number of cases (n=7). Current users of oral contraceptives from Fowke et al. were excluded from this analysis (2 cases and 1 control) to be consistent with the original exclusion criteria from the studies by Muti et al. and Meilahn et al.. In the case-control study by Fowke et al., urine samples were collected either before or after surgery/ancillary treatment and different effects of the 2:16α-OHE_1 on breast cancer by the timing of urine collection were reported in the original publication. Thus, cases with post-treatment urine collection from Fowke et al. were excluded (n=14). After applying these additional exclusions, the present combined analysis includes 731 premenopausal women (183 cases/548 controls).

The distribution of covariates was compared among cases and controls using either the parametric t-test or non-parametric Wilcoxon rank sum for continuous variables, or the chi-

square test for categorical variables. Additionally, study adjusted p-values for the association between individual covariates and case/control status were generated using conditional logistic regression models that included dummy variables for the individual studies.

The original study-specific findings were replicated prior to conducting this combined analysis to ensure accurate information in the datafiles. Preliminary analyses comparing the use of the original matched sets versus common matching criteria based on 5-year age strata yielded similar estimates. Therefore, study specific and combined unadjusted and adjusted odds ratios (ORs) and 95% Confidence Intervals (CIs) were estimated by conditional logistic regression (Breslow 1980) matched on 5-year age groups (<35, 35-39, 40-44, 45-49, 50-54, >55). Four different models were performed for each metabolite: (1) unadjusted, (2) adjusted for known breast cancer risk factors, (3) adjusted for study only, and (4) adjusted for known breast cancer risk factors in addition to study. Age at the time of enrollment in the original studies was taken into account by stratifying the models by 5-year age groups.

Multivariable models were adjusted for known breast cancer risk factors including family history of breast cancer in a first degree relative (yes/no), history of benign breast disease (yes/no), body mass index (kg/m^2), smoking (never/former/current), oral contraceptive use (never/former/unknown), age at menarche and a combined pregnancy variable (never pregnant, age at first pregnancy <20, age at first pregnancy 20-29, and age at first pregnancy ≥30). These factors were chosen *a priori* as adjustment variables either due to their established associations with breast cancer or because they are important characteristics of the Gail Model (Gail 1989) used to assess breast cancer risk. Study adjusted models included separate dummy variables for each study, to account for potential differences in overall study design and study populations.

Separate trend tests for each estrogen metabolite were performed using the midpoint for each metabolite category.

Urine samples for all premenopausal subjects in the study by Muti et al. were collected during the luteal phase of the menstrual cycle (20-24th day). Cases and controls in the original study by Meilahn et al. were matched on phase of menstrual cycle (follicular: within 15 days of the start of the last menstrual cycle, or luteal: more than 15 days). Information on menstrual phase at urine collection was not available in the study by Fowke et al.. In the present analysis, menstrual cycle phase at urine collection was categorized as (follicular $\leq$ 14 days/luteal $\geq$ 15 days/ unknown), based on an assumed average cycle length of 28 days. Additional adjustment for menstrual phase at the time of urine collection did not alter estrogen metabolite estimates nor was menstrual phase at the time of urine collection a significant predictor in the study adjusted models; this variable was not included in the final models.

Heterogeneity between studies was evaluated graphically by visual examination of study specific estimates using a forest plot and by the Cochran's Q-statistic chi-square test (Petitti 2000). In addition, the study-specific median levels of each metabolite among the control populations were evaluated. The study by Fowke et al. conducted in Shanghai, China was significantly different than the studies by Meilahn et al. and Muti et al. The metabolite levels among the Shanghai study population were generally in the lowest tertile of the common cutpoints, with lower levels of all metabolites occurring in this Asian population. Due the observed statistical differences in study specific median levels and estimates, and the potential biological differences in estrogen metabolism in Asian versus Caucasian populations, a sensitivity analysis was performed in which analyses were repeated without the data from the Shanghai study. In addition, the Fowke et al. study utilized a case-control study design whereas

the studies by Muti et al. and Meilahn et al. were nested case-control studies in which the urine sample was collected before diagnosis and before treatment for breast cancer.

Previous research suggests that estrogen metabolism may vary according to levels of body mass index (BMI) (Modugno 2006, Fishman 1975, Schneider 1983) and by smoking status (Sowers 2006). Subgroup specific models were conducted to assess whether the association between estrogen metabolites and breast cancer varied by BMI (<25, ≥ 25 kg/m^2) and smoking status (current, non-current). In a combined model, a dummy variable for BMI and a categorical interaction term for both BMI and the metabolite were included. Separate combined models were performed for each metabolite exposure. Wald X^2 tests were used to test the overall effects of the interaction. Interactions between smoking status and each metabolite were assessed using a similar approach.

Each metabolite also was evaluated in relation to breast cancer according to hormone receptor status by performing separate age-adjusted multinomial logistic regression models. The outcome variable for the estrogen receptor models included four categories: controls (reference), estrogen receptor positive (ER+) cases, estrogen receptor negative (ER-) cases, and estrogen receptor status unknown (ER unk) cases. Similarly, for the progesterone receptor models, the outcome variable included four categories: controls (reference), progesterone receptor positive (PR+) cases, progesterone receptor negative (PR-) cases, and progesterone receptor status unknown (PR unk) cases. These multinomial logistic regression models included adjustment for age using three categories (≤ 39, 40-44, ≥ 45), due to the smaller number of cases available to evaluate breast cancer by hormone receptor status.

Potential interactions between the individual metabolite exposures and study were assessed by creating separate categorical interaction terms for each metabolite (using dummy

variables for both the study and metabolite categorical variables). Multiparameter Wald X^2 tests were used to test the overall effects of study, metabolite and the interaction. Interactions between age and the 2-OHE_1, 16α-OHE_1, and 2:16α-OHE_1 metabolites also were assessed using a similar approach.

All analyses were performed using SAS (Version 9.1, Cary, NC, USA) and STATA 10. Tests of significance were two-sided. Data were analyzed in Pittsburgh, PA and the datafiles received did not include personal identifiers. Each study participating in this present analysis was approved by the Institutional Review Board or appropriate ethical committee at the respective institution, and participants provided informed consent.

4.4 RESULTS

Unadjusted study-specific ORs and 95% CIs for the association between tertiles of each metabolite and breast cancer are shown in Table 9. Study- specific odds ratio estimates for the association between the 2:16α-OHE_1 ratio and breast cancer were in a protective direction for the two nested case-control studies by Meilahn et al. and Muti et al.; although not statistically significant [2:16α-$OHE_1 \geq 2.67$ vs. < 1.76 (Meilahn: OR=0.76; 95% CI: 0.34, 1.69) and (Muti: OR=0.65; 95% CI: 0.33, 1.29)]. However, for the study by Fowke et al., study-specific estimates for 2:16α-OHE_1 were non-significantly elevated (2:16α-$OHE_1 \geq 2.67$ vs. < 1.76: OR=1.34; 95% CI: 0.19, 9.75). The forest plot shown in Figure 7 depicts the study-specific estimates for the association between the 2:16α-OHE_1 ratio among premenopausal women and breast cancer. The odds ratio estimate for Fowke et al. is in the opposite direction of the Meilahn et al. and Muti et al. studies, however the Cochrane chi-square test of heterogeneity was not statistically significant

(X^2=0.48; p-value=0.79). Study-specific estimates for the association between the individual metabolites 2-OHE_1 and 16α-OHE_1 also are presented in Table 9. No significant study-specific associations were detected with 2-OHE_1, 16α-OHE_1 or the 2:16α-OHE_1 ratio.

Descriptive characteristics of this combined study population by case/control status are summarized in Table 10. The study sample was mostly Caucasian (68.9% of cases and 86.9% of controls), with an average age of 42.8 ± 4.9 years (range, 33-54) for cases and 42.9 ± 4.9 years (range, 34-56) for controls. Median levels of 2-OHE_1 and 2:16α-OHE_1 were significantly lower among cases as compared to controls [(2-OHE_1: 17.9 ng/ml vs. 21.7 ng/ml, unadjusted p-value= <0.001); 2:16α-OHE: 1.8 vs. 2.2, unadjusted p-value: <0.001)] whereas no difference was observed for the 16α-OHE metabolite (unadjusted p-value: 0.26) (Table 11).

Table 12 summarizes the ORs and corresponding 95% CIs from unadjusted and adjusted models estimating the odds of breast cancer in relation to the tertiles of 2-OHE_1, 16α-OHE_1 and the 2:16α-OHE_1 ratio (N=183 cases and 548 controls). Among women in the highest tertile of the 2:16α-OHE_1 ratio, the unadjusted OR for breast cancer was 0.51 (95% CI: 0.33, 0.78) as compared to those in the lowest tertile. This estimate remained relatively unchanged with adjustment for known breast cancer risk factors. However, these results attenuated and were no longer statistically significant in the models adjusted for study (OR=0.81, 95% CI: 0.49, 1.32) or adjusted for both study and known breast cancer risk factors (OR=0.74, 95% CI: 0.44, 1.23). Similar findings were observed with the 2-OHE_1 metabolite. Associations between 16α-OHE_1 and breast cancer were not statistically significant in any of the models, although study adjusted analyses suggested a non-significant increase in breast cancer risk. The tests for trend suggested a significant inverse trend with both 2-OHE_1 and the 2:16α-OHE_1 ratio, but this trend was no longer significant after adjustment for study. Although the main effect of study was statistically

significant in each of the metabolite main models, no tests for interaction between study and any metabolite were statistically significant (p-value > 0.10 for each). No significant interactions between age and the 2-OHE_1, 16α-OHE_1, or 2:16α-OHE_1 metabolites were observed, p-value > 0.10 (data not shown).

Table 13 summarizes the results from the sensitivity analyses limited to Caucasian women (N=126 cases and 476 controls). None of the individual metabolites 2-OHE_1 and 16α-OHE_1 was significantly associated with breast cancer in these models, although the direction of the estimated effects was consistent with the study hypotheses. No significant trends were observed with any of the metabolites. There was no significant heterogeneity between the two Caucasian studies (p-value > 0.18) nor was there evidence of interaction between study and any metabolites (p-value >0.38 for each) or interaction between age and each of the metabolites (p-value > 0.23 for each) (data not shown).

The age-adjusted multinomial models estimating the relative risk (RR) of ER status by tertile of estrogen metabolites, as compared to controls, are summarized in Table 14. Separate RR estimates compare each ER subtype (ER+, ER- and ER unknown) to the control group, with the lowest tertile of each metabolite as the exposure reference group. The only significant association of estrogen metabolite and ER subtype was that women in the highest tertile of 2:16α-OHE_1 ratio were less likely to be ER- cases compared to controls (RR=0.33; 95% CI: 0.13, 0.84); although these findings are based on small numbers. A similar RR=0.40 in the 1.76-2.66 group was borderline significant. Results for cases with unknown receptor status were similar to those from the ER- models. Comparable multinomial models estimating the association between 2-OHE_1, 16α-OHE_1, and 2:16α-OHE_1 and breast cancer according to PR status yielded no statistically significant associations (data not shown).

No significant associations were observed in subgroup specific analyses of BMI (< 25 kg/m^2 and ≥ 25 kg/m^2) and smoking status (current vs. non-current smoker), with the exception of a modest non-statistically significant reduction in breast cancer among non-current smokers with 2:16α-OHE_1 ≥ 2.76 vs. <1.76 (OR=0.57, 95% CI: 0.31-1.06) (data not shown). Overall tests of interaction were not statistically significant (Wald test p-value > 0.10 for each), with the exception of borderline significance for the interaction between 2-OHE_1 and BMI (Wald test p-value=0.08) and 2-OHE_1 and smoking status (Wald test p-value=0.07) (data not shown).

4.5 DISCUSSION

In this combined analysis we did not observe a significant association between the 2:16α-OHE_1 ratio and invasive breast cancer among premenopausal women. Unadjusted models and models adjusted for known breast cancer risk factors suggested that higher levels of the 2:16α-OHE_1 ratio were inversely associated with breast cancer; however, this reduction in risk was attenuated with adjustment for study. A similar pattern was observed with the 2-OHE_1 metabolite, in that the apparent protective effect with higher levels of 2-OHE_1 was no longer observed once the study variable was included as an adjustment factor in the model.

To date, six studies have included premenopausal cases in their evaluation of estrogen metabolites and breast cancer, with varied results reported across the studies (Fowke 2003, Ho 1998, Kabat 1997, Kabat 2006, Meilahn 1998, Muti 2000). The original studies by Muti et al. and Meilahn et al. suggested a reduction in risk of premenopausal breast cancer among women in the highest tertile of the 2:16α-OHE_1; however, the original findings were not statistically significant. More recently, Kabat et al. (2006) reported a strong protective effect on invasive

breast cancer among premenopausal women with a 2:16α-OHE_1 value ≥ 2.3 as compared to those in the category ≤ 1.4 (OR=0.50; 95% CI: 0.25-1.01). Other published studies have included premenopausal cases but did not provide estimates separately for premenopausal women (Fowke 2003, Ho 1998, Kabat 1997). Overall, this research area has been limited both by the number of studies conducted and by the small number of premenopausal cases included in those analyses. Although the prior studies suggest a protective effect with increasing levels of 2:16α-OHE, the association between estrogen metabolites and premenopausal breast cancer remains unclear.

The objective of this combined analysis was to improve our understanding of the relationship between estrogen metabolites and breast cancer with a larger, combined sample size of premenopausal cases. In the original study by Muti et al., the investigators reported a 42% reduction in premenopausal breast cancer risk among women in the highest quintile of 2:16α-OHE as compared to those in the lowest quintile (OR=0.58, 95% CI: 0.25-1.34). Similarly, Meilahn et al. reported a protective effect in the original study, albeit not statistically significant ($OR_{\text{Tertile 3 vs. Tertile 1}}$=0.74, 95% CI: 0.35-1.62). In this analysis, the direction of the effect suggested a modest decrease in breast cancer among women in the highest tertile of the 2:16α-OHE compared to those in the lowest; however, these results were not statistically significant.

There are potential explanations for the null findings presented in this analysis including the limitation of a fixed sample size based on the overall small number of studies which have evaluated this association and the number of studies which agreed to participate in this combined analysis. Despite these limitations, one cannot exclude the possibility that a null finding reflects the true association between premenopausal urinary estrogen metabolite levels and breast cancer.

Although hormonally related factors may impact ER+ tumors differently than ER- tumors (Huang 2000, Cotterchio 2003, Ma 2006), findings have been inconsistent. Additionally, breast

cancer risk factor profiles may differ by hormone receptor subtype and the overall characteristics of the hormone-specific breast tumors also may vary (Althuis 2004). These findings support the notion of different etiologic mechanisms underlying the breast cancer subtypes and emphasize the importance of evaluating breast cancer tumor subtypes separately rather than as one combined disease. We attempted to investigate the association between each metabolite (2-OHE_1, 16α-OHE_1, 2:16α-OHE_1) and invasive breast cancer by ER and PR status. To our knowledge, only one other study (Kabat 2006) has evaluated the association of estrogen metabolites and invasive breast cancer among premenopausal women by ER status. In the case-control study by Kabat et al. the odds of breast cancer was significantly reduced with increasing levels of the 2:16α-OHE_1 ratio among ER+ cases (p-trend=0.02). Among women with a 2:16α-OHE_1 value $\geq$ 2.3, the odds of ER+ breast cancer was 0.32 times that of women with a 2:16α-OHE_1 value of $\leq$ 1.4 (95% CI: 0.12-0.84). A protective effect was also observed among ER- cases; however, the OR of 0.52 did not reach statistical significance. These results were based on a fairly small number of cases and need to be replicated in larger studies, as the authors indicated in their original publication.

In the present analysis, no significant associations by hormone receptor status were detected. Although results from the multinomial models suggest a reduction in the relative risk of ER- breast cancer among those in the highest tertile of 2:16α-OHE_1 compared to the lowest tertile, this finding was based on a fairly small number of cases and controls and should be interpreted with caution. Additionally, the estimates provided in Table 14 are largely driven by the ER+ and ER- cases in the study by Muti et al.. The majority of premenopausal cases within the Guernsey study were classified as unknown type. This may in part be due to chance, to tumor classification policies at the time of the study resulting in a large unknown category, or the lower

presence of measurable estrogen receptors among premenopausal women. Overall, the interpretation of our results is limited by both the small number of cases when separated by both ER status and estrogen metabolite categories, as well as the large number of unknown tumor types. Additionally, it is difficult to directly compare our results to those of Kabat 2006 since different analytical approaches were employed. In addition to ER status, we also explored whether the affects of each metabolite on breast cancer varied by PR status, which has not been evaluated in prior studies. The interpretation of the findings from the PR multinomial logistic regression models is also limited by the small number of cases classified by PR status in our combined study. Although it is difficult to draw conclusions from the hormone receptor analyses presented in this combined study, our results suggest potential differing effects by ER status and warrant further investigation in larger studies with more complete information on hormone receptor status.

Differences in estrogen metabolite levels by study were also assessed. No significant interactions between any of the estrogen metabolites and study were observed. However, when all three studies were combined, the main effect of study was significant, and in particular, the indicator variable for the Fowke et al. study was significantly different when compared to the indicator variable for the Muti et al. study. Furthermore, the protective effects observed in the unadjusted models attenuated with adjustment for study. Although there was no evidence of interaction by study, study appears to be an important confounding factor. This suggests that there are important differences across the studies which are important to consider such as differences in study design, laboratory variability, and the distribution of the estrogen metabolites among various ethnic populations, in particular Caucasian and Asian women.

Metabolite levels have been shown to vary by race (Aldercreutz 1994, Jernstrom 2003, Ursin 2001, Taioli 1996, Matthews 2004). The range of the metabolite values in the Shanghai study by Fowke et al. overlapped with those in the ORDET and Guernsey studies yet the overall median levels were significantly lower among the Shanghai population (Median 2:16α-OHE_1 among controls: Fowke et al. 0.85; Meilahn et al. 2.1; Muti et al. 2.5) (p-value <0.0001). This is not surprising, as higher levels of endogenous estrogens levels have been reported among Caucasian women as compared to Asian women (Bernstein 1990, Key 1990, Sowers 2006), and estrogen metabolite levels have also been shown to differ by race. An earlier study by Aldercreutz et al. among premenopausal women reported lower 2-OHE_1 and 16α-OHE_1 levels among Asian women compared to Caucasian women (Aldercreutz 1994). Mean values from the Aldercreutz study cannot be directly compared to those in our combined analysis due to the difference in laboratory methods used to measure the metabolites. Overall, limited research has been conducted on racial differences in estrogen metabolites among premenopausal women.

Studies of hormone levels among premenopausal women are often difficult to carry out due to the changes in circulating estrogen levels throughout the menstrual cycle and the variability in the length of the cycle (Bernstein 2002). Whether urinary estrogen metabolite levels vary by the timing of urine collection during different phases of the menstrual cycle remains unknown. Some studies suggest a potential difference (Xu 1999) while others do not (Westerlind 1999). In this analysis, adjustment for menstrual phase at the time of urine collection did not alter model estimates in this combined analysis. Given that urine samples in the ORDET study were collected during the luteal phase and that in the Guernsey study, menstrual phase was an original matching criterion, these original study design features may explain our observed lack of variability in estrogen metabolites by menstrual phase.

This combined analysis included a total of 183 premenopausal invasive breast cancer cases, a larger number than previous studies published on this topic. Prior to this combined analysis, the largest study of estrogen metabolites and premenopausal breast cancer by Kabat et al. (2006) included a total of 105 invasive cases. The analysis comprising of the two Caucasian studies (N=126) includes a slightly larger sample size of premenopausal cases in relation to the Kabat 2006 study. Additional strengths of this analysis include the use of original data to evaluate 2-OHE_1, 16α-OHE_1, and the 2:16α-OHE_1 ratio in relation to premenopausal breast cancer using common cutpoints, as well as the analysis by ER and PR status and other relevant BMI and smoking subgroups, albeit the number of cases and controls were fairly small. Although we did not detect significant associations with these analyses, this study identifies a need to investigate these potential associations in larger studies of premenopausal women. The studies by Muti et al. and Meilahn et al. were nested case-control studies in which the urine sample was collected years before the cancer diagnosis, allowing temporal inference to be drawn from the results from those combined analyses.

There are some limitations to this study that should be considered when interpreting our findings. Information was not available on the genotypes involved in estrogen metabolism which may affect metabolite levels. Genetic polymorphisms may influence the particular estrogen metabolic pathway favored (Taioli 1999); however, this component could not be assessed in this combined analysis. The objective of this analysis was to evaluate the 2-OHE_1 and 16α-OHE_1 metabolites, but additional estrogen metabolites, such as 4-hydroxyestrone, may also contribute to the carcinogenic process. Furthermore, we could not consider the potential modifying role of dietary factors on estrogen metabolites (Lord 2002) or changes in the known breast cancer risk factors over time. This is particularly relevant for the nested case-control

studies in which the exposures could have changed during the study period. A general limitation to the previous published studies in this area includes the use of one urinary measurement of the estrogen metabolites. Many of the limitations to this combined analysis and to the original study designs are common challenges in studies of estrogen metabolites.

Despite these limitations, the aim of this analysis was to clarify the role of the 2:16α-OHE_1 metabolite on breast cancer by re-evaluating primary data from existing studies. Future studies addressing this research question should address the above limitations and consider other methodological issues, such as racial/ethnic differences in estrogen metabolite levels and whether the association between estrogen metabolites and breast cancer differs by race. Additionally, it remains unclear whether a single measurement of estrogen metabolites is representative of long-term levels, or whether estrogen metabolite levels measured at a particular time point, or at multiple time points, may be more informative with regard to breast cancer development. To date, no previous studies have assessed estrogen metabolite levels among premenopausal women over a long duration or during different periods of life. These issues also apply to studies examining this association among postmenopausal women.

The role of ovarian hormones, particularly estrogen, on breast carcinogenesis is complex. Furthermore, the impact of estrogen metabolites on this process has yet to be understood. Estrogen metabolites are affected by numerous factors, including lifestyle and dietary factors, and genetic polymorphisms involved in estrogen metabolism pathways, thus challenging our understanding of the role of these biological factors in breast cancer development. Many of the known breast cancer risk factors relate to a woman's cumulative lifetime exposure to estrogen, yet few of these factors may be modified. Understanding the potential role of estrogen

metabolites in the carcinogenic process will not only help elucidate the underlying mechanism of estrogen exposure but may also offer the potential to identify high-risk premenopausal women.

4.6 TABLES AND FIGURES

Table 8 Characteristics of Participating Studies: Combined Analysis of Estrogen Metabolites and Premenopausal Breast Cancer

Study	Cases/ Controls	Study Population & Location	Age Range	Case selection	Control Selection	Original Matching Criteria
Meilahn et al. (1998)	55/184	Guernsey III cohort, Guernsey Island, 1977-85	34-54 years	Primary clinically diagnosed	Randomly selected from cohort (those alive and free of breast cancer at end of cohort & with available urine)	Age ± 2 yrs, baseline exam ±1yr, menstrual phase 1:3 matching
Muti et al. (2000)	71/292	Hormones and Diet Etiology of Breast Cancer Study (ORDET), 1987-1992 Varese Province, Northern Italy	35-56 years	Linkage with Lombardy Cancer Registry	Randomly selected from cohort among those alive at time of diagnosis of matched case	Age ±5 years, time of blood draw, recruitment center , recruitment date ± 180 days 1:4 matching
Fowke et al. (2003)	59/73	Shanghai, China	33-51 years	Incident cases from tumor or hospital registries	Randomly selected from Shanghai population registry	Age ± 3 years and date of sample collection ± 30 days 1:1 matching

Table 9 Study Specific Odds Ratio Estimates and 95% Confidence Intervals: Association between Tertiles[1] of the 2-OHE_1, 16α-OHE_1 and 2:16α-OHE_1 Metabolites and Premenopausal Breast Cancer

	Fowke et al. (N=129)		Meilahn et al. (N=239)		Muti et al. (N=363)	
Estrogen Metabolite	**Cases/ Controls**	**Unadjusted OR (95% CI)**	**Cases/ Controls**	**Unadjusted OR (95% CI)**	**Cases/ Controls**	**Unadjusted OR (95% CI)**
2-OHE_1						
< 15.33	53/58	1.00	24/82	1.00	9/40	1.00
15.33-38.75	4/13	0.32 (0.10, 1.06)	23/76	1.06 (0.55, 2.05)	27/95	1.30 (0.56, 3.03)
≥ 38.76	0/0	N/A*	8/26	1.08 (0.43, 2.74)	35/153	1.01 (0.44, 2.29)
16α-OHE_1						
< 7.97	30/33	1.00	22/82	1.00	11/65	1.00
7.97-17.20	23/31	0.83 (0.39, 1.74)	19/71	1.04 (0.52, 2.09)	24/82	1.71 (0.77, 3.80)
≥ 17.21	4/7	0.60 (0.16, 2.25)	14/31	1.74 (0.78, 3.88)	36/141	1.53 (0.72, 3.23)
2:16α-OHE_1						
< 1.76	49/66	1.00	20/66	1.00	18/51	1.00
1.76-2.66	6/4	2.63 (0.64, 10.85)	23/68	1.06 (0.53, 2.12)	24/111	0.63 (0.31, 1.27)
≥ 2.67	2/2	1.34 (0.19, 9.75)	12/50	0.76 (0.34, 1.69)	29/126	0.65 (0.33, 1.29)

Note: Estimates generated using conditional logistic regression matched on 5-year age groups. Urinary 2-OHE_1 and 16α-OHE_1 metabolites (ng/ml) are standardized to urinary creatinine levels (mg/dl).

1=Common tertile categories based on the distribution of the individual metabolites among all controls in the combined study population.

*NA = unable to calculate estimate.

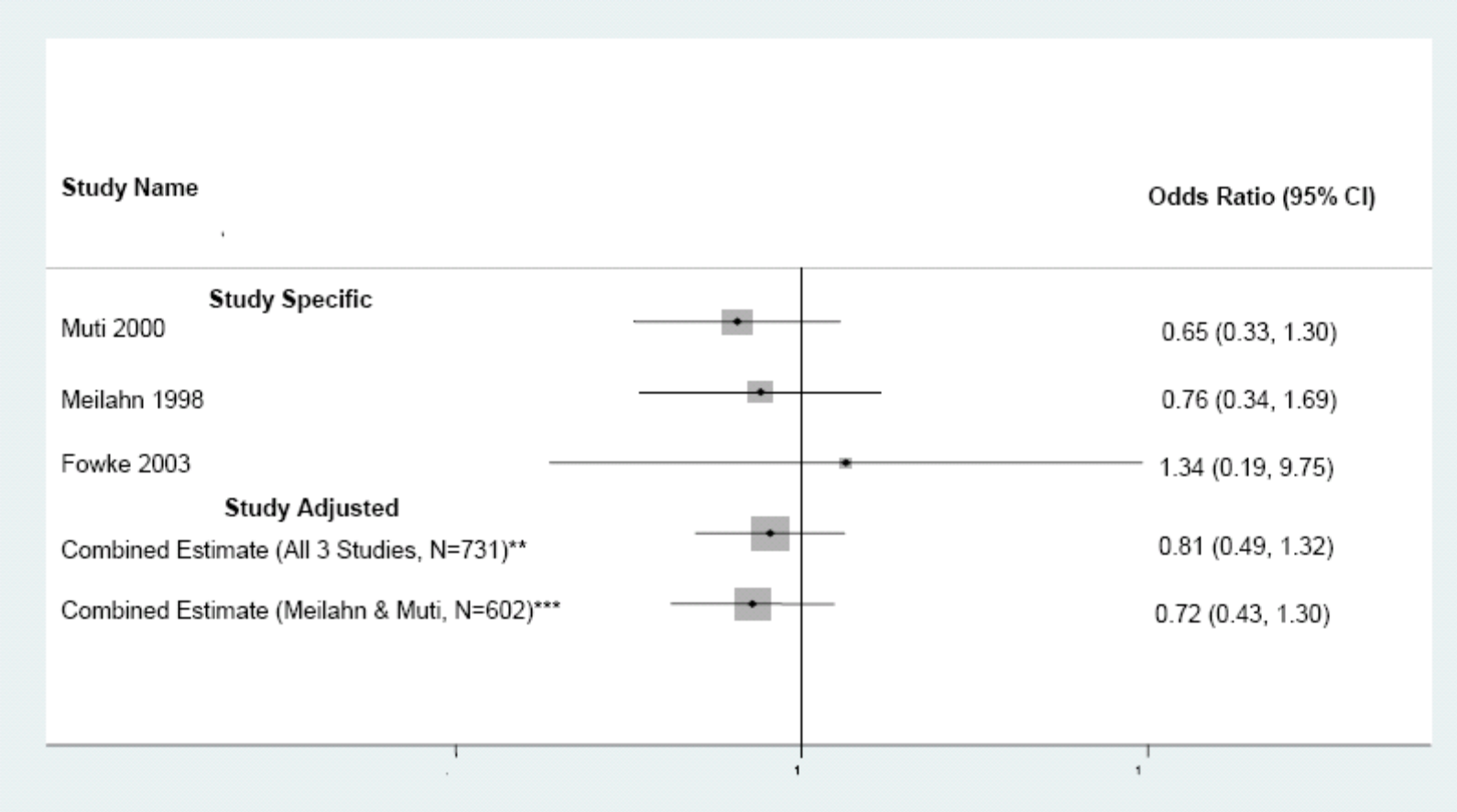

Figure 7 Odds Ratio Estimates: Association of the 2:16α-OHEratio$_1$ (highest tertile vs. lowest tertile) and Breast Cancer†

†Unadjusted study specific odds ratio and 95% confidence limits shown refer to the highest tertile category of the 2:16α-OHE$_1$ ratio compared to the lowest (reference group): ≥ 2.67 vs. < 1.76. The common tertile categories were determined based on the distribution of the 2:16α-OHE$_1$ ratio among controls from all studies combined. Estimates generated using conditional logistic regression matched on 5 year age strata.
**Includes data from all three studies (N=731).
***Includes data only from Meilahn et al. and Muti et al. (N=602)

Table 10 Descriptive Characteristics of the Combined Analysis Study Sample (N=731)

Variable	**Cases** (n=183)	**Controls** (n=548)	**P-value**[1]	**P-value**[2] (adjusted by study)
Study Meilahn et al. Muti et al. Fowke et al.	 55 71 57	 184 292 72	-	-
Age (years)*	42.8 ± 4.9	42.9 ± 4.9	0.92	0.70
Ethnicity, N (%) Caucasian Asian	 126 (68.9) 57 (31.1)	 476 (86.9) 72 (13.1)	 <.0001	-
Family History of Breast Cancer, N (%)	11 (6.0)	25 (4.6)	0.44	0.22
History of Benign Breast Disease, N (%)	46 (25.1)	123 (22.4)	0.37	0.07
Body Mass Index (kg/m_2)*	23.8 ± 3.5 median: 23.6	25.4 ± 4.4 median: 23.8	0.31^	0.69
Waist-to-hip ratio **	0.80 ± 0.06	0.78 ± 0.06	0.02	0.07
Smoking				
Never	124 (67.8)	324 (59.1)		
Former	17 (9.3)	80 (14.6)		
Current	25 (13.7)	109 (19.9)		
Unknown	17 (9.2)	35 (6.4)	0.03	0.19
Alcohol Consumption				
Yes	83 (45.4)	188 (34.3)		
No	45 (24.6)	172 (31.4)		
Unknown	55 (30.0)	188 (34.3)	0.03	-
Oral Contraceptive Use				
Yes	76 (41.5)	241 (44.0)		
No	120 (65.6)	305 (55.7)	0.19	0.51
Age at menarche > 13 years, N (%)	85 (46.4)	220 (40.4)	0.13	0.65
Nulliparous, N (%)	18 (9.8)	53 (9.7)	0.95	0.53
Age at First Pregnancy*	26.2 ± 4.3	25.3 ± 4.2	0.02	0.88
Estrogen Receptor, N (%) Positive Negative Unknown	 71 (38.7) 42 (23.0) 70 (38.3)	-	-	-
Progesterone Receptor, N (%) Positive Negative Unknown	 80 (43.7) 29 (15.8) 74 (40.4)	-	-	-

Note: Percentages may not sum to 100 due to missing values. Estrogen metabolites missing for 5 controls.
*Mean ± SD
**Waist to hip ratio and alcohol consumption data only available in Muti et al. and Fowke et al.
1=t-test p-value for continuous variables or chi-square p-value for categorical variables, unless otherwise noted 2=Wald test p-value adjusted for study
^ Wilcoxon Rank Sum p-value, †Among parous women

Table 11 Median Estrogen Metabolite Levels by Case/Control Status^, (N=731)

Estrogen Metabolite*	Cases (n=183)	Controls (n=543)	P-value†
2-OHE_1	17.9 (0.50-309.9)	21.7 (0.5-351.8)	<0.001
16α-OHE_1	10.8 (2.2-145.5)	11.3 (0.96-181.8)	0.26
2:16α-OHE_1	1.8 (0.3, 6.5)	2.2 (0.28-7.6)	<0.001

Note: Urinary 2-OHE_1 and 16α-OHE_1 Metabolites (ng/ml) are standardized to urinary creatinine levels (mg/dl).
^Median (Range), *Estrogen metabolite data missing for 5 controls
† Wilcoxon rank sum p-value

Table 12 The Odds of Premenopausal Breast Cancer by Tertiles of Urinary Estrogen Metabolites (2-OHE_1, 16α-OHE_1 and 2:16α-OHE_1), (N=731)

Estrogen Metabolites§	Case/ Control (183/543)	Unadjusted OR (95% CI)	Adjusted* OR (95% CI)	Adjusted** OR (95% CI) (by study only)	Adjusted*** OR (95% CI)
2-OHE_1					
< 15.33	86/180	1.00	1.00	1.00	1.00
15.33-38.75	54/184	0.63 (0.42, 0.94)	0.64 (0.42, 0.97)	0.93 (0.59, 1.47)	0.94 (0.58, 1.50)
≥ 38.76	43/179	0.51 (0.33, 0.78)	0.53 (0.34, 0.84)	0.86 (0.50, 1.48)	0.85 (0.49, 1.48)
P-values†					
p-trend		0.005	0.013	0.61	0.58
Study (Main Effect)		-	-	< 0.001	< 0.001
Interaction (2OHE*Study)		-	-	0.28	0.27
16α-OHE_1					
< 7.97	63/180	1.00	1.00	1.00	1.00
7.97-17.20	66/184	1.06 (0.71, 1.59)	1.04 (0.69, 1.58)	1.15 (0.76, 1.75)	1.15 (0.75, 1.77)
≥ 17.21	54/179	0.89 (0.58, 1.36)	0.92 (0.59, 1.42)	1.27 (0.80, 2.04)	1.29 (0.80, 2.08)
P-value†					
p-trend		0.49	0.63	0.34	0.38
Study (Main Effect)		-	-	< 0.001	< 0.001
Interaction (16OHE*Study)		-	-	0.46	0.52
2:16α-OHE_1					
< 1.76	87/183	1.00	1.00	1.00	1.00
1.76-2.66	53/183	0.61 (0.41, 0.92)	0.63 (0.41, 0.96)	0.92 (0.58, 1.47)	0.89 (0.55, 1.42)
≥ 2.67	43/178	0.51 (0.33, 0.78)	0.50 (0.32, 0.78)	0.81 (0.49, 1.32)	0.74 (0.44, 1.23)
P-value†					
p-trend		0.001	0.002	0.39	0.25
Study (Main Effect)		-	-	< 0.001	< 0.001

Note: 2-OHE_1 and 16α-OHE_1 Metabolites (ng/ml) are standardized to urinary creatinine levels (mg/dl). Estimates generated using conditional logistic regression matched on 5 year age strata. Data on 2-OHE_1 and 16α-OHE_1 missing for 5 controls (2:16α-OHE_1 missing for 4 controls).

Abbreviations: OR=Odds Ratio, 95% CI=Confidence Intervals

§=Categories based on distribution of 2:16α-OHE_1 among the controls

*Models adjusted for known breast cancer risk factors including: family history of breast cancer (yes/no), history of benign breast disease (yes/no/unknown), age at first menstrual period (continuous), body mass index (kg/m^2, continuous), oral contraceptive use (yes/no), smoking (yes/no/unknown) and pregnancy combined variable (never pregnant, age at first pregnancy <20, age at first pregnant 20-29, age at first pregnant ≥ 30 years).

**Models adjusted for study only.

***Models adjusted for study in addition to the known breast cancer risk factors mentioned above.

† P-values shown: (1) p-value for test of trend using the midpoint of each metabolite category, (2) Wald X^2 p-value for overall effect of study in models shown above and (3) Wald X^2 p-value for test for interaction between the metabolite and study from the model (not shown) including: the specific metabolite (categorical), study variable (categorical) and the metabolite by study interaction term.

Table 13 The Odds of Premenopausal Breast Cancer by Tertiles of Urinary Estrogen Metabolites (2-OHE1, 16α-OHE1 and 2:16α-OHE1) for Caucasian Women, (N=602)

Estrogen Metabolites[§]	Case/ control (126/476)	Unadjusted OR (95% CI)	Adjusted* OR (95% CI)	Adjusted** OR (95% CI) (by study only)	Adjusted*** OR (95% CI)
2-OHE$_1$					
< 15.33	33/122	1.00	1.00	1.00	1.00
15.33-38.75	50/171	1.11 (0.67, 1.84)	1.20 (0.71, 2.05)	1.15 (0.69, 1.92)	1.22 (0.71, 2.07)
≥ 38.76	43/179	0.91 (0.54, 1.53)	0.94 (0.54, 1.63)	0.99 (0.56, 1.74)	0.98 (0.55, 1.75)
P-values[†]					
p-trend		0.53	0.55	0.77	0.68
Study (Main Effect)		-	-	0.44	0.70
Interaction (2OHE*Study)		-	-	0.81	0.96
16α-OHE$_1$					
< 7.97	33/147	1.00	1.00	1.00	1.00
7.97-17.20	43/153	1.28 (0.77, 2.12)	1.34 (0.79, 2.27)	1.32 (0.79, 2.21)	1.34 (0.78, 2.26)
≥ 17.21	50/172	1.33 (0.81, 2.18)	1.43 (0.85, 2.40)	1.48 (0.88, 2.50)	1.54 (0.90, 2.64)
P-value[†]					
p-trend		0.35	0.26	0.19	0.18
Study (Main Effect)		-	-	0.19	0.19
Interaction (16OHE*Study)		-	-	0.39	0.64
2:16α-OHE$_1$					
< 1.76	38/117	1.00	1.00	1.00	1.00
1.76-2.66	47/179	0.79 (0.48, 1.29)	0.78 (0.47, 1.30)	0.80 (0.48, 1.31)	0.79 (0.47, 1.31)
≥ 2.67	41/176	0.70 (0.42, 1.17)	0.64 (0.38, 1.09)	0.72 (0.43, 1.20)	0.65 (0.38, 1.11)
P-value[†]					
p-trend		0.17	0.10	0.21	0.12
Study (Main Effect)		-	-	0.45	0.73

Note: 2-OHE$_1$ and 16α-OHE$_1$ metabolites (ng/ml) are standardized to urinary creatinine levels (mg/dl). Estimates generated using conditional logistic regression matched on 5 year age strata. Information on 2-OHE$_1$ and 16α-OHE$_1$ missing for 4 controls. Abbreviations: OR=Odds Ratio, 95% CI=Confidence Intervals

§=Categories based on distribution of 2:16α-OHE$_1$ among the controls

*Models adjusted for known breast cancer risk factors including: family history of breast cancer (yes/no), history of benign breast disease (yes/no/unknown), age at first menstrual period (continuous), body mass index (kg/m^2, continuous), oral contraceptive use (yes/no), smoking (yes/no/unknown) and pregnancy combined variable (never pregnant, age at first pregnancy <20, age at first pregnant 20-29, age at first pregnant ≥ 30 years).

**Models adjusted for study only.

***Models adjusted for study in addition to the known breast cancer risk factors mentioned above.

† P-values shown: (1) Wald X^2 p-value for test of trend using the midpoint of each metabolite category, (2) Wald X^2 p-value for overall effect of study in models shown above and (3) Wald X^2 p-value for test for interaction between the metabolite and study from the model (not shown) including the specific metabolite (categorical), study variable (categorical) and the metabolite by study interaction.

Table 14 Relative Risk of Estrogen Receptor Status by Tertiles of Estrogen Metabolites (2-OHE1, 16α-OHE1 and 2:16α-OHE1) as compared to controls, (N=602)*

		ER positive		ER negative		ER unknown	
Estrogen Metabolites	**Controls (N=476)**	**Cases (N=39)**	**RR (95% CI)**	**Cases (N=31)**	**RR (95% CI)**	**Cases (N=56)**	**RR (95% CI)**
2-OHE$_1$							
< 15.33	122	6	1.00	4	1.00	23	
15.33-38.75	171	13	0.93 (0.33, 2.62)	15	1.89 (0.59, 6.03)	22	1.08 (0.55, 2.10)
≥ 38.76	179	20	0.87 (0.33, 2.33)	12	1.00 (0.30, 3.36)	11	1.35 (0.57, 3.20)
16α-OHE$_1$							
< 7.97	147	8	1.00	4	1.00	21	1.00
7.97-17.20	153	14	1.42 (0.56, 3.64)	12	2.54 (0.78, 8.22)	17	0.99 (0.48, 2.04)
≥ 17.21	172	17	1.01 (0.41, 2.49)	15	2.11 (0.66, 6.76)	18	2.31 (1.07, 4.97)
2:16α-OHE$_1$							
< 1.76	117	6	1.00	12	1.00	20	1.00
1.76-2.66	179	14	1.06 (0.33, 3.39)	10	0.40 (0.16, 1.00)	23	1.05 (0.53, 2.09)
≥ 2.76	176	19	1.25 (0.46, 3.38)	9	0.33 (0.13, 0.84)	13	0.78 (0.36, 1.70)

Note: Urinary 2-OHE$_1$ and 16α-OHE$_1$ metabolites (ng/ml) are standardized to urinary creatinine levels (mg/dl). Data on 2-OHE$_1$ and 16α-OHE$_1$ are missing for 4 controls. Odds ratio estimates generated from multinomial models with controls as the comparison group. Estimates were generated using data from the Caucasian studies: Meilahn et al. and Muti et al.

*All multinomial models adjusted for study (categorical) and age (≤ 39, 40-44, ≥ 45)

5.0 URINARY ESTROGEN METABOLITES AND POSTMENOPAUSAL BREAST CANCER: A COMBINED ANALYSIS OF INDIVIDUAL LEVEL DATA

To be submitted for publication

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5.1 ABSTRACT

Objective: Circulating estrogens among postmenopausal women are associated with breast cancer; however, the role of estrogen metabolites, particularly 2-hydroxyestrone (2-OHE_1), 16α-hydroxyestrone (16α-OHE_1), and their ratio (2:16α-OHE_1), in breast carcinogenesis remains unclear. The objective of this analysis was to evaluate urinary 2-OHE_1 and 16α-OHE_1, and their ratio (2:16α-OHE_1) in relation to postmenopausal breast cancer by conducting a combined analysis of previously published studies.

Methods: This combined analysis of four previously published studies yielded a study sample of 966 postmenopausal women, including 319 invasive cases and 647 controls. Urinary estrogen metabolite levels were measured using an ELISA assay. Both study-specific and combined unadjusted and multivariable adjusted odds ratios (ORs) and 95% confidence intervals (CIs) were estimated using conditional logistic regression matching on 5-year age groups. Sensitivity analyses also were conducted combining only the Caucasian nested case-control studies. All statistical tests were two-sided.

Results: Unadjusted median levels of the 2:16α-OHE_1 were significantly lower among cases (1.8) compared to controls (2.0) (p=0.0010). The inverse association between higher levels of the 2:16α-OHE_1 ratio (≥ 2.46 vs. < 1.53) and breast cancer (OR=0.56, 95% CI: 0.39, 0.79) was attenuated after adjustment for study (($OR_{\geq 2.46 \text{ vs. } < 1.53}$=0.87, 95% CI: 0.58, 1.29). Sensitivity analyses limited to the Caucasian nested case-control studies yielded similar results.

Conclusions: The results of this combined analysis do not support a reduction in postmenopausal breast cancer risk associated with higher urinary 2:16α-hydroxyestrone metabolite levels.

5.2 INTRODUCTION

Breast cancer remains a significant public health concern as it continues to contribute to both the morbidity and mortality of women in the United States, with 182,460 new invasive cases estimated in 2008 (ACS 2008). Among postmenopausal women, known breast cancer risk factors include age at natural menopause, prior false-positive mammogram, use of hormone therapy, and obesity (Barlow 2006). Postmenopausal obesity is thought to increase breast cancer risk due to the aromatization of androgens to estrone in adipose tissue (McTiernnan 2003, Vainio 2002, Colditz 1993), and among postmenopausal women, estrone is the main source of endogenous estrogen and estrogen metabolites (Lippert 2000). Higher levels of circulating endogenous estrogen have been associated with an increased breast cancer risk among postmenopausal women (EHBCCG 2002, Eliasssen 2006, Cauley 1999, Zeleniuch-Jacquotte, Missmer 2004, Helzlsouer 1994, Toniolo 1995, Berrino 1996, Dorgan 1996, Thomas 1997); however, whether estrogen metabolites are associated with postmenopausal breast cancer remains unclear. Understanding the role of estrogen metabolites among postmenopausal women may help elucidate underlying mechanisms of breast cancer and may also offer a means of prevention.

Estrogens are involved in the proliferation of human breast epithelial cells and may influence carcinogenesis indirectly through mitotic effects (Preston-Martin 1990, Fiegelson 1996) or by inducing genotoxic effects as a result of oxidative damage (Yager 2000). An alternative mechanism has also been proposed in which estrogen, through the formation of depurinating estrogen adducts, acts as a cancer initiator (Cavalieri 2006). Estrogen metabolism occurs by the oxidation pathway (Lippert 1999). Among postmenopausal women, estrone is metabolized via two main pathways involving hydroxylation sites C2, C4 or C-16, leading to the

formation of either A-ring or D-ring metabolites (Mueck 2000, Lippert 2000). Main metabolites of the A-ring pathway include 2-hydroxyestrone (2-OHE_1) and 4-hydroxyestrone (4-OHE), while 16-alpha-hydroxyestrone (16α-OHE_1) and estriol result from the D-ring pathway (Mueck et al 2002).

Although both 2-OHE_1 and 16α-OHE_1 have estrogenic properties, they vary in regard to their ability to bind to the estrogen receptor as well as the nature of their estrogenic properties. The 16α-OHE_1 metabolite has been shown to have higher estrogen properties based on its ability to covalently bind to the estrogen receptor (Swaneck 1988) while also sharing properties similar to those of estradiol (Lippert 2003, Seeger 2006), whereas the 2-OHE_1 metabolite exhibits lower estrogen activity, in part due to the reduced affinity to estrogen receptor binding as well as reduced cell proliferative activity (Schneider 1984). Furthermore, these metabolites result from mutually exclusive pathways (Zhu 1998), rendering the ratio of 2-OHE_1:16α-OHE_1 a useful measure of exposure to active metabolites.

To date, several studies have evaluated the relationship between estrogen metabolite levels and breast cancer risk among postmenopausal women with metabolites measured in either urine (Fowke 2003, Ho 1998, Kabat 1997, Kabat 2006, Meilahn 1998, Modugno 2006, Muti 2000, Ursin 1999, Wellejus 2005), serum (Cauley 2003, Modugno 2006) or plasma (Eliassen 2008). While the results of some studies support an overall association of a reduction in breast cancer risk with a higher 2:16α -OHE_1 ratio (Kabat 1997, Ho 1998), other studies suggest either a modest association or a lack of statistical significance (Meilahn 1998, Muti 2000, Wellejus 2005), or show no association at all (Ursin 1999, Cauley 2003, Eliassen). Due to the varied results of published studies to date, the relationship between 2-OHE_1, 16α-OHE_1 and 2:16α-OHE_1 and postmenopausal breast cancer remains unclear.

The aim of this combined analysis of primary data from published studies was to evaluate whether the 2-OHE_1 and 16α-OHE_1 metabolites, and the 2:16α-OHE_1 ratio, are associated with postmenopausal breast cancer. We hypothesized that higher levels of the 2-OHE_1 metabolite would be associated with a protective effect while higher levels of the 16α-OHE_1 metabolite would be associated with an increase in breast cancer risk among postmenopausal women. We also hypothesized that a higher 2:16α-OHE_1 ratio would be inversely associated with postmenopausal breast cancer risk.

5.3 METHODS

5.3.1 Study design

We conducted a combined analysis of individual level data from four previously published studies, including three nested case-control studies conducted in Northern Italy (Muti et al.), Guernsey Island (Meilahn et al.) and Denmark (Wellejus et al.), and one case-control study conducted in Shanghai, China (Fowke et al.). Primary data from the participating studies were obtained and potential heterogeneity in exposure distributions assessed prior to generating a summary estimate of the combined data. Table 15 provides a brief description of the participating studies with additional details provided in the original publications (Muti 2000, Meilahn 1998, Fowke 2003, Wellejus 2005).

5.3.2 Study Identification and Selection

Original research studies that evaluated the relationship between estrogen metabolites and postmenopausal breast cancer were identified by searching the National Library of Medicine and National Institutes of Health Pubmed database. The search strategy involved the following keyword search terms: (1) estrogen metabolites (n=2901 articles) and (2) estrogen metabolites with additional limits to females and English language (n=974 articles), (3) estrogen metabolites AND breast cancer (n=376 articles). Each of the 376 citations and abstracts were reviewed, and 12 articles were identified as having examined estrogen metabolites in relation to breast cancer with 8 studies including postmenopausal women. Reference lists from retrieved articles were also reviewed in order to identify additional eligible articles; no additional studies were identified. This search is current as of July 15, 2008.

Articles were considered eligible for review if they met the following pre-determined inclusion criteria: (1) an original research study, (2) inclusion of postmenopausal women, (3) exposure measured as 16α-hydroxyestrone (16α-OHE), 2-hydroxyestrone (2-OHE), and/or the ratio of these two main estrogen metabolites, (4) urine as the sample source, (5) breast cancer assessed as the main outcome and (6) sample size of at least 50 subjects. These *a priori* criteria were selected to maximize the comparability across studies.

5.3.3 Selection results

Among the twelve published studies, eight studies (Fowke 2003, Kabat 1997, Ho 1998, Kabat 2006, Meilahn 1998, Muti 2000, Ursin 1999, Wellejus 2005) included postmenopausal cases in their original study, and fulfilled the above eligibility criteria for inclusion in this combined

analysis of individual data relating to urinary estrogen metabolites and postmenopausal breast cancer. Of these eight eligible published studies, three were nested case-control studies (Meilahn, Muti, Wellejus), and five were case-control studies (Fowke, Kabat 1997, Kabat 1998, Ho 1998, Ursin 1999, Wellejus 2005). Investigators from the eligible studies were contacted and invited to participate in this combined analysis. Multiple invitations were extended in an attempt to enlist the participation of all eligible studies. Of these, five studies, three nested case-control studies (Muti et al., Meilahn et al., Wellejus et al.) and two case-control studies (Fowke et al., Ursin et al) agreed to participate. Data from the case-control study by Ursin et al. was not included in the final combined study sample due to the inclusion of prevalent breast cancer cases.

5.3.4 Data Collection and Extraction

The participating studies utilized structured questionnaires to ascertain information on demographics, lifestyle and anthropometric factors, hormone use, reproductive history, and other known breast cancer factors. Investigators were asked to submit their study-specific datafile along with a copy of the original questionnaire, a description of the variables and study methods, and an electronic datafile which included the main exposures of interest, the urinary 2-OHE_1 and 16α-OHE_1 metabolites, along with important breast cancer covariates. Definitions of menopausal status were provided by each study. In the study by Muti et al., women without menstrual bleeding for at least 12 months were considered postmenopausal. Wellejus et al. defined women as either known or probable postmenopausal. Known postmenopausal status included women with 1) no hysterectomy and no menstruation in the 12 months prior to study entry, or (2) a bilateral oophorectomy or (3) age at last menstruation lower than age at hysterectomy. Probable postmenopausal status included women with 1) menstruation during the 12 months prior to study

entry and current use of HRT, or 2) hysterectomy with unilateral (or unknown) oophorectomy or 3) age at last menstruation same as age at hysterectomy. Women in the study by Meilahn et al. were classified as postmenopausal if they reported (1) undergoing natural menopause, or (2) a hysterectomy with unilateral oophorectomy. Each datafile was checked for consistency and uniformity with previously published results. Discrepancies identified were resolved with the study investigators. Policies for data publication and authorship were reviewed and accepted by study investigators.

Completeness of Data

All variables requested were coded with a common format for this analysis with the exception of education and socioeconomic status, due to differences in classification systems. Information on waist-to-hip ratio and alcohol consumption was available in three studies (Wellejus, Fowke, Muti) and family history of breast cancer was not available in the Danish study (Wellejus). Data on estrogen receptor (ER) and progesterone receptor (PR) status was available for 90.6 % and 40.8% of cases, respectively. PR data was not available in the Guernsey data (Meilahn) and was classified as missing for 74.7% of cases in the Danish study (Wellejus 2005). Due to incomplete information on tumor staging, we were unable to assess metabolite levels by stage of disease. Overall, the proportion of missing values for each covariate is low with the exception of those noted above.

5.3.5 Laboratory measurement of main exposure

Spot urine samples were collected for Meilahn et al., Fowke et al., and Wellejus et al. and an overnight spot urine sample was collected in the study by Muti et al. The use of a spot urine collection has been shown to be a reliable sample source for the measurement of urinary estrogen

metabolites (Chen 1999) with no reported significant differences between spot urine samples or multiple urine collections over a 24-hour period (Westerlind 1999). Additionally, the potential affect of long-term sample storage on metabolite levels was assessed in the original studies by Meilahn et al. and Wellejus et al.; no variations in metabolite levels were reported. All participating studies measured urinary 2-OHE and 16α-OHE using a commercially available competitive, solid-phase enzyme immunoassay kit (ESTRAMET) produced by Immunacare Corp (Bethlehem, PA, USA). With this assay kit, the binding of the monoclonal antibodies to estrogen metabolites (antigen) is captured directly on the solid phase. The urinary forms of 2-OHE and 16-OHE are found as glucuronide conjugates and require the removal of sugars before the monoclonal antibodies in the assay kit can detect the urinary metabolites. Studies by Fowke et al., Muti et al, and Wellejus et al. utilized the more recent ELISA kit (Bradlow 1998), whereas Meilahn et al. used the original assay developed by Klug (Klug 1994). The sensitivity of the modified kit is approximately 0.625 ng/ml for the 16α-OHE and 2-OHE metabolites (Bradlow 1998, Falk 2000). The reported mean within assay variability of this kit is approximately 4% while the mean between assay variability is about 10%. The recent assay kit includes modifications to the antibody concentrations, enzyme concentrations and standards that allow for an increased sensitivity level among postmenopausal women (Bradlow 1998, Falk 2000).

5.3.6 Statistical analysis

The 2-OHE_1 and 16α –OHE_1 metabolites (ng/ml) were divided by the urinary creatinine concentration (mg/dl) as a means of standardizing the metabolite values by total volume of urine. The 2:16α-OHE_1 variable in this analysis is based on the ratio of these two individual estrogen metabolites. 2-OHE_1 and 16α-OHE_1 metabolite data was available on 98.7% of this study

sample. The estrogen metabolite exposures (2-OHE_1, 16α-OHE_1 and the 2:16α-OHE_1 ratio) were all non-normally distributed and were analyzed as categorical variables (tertiles) based on the distribution among the total combined control population.

The main outcome of this analysis is invasive breast cancer. *In situ* breast cancer cases were excluded from the present analysis due to the limited number of cases (n=6). Current users of oral contraceptives from Fowke et al. were excluded from this analysis (n=2) to be consistent with the original exclusion criteria from the other studies. In the case-control study by Fowke et al., urine samples were collected either before or after surgery/ancillary treatment and different effects of the 2:16α-OHE_1 on breast cancer by the timing of urine collection were reported in the original publication. Thus, cases with post-treatment urine collection from Fowke et al. were excluded (n=18). Additionally, the present analysis was limited to women not currently on hormone replacement therapy (HRT) at the time of entry into the original studies due to noted differences in the 2:16α-OHE ratio by postmenopausal HRT status (Armamento-Villareal 2004, Wellejus 2005). Participants in the study by Wellejus et al. who were currently on HRT at cohort entry were excluded from these analyses (234 cases/234 controls). All other participating studies excluded women currently on HRT as part of their original enrollment criteria. Lastly, information on estrogen metabolites was missing for 13 women (3 cases/10 controls). After applying these additional exclusions, the present combined analysis includes 966 postmenopausal women (319 cases/647 controls).

The distribution of covariates was compared among cases and controls using either the parametric t-test or non-parametric Wilcoxon rank sum for continuous variables, or the chi-square test for categorical variables. Additionally, study adjusted p-values for the association

between individual covariates and case/control status were generated using conditional logistic regression models which included dummy variables for the individual studies.

The original study-specific findings previously published were replicated prior to conducting this combined analysis to ensure accurate information in the datafiles. Additionally, preliminary analyses were performed to assess the use of original matched sets versus creating common matching criteria using 5-year age strata (<50, 50-54, 55-59, 60-64, 65-69,70-74, ≥ 75). Both analytical approaches yielded similar estimates, and thus 5-year age groups were used as the matching term in the present analyses. Unadjusted and adjusted odds ratios (ORs) and 95% Confidence Intervals (CIs) were estimated by conditional logistic regression (Breslow 1980) matched on 5-year age groups. The association between each metabolite and breast cancer was assessed using separate conditional logistic regression models. Four different models were performed using the combined data: (1) unadjusted, (2) adjusted for known breast cancer risk factors, (3) adjusted for study only, and (4) adjusted for known breast cancer risk factors in addition to study. In all models, age at the time of enrollment in the original studies was taken into account by stratifying the models by 5-year age groups.

Multivariable models included adjustment for known breast cancer risk factors including family history of breast cancer in a first degree relative (yes/no), history of benign breast disease (yes/no), body mass index (kg/m^2), smoking (never/former/current), oral contraceptive use (never/former/unknown), age at menarche (<13 years/≥ 13 years/unknown) and a combined pregnancy variable (never pregnant, age at first pregnancy <20, age at first pregnancy 20-29, and age at first pregnancy ≥30). These factors were chosen *a priori* as adjustment variables either due to their established associations with breast cancer or because they are important characteristics of the Gail Model (Gail 1989) used to assess breast cancer risk. The conditional

logistic regression models were adjusted by study using separate dummy variables for each study to account for potential differences in overall study design and study populations. Separate trend tests for each estrogen metabolite were performed using the midpoint for each metabolite category.

Heterogeneity between studies was evaluated graphically by visual examination of study specific estimates using a forest plot and by the Cochran's Q-statistic chi-square test (Petitti 2000). In addition, the study-specific median levels of each metabolite among the control populations were evaluated. The study by Fowke et al. conducted in Shanghai, China was significantly different than the studies by Meilahn et al. and Muti et al., with lower levels of all metabolites among this Asian population. Considering the observed statistical differences in study specific median levels and estimates, as well as the potential biological differences in estrogen metabolism in Asian versus Caucasian populations, a sensitivity analysis was also performed in which the conditional logistic regression models were repeated without the data from the Fowke et al. Shanghai study. Additionally, the Fowke et al. study utilized a case-control study design whereas the studies by Muti et al., Meilahn et al. and Wellejus et al. were nested case-control studies in which the urine sample was collected before breast cancer diagnosis. The sensitivity analysis including data from the three nested case control studies included 910 postmenopausal women (300 cases/610 controls). Additionally, using data from the nested case-control studies, all models were re-estimated among women ages 50-65 as this was a common age group across the studies (N=833).

Previous research suggests that estrogen metabolism may vary according to levels of body mass index (BMI) (Modugno 2006, Fishman 1975, Schneider 1983) and by smoking status (Sowers 2006). Subgroup specific models were conducted to assess whether the association

between estrogen metabolites and breast cancer varied by BMI (<25, ≥ 25 kg/m^2) and smoking status (current, non-current). In a combined model, a dummy variable for BMI and a categorical interaction term for BMI and the metabolite were included. Separate combined models were performed for each metabolite exposure. Wald X^2 tests were used to test the overall effects of the interaction. Interactions between smoking status and each metabolite were assessed using a similar approach.

Each of the individual metabolites 2-OHE$_1$ and 16α-OHE$_1$, and the 2:16α-OHE$_1$ ratio, were also evaluated in relation to breast cancer according to hormone receptor status by performing separate age-adjusted multinomial logistic regression models. The outcome variable for the ER models included four categories: controls (reference), estrogen receptor positive (ER+) cases, estrogen receptor negative (ER-) cases, and estrogen receptor status unknown (ER unk) cases. Similarly, for the progesterone receptor models, the outcome variable included four categories: controls (reference), progesterone receptor positive (PR+) cases, progesterone receptor negative (PR-) cases, and progesterone receptor status unknown (PR unk) cases. These multinomial logistic regression models included adjustment for age using four categories (≤ 54, 55-59 60-64, ≥ 65) due to the smaller number of cases when evaluating breast cancer cases by hormone receptor status.

Potential interactions between the individual metabolite exposures and study were assessed by creating separate categorical interaction terms for each metabolite (using dummy variables for both the study and metabolite categorical variables). Multiparameter Wald X^2 tests were used to test the overall effects of study, metabolite and the interaction. Interactions between age (continuous) and the 2-OHE$_1$, 16α-OHE$_1$, and 2:16α-OHE$_1$ metabolites also were assessed using a similar approach.

All analyses were performed using SAS (Version 9.1, Cary, NC, USA) and STATA 10. Tests of significance were two-sided. Data were analyzed in Pittsburgh, PA and the datafiles received did not include personal identifiers. Each study participating in this present analysis was approved by the Institutional Review Board or appropriate ethical committee at the respective institutions, and participants provided informed consent.

5.4 RESULTS

Table 16 summarizes the unadjusted study specific odds ratio estimates and 95% confidence intervals for the association between tertiles of each metabolite and postmenopausal breast cancer. Higher levels of the 2:16α-OHE_1 ratio ($\geq$ 2.46 vs. < 1.53) were associated with a reduction in breast cancer risk in the studies by Fowke et al. (OR=0.38, 95% CI: 0.04, 3.50), Meilahn et al. (OR=0.66, 95% CI: 0.23, 1.88), and Wellejus et al. (OR=0.76, 95% CI: 0.42, 1.39); albeit these results were not statistically significant. The study-specific estimates for Muti et al. were in the positive direction, suggesting an increased risk with higher levels of the 2:16α-OHE_1 ratio ($OR_{\geq 2.46 \text{ vs. } < 1.53}$=1.63, 95% CI: 0.64, 4.12). These study specific estimates are depicted in the forest plot shown in Figure 8. Despite the apparent difference in the direction of effect, the Cochrane chi-square test of heterogeneity was not statistically significant (X^2=02.73, p-value=0.44).

Characteristics of the combined study population by case/control status are summarized in Table 17. The majority of the study sample was Caucasian (94.0% of cases and 94.3% of controls), with an average age of 57.7 ± .48 years for cases and 57.8 ± 5.1 years for controls. Unadjusted median estrogen metabolite levels differed between cases and controls, with cases

having lower levels of the 2-OHE_1, 16α-OHE_1, and the 2:16α-OHE_1 metabolites compared with controls (Table 18; p-value <0.001 for each).

Table 19 presents the ORs and corresponding 95% CIs estimating the odds of breast cancer in relation to the individual 2-OHE_1 and 16α-OHE_1 metabolites and the 2:16α-OHE_1 ratio (319 cases/647 controls). Among women in the highest tertile of the 2:16α-OHE_1 ratio, the odds of breast cancer was significantly reduced (OR=0.56, 95% CI: 0.39, 0.79) as compared to those in the lowest tertile; however this reduction in risk attenuated with adjustment for study (OR=0.87, 95% CI: 0.58, 1.29) or adjustment for both study and known breast cancer risk factors (OR=0.98, 95% CI: 0.66, 1.98). A similar pattern was observed with the 2-OHE_1 metabolite. Although the protective effect observed with higher levels of the 2-OHE_1 metabolite ($OR_{\geq 8.50 \text{ vs.} < 2.99}$=0.28, 95% CI: 0.20, 0.41) was no longer statistically significant with adjustment for study ($OR_{\geq 8.50 \text{ vs.} < 2.99}$= 0.69, 95% CI: 0.40, 1.19) and/or known breast cancer risk factors. Results from the tests for trend from the unadjusted models suggested a significant inverse trend (p<.0001) with 2-OHE_1, 16α-OHE_1, and the 2:16α-OHE_1 ratio but this trend was no longer apparent after adjustment for study (p>0.10). The main effect of study in each of the metabolite main models was statistically significant as indicated by the Wald test p-values. None of the interactions between study and metabolite were statistically significant, p-value >0.10. None of the interactions between age and each of the metabolites were statistically significant, p-value > 0.10 (data not shown).

Table 20 summarizes the results from the sensitivity analyses which includes data from the three Caucasian nested case-control studies (300 cases/610 controls). Results from both the unadjusted and adjusted models were similar to those presented in Table 19. The observed inverse relationship with higher levels of the 2:16α-OHE_1 in unadjusted models attenuated with

adjustment for either study or both study and known breast cancer risk factors. No significant associations with either the 2-OHE_1, 16α-OHE_1, or their ratio were observed in the subgroup specific analyses of BMI (< 25 kg/m^2 and ≥ 25 kg/m^2) or smoking status (current vs. non-current smoker) (data not shown). Overall tests of interaction were not statistically significant (Wald test p-value ≥ 0.15 for each) (data not shown). Additionally, results from the models repeated among women ages 50-65 were relatively similar to those presented in Table 19 (data not shown).

The relative risk of ER status by tertiles of estrogen metabolites as compared to controls, are summarized in Table 21. No significant differences were observed in the relative risk (RR) of ER+ or ER- tumors by tertiles of 2:16α-OHE_1, with observed estimates in the same direction (ER+: $RR_{\text{Tertile 3 vs Tertile 1}}$=0.67, 95% CI: 0.43, 1.04; ER-: $RR_{\text{Tertile 3 vs. Tertile 1}}$=0.59, 95% CI: 0.31, 1.12). No significant associations were observed with the 16α-OHE_1 metabolite, although the direction of the estimate suggests a negative association for ER- cases. No significant associations were observed for the analyses by PR status (data not shown).

5.5 DISCUSSION

In this combined analysis of urinary 2-OHE_1 and 16α-OHE_1 metabolites and breast cancer, we did not observe significant associations with either of the individual metabolites or their ratio and breast cancer. Unadjusted models suggested that higher levels of the 2-OHE_1 metabolite and the 2:16α-OHE_1 ratio might be protective; however this apparent inverse relationship attenuated with adjustment for study.

To date, 12 studies have evaluated estrogen metabolites in relation to breast cancer among postmenopausal women, of which seven have been nested case-control studies (Meilahn 1998, Modugno 2006, Muti 2000, Cauley 2003, Modugno 2006, Wellejus 2005, Eliassen 2008) and five were case-control studies (Fowke 2003, Ho 1998, Kabat 1997, Kabat 2006, Ursin 1999). Previous case-control studies have either suggested a potential reduction (Kabat 1997, Ho 1998, Kabat 2006) in breast cancer risk with a higher 2:16α -OHE_1 ratio or no association (Ursin 1999), while results from most nested case-control studies suggest no association. Differences in the inconsistency of results between case-control and nested-case control studies may be due to the inability to establish temporal inference in case-control studies. Furthermore, nested case-control studies have evaluated this association with estrogen metabolites measured in urine (Muti 2000, Meilahn 1998 Wellejus 2005), serum (Cauley 1999) and plasma (Eliassen 2008), and despite the difference in biological specimen, the results from these studies have mostly been null among women not currently on HRT.

The objective of this analysis was to improve our understanding of the association between urinary estrogen metabolites and breast cancer among postmenopausal women not currently on HRT, with the use of a larger combined sample size. The original results from the participating nested case-control studies indicated either no association with higher levels of 2-OHE_1 or 16α-OHE_1 among non-users of HRT in the study by Wellejus et al. (Wellejus 2005), or a potential decrease in breast cancer risk with higher levels of the 2:16α-OHE_1 ratio in the Guernsey study by Meilahn et al. (unadjusted $OR_{\text{tertile 3 vs. 1}}$=0.71, 95% CI: 0.29-1.75), or no association with 2:16α-OHE_1 indicated by OR values above 1 in the study by Muti et al. (unadjusted $OR_{\text{quantile 5 vs. quantile 1}}$)=1.17, 95% CI: 0.49-2.75). In our analyses limited to nested case-control studies (300 cases, 610 controls), we did not observe a statistical significant

association with higher levels of the 2:16α-OHE_1 ratio. Furthermore, the odds ratio estimates in both study adjusted and multivariable adjusted models surrounded 1 and thus do not lend evidence in support of an inverse relationship between 2:16α-OHE_1 and breast cancer.

Hormonally related factors are suggested to impact ER+ tumors differently than ER- tumors (Huang 2000, Cotterchio 2003), with estrogens suggested to have a stronger association with ER+ tumors compared to ER- tumors (Missmer 2004), though findings across studies have been inconsistent. Furthermore, the association with estrogen metabolites and ER specific subtypes remains unclear. Previous studies of estrogen metabolites have observed significant associations with either ER+ or ER- tumors, with differences in the direction of effect. Although higher levels of the 2-OHE_1 metabolite were significantly associated with a reduced relative risk of both ER+ and ER- tumors in this combined analysis and results for the 2:16α:OHE_1 ratio followed a similar pattern, the direction and magnitude of effect were similar for both ER subtypes, suggesting no difference in the overall effect of metabolites on ER specific tumors. Few studies have evaluated the association between estrogen metabolites and breast cancer by ER or PR status, of which two were nested case-controls (Wellejus 2005, Eliassen 2008) and two were retrospective case-control studies (Kabat 1997, Kabat 2006). In the original study by Wellejus et al., no significant associations were observed between 2-OHE_1, 16α-OHE_1, or the 2:16α-OHE_1 and ER specific breast cancer among women not currently on hormone replacement therapy, although the direction of the estimates suggested a reduction in ER- breast cancer with higher levels of the 2:16α-OHE_1 ratio (Wellejus 2005). Results from a recent nested case-control study within the Nurses Health Study suggested significant positive associations with both the 2-OHE_1 metabolite and 2:16α-OHE_1 (measured in plasma) and ER-/PR- tumors (Eliassen 2008). Results from a small case-control study of both pre- and postmenopausal women (n=106)

reported higher mean levels of the 2:16α-OHE_1 ratio among those with ER+ tumors compared to ER- tumors (Kabat 1997). A larger case-control study including 164 postmenopausal breast cancer cases observed a moderately statistically significant inverse association between the 2:16α-OHE_1 ratio and ER negative breast cancer (OR=0.38, 95% CI: 0.15-1.01) and no association among ER- cases (OR=1.05, 95% CI: 0.53-1.06) (Kabat 2006). The results from the previous studies cannot be directly compared due to differences in analytical methods as well as type of biological sample (plasma vs. urinary measures), but nonetheless, the inconsistent findings across the studies demonstrates the need for more research in this area, particularly in studies with a larger number of postmenopausal breast cancer cases. Furthermore, in this combined study, we were unable to assess combinations of ER and PR status due to the large number of cases with unknown PR status. Future studies should include more complete information on PR status.

Few studies have reported significant differences in the association between estrogen metabolites and breast cancer among subgroups and results often vary across studies, partly due to differences in subgroup definitions. In this combined analysis, no significant associations were detected among subgroups of BMI and smoking, and furthermore, the direction of effect was not consistent within subgroups. Postmenopausal obesity has been associated with an increase in breast cancer risk (EHBCCG 2003, van den Brandt 2000, Key 2003) due to the aromatization of steroids to estrone in adipose tissue (McTiernan 2003, Vainio 2002, Colditz 1993) and estrogen metabolites have been suggested to differ by body mass index (Sowers 2006); however, in this combined analysis no significant associations were observed in either subgroup of BMI (< 25 kg/m^2 or ≥ 25 kg/m^2).

In this combined analysis, no significant interactions were observed between any of the estrogen metabolites and study. However, the main effect of study was significant, and furthermore, adjustment for study attenuated the protective effects observed in the unadjusted models. Although study may not modify the association between estrogen metabolites and breast cancer, it appears to confound the relationship. This is likely due to differences in study characteristics, study populations or the differences in the distribution of metabolites.

Estrogen metabolite levels in both the studies by Fowke et al. and Wellejus et al. were mostly distributed among the lowest tertile of common cutpoints. The lower estrogen metabolite levels among the Shanghai population are not unexpected as lower levels of circulating estrogens have been reported among Asian populations compared with Caucasian women (Aldercreutz 1994). Estrogen metabolite levels among the Danish study were also lower as compared to the other two Caucasian studies (Muti, Meilahn), suggesting that the normal range of the metabolites are unknown. In general, the range of metabolite values overlapped across studies yet the overall median levels were significantly different. Although few studies have evaluated racial differences in estrogen metabolites (Ursin 2001, Jernstrom 2003, Falk 2005**,** Taioli 1996), this combined analysis highlights observed variability in the metabolite levels both across and within racial/ethnic populations and emphasizes the complexity in studying these metabolites.

There are several strengths to this combined analysis including the use of primary individual level data, the ability to assess the relationship between the estrogen metabolites and breast cancer using common cutpoints, the large number of postmenopausal breast cancer cases, the completeness of information on estrogen receptor status (90.6%), and the ability to adjust for most known breast cancer risk factors. Additionally, the nested case-control studies measured

estrogen metabolite levels at study entry and thus levels were not affected by disease status or by treatment.

Limitations to this combined analysis include the inability to consider additional factors which may affect estrogen metabolite levels such as genetic polymorphisms (Taioli 1999) and dietary factors (Lord 2002). Although the focus of this analysis was to assess the role of the 2-OHE_1 and 16α-OHE_1 metabolites, additional metabolites such as 4-hydroxyestrone and the adducts which form from the quinone by-products, may also be important factors in breast carcinogenesis (Cavalieri 2006, Yager 2000). Despite multiple attempts to include all previously published eligible studies, we were not able to obtain data from three previous case-control studies of estrogen metabolites and postmenopausal breast cancer. While we did not have information on mammography screening practices, we were able to adjust for benign breast disease in the multivariable models. General limitations to the original studies included in this combined analysis include the measurement of estrogen metabolites during one time point, and the inability to assess changes in known breast cancer risk factors over time due to the nested case-control design. Urinary estrogen metabolites measured at a single point in time may not accurately reflect a woman's normal levels. Within the study by Eliassen et al., a reproducibility study of metabolite levels among postmenopausal women indicated that metabolite levels were comparable over a three year period (Eliassen 2008). However, more research is needed to better understand estrogen metabolites levels during different time periods.

This combined analysis of previously published studies does not support an association between urinary estrogen metabolites and postmenopausal breast cancer. Future studies should investigate estrogen metabolites measured at multiple time points or during different periods of life, and whether this affects the association between estrogen metabolites and breast cancer risk.

Additionally, future studies should address the limitations of the existing studies and aim to include information on genetic polymorphisms, dietary factors, and race, all of which may affect estrogen metabolite levels. Improving our understanding of estrogen metabolites and their influence on breast cancer risk may offer a means of prevention or early detection, and may also provide additional insight in regards to the role of estrogen in breast cancer development.

5.6 TABLES AND FIGURES

Table 15 Characteristics of Participating Studies: Combined Analysis of Estrogen Metabolites and Postmenopausal Breast Cancer

Study	Cases/ Controls	Study Population & Location	Age Range	Case selection	Control Selection	Original Matching Criteria
Nested Case-Control						
Meilahn et al. (1998)	39/139	Guernsey III cohort, Guernsey Island, 1977-85	48-79 years	Primary clinically diagnosed	Randomly selected from cohort (those alive and free of breast cancer at end of cohort & with available urine)	Age ± 2 yrs, baseline exam ±1yr, menstrual phase
Muti et al. (2000)	71/282	Hormones and Diet Etiology of Breast Cancer Study (ORDET), 1987-1992 Varese Province, Northern Italy	42-69 years	Linkage with Lombardy Cancer Registry	Randomly selected from cohort among those alive at time of diagnosis of matched case	Age ±5 year, time of blood draw, recruitment center , recruitment date ± 180 days
Wellejus et al. (2005)	193/199	Greater Copenhagen or Aarhus areas 1993-2000	50-65 years	Linkage with the Danish Cancer Registry and the Danish Breast Cancer Cooperative Group Registry	Cancer free at exact age at diagnosis of case	Age at entry (6 month intervals), HRT status postmenopausal status (known/probably) 1:1 matching
Case-control						
Ursin et al. (1999)	66/76	Los Angeles, Ca USA	53-70 Years	LA County Cancer Surveillance Program (SEER) Diagnosed 1987-1989, or in 1992 *prevalent cases in this ancillary study	Population based Neighborhood controls from the same area as the cases	Age ± 3 years, ethnicity, & neighborhood 1:1 matching *Matching not retained in this ancillary study
Fowke et al. (2003)	19/37	Shanghai China	47-64 Years	Incident cases from tumor or hospital registries	Randomly selected from Shanghai population registry	Age ± 3 years and date of sample collection ± 30 days

Table 16 Study Specific Unadjusted Odds Ratio Estimates and 95% Confidence Intervals: Association between Tertiles[1] of the 2-OHE_1, 16α-OHE_1 and 2:16α-OHE_1 Metabolites and Postmenopausal Breast Cancer (N=966)

Estrogen Metabolite	Fowke et al. (N=19/37)		Meilahn et al. (N=39/139)		Muti et al. (N=71/282)		Wellejus et al. (N=193/199)	
	Cases/ Controls	OR (95%CI)	Cases/ Controls	OR (95%CI)	Cases/ Controls	OR (95%CI)	Cases/ Controls	OR (95%CI)
2-OHE_1								
< 2.99	9/6	1.00	3/6	1.00	5/27	1.00	167/174	1.00
2.99-8.49	9/25	0.30 (0.09, 1.07)	25/82	0.59 (0.14, 2.50)	28/90	1.68 (0.59, 4.77)	21/24	0.91 (0.49, 1.69)
≥ 8.50	1/6	0.13 (0.01, 1.32)	11/51	0.38 (0.08, 1.77)	38/155	1.33 (0.48, 3.70)	2/1	2.08 (0.19, 23.09)
16α-OHE_1								
< 1.50	1/1	1.00	4/7	1.00	8/37	1.00	144/169	1.00
1.50 – 4.47	12/22	0.68 (0.04, 11.71)	16/60	0.42 (0.11, 1.63)	30/109	1.26 (0.53, 2.99)	43/27	1.86 (1.10, 3.16)
≥ 4.48	6/14	0.61 (0.03, 11.67)	19/72	0.41 (0.11, 1.59)	33/126	1.22 (0.52, 2.88)	3/3	1.17 (0.23, 5.86)
2:16α-OHE_1								
< 1.53	14/29	1.00	16/58	1.00	6/37	1.00	90/85	1.00
1.53-2.45	4/3	2.23 (0.43, 11.50)	17/48	1.24 (0.57, 2.69)	26/87	1.79 (0.68, 4.69)	75/83	0.85 (0.55, 1.31)
≥ 2.46	1/5	0.38 (0.04, 3.50)	6/33	0.66 (0.23, 1.88)	39/146	1.63 (0.64, 4.12)	25/31	0.76 (0.42, 1.39)

Note: Estimates generated using conditional logistic regression matched on 5-year age groups. Urinary 2-OHE_1 and 16α-OHE_1 metabolites (ng/ml) are standardized to urinary creatinine levels (mg/dl).
1=Common tertile categories based on the distribution of the individual metabolites among all controls in the combined study population.

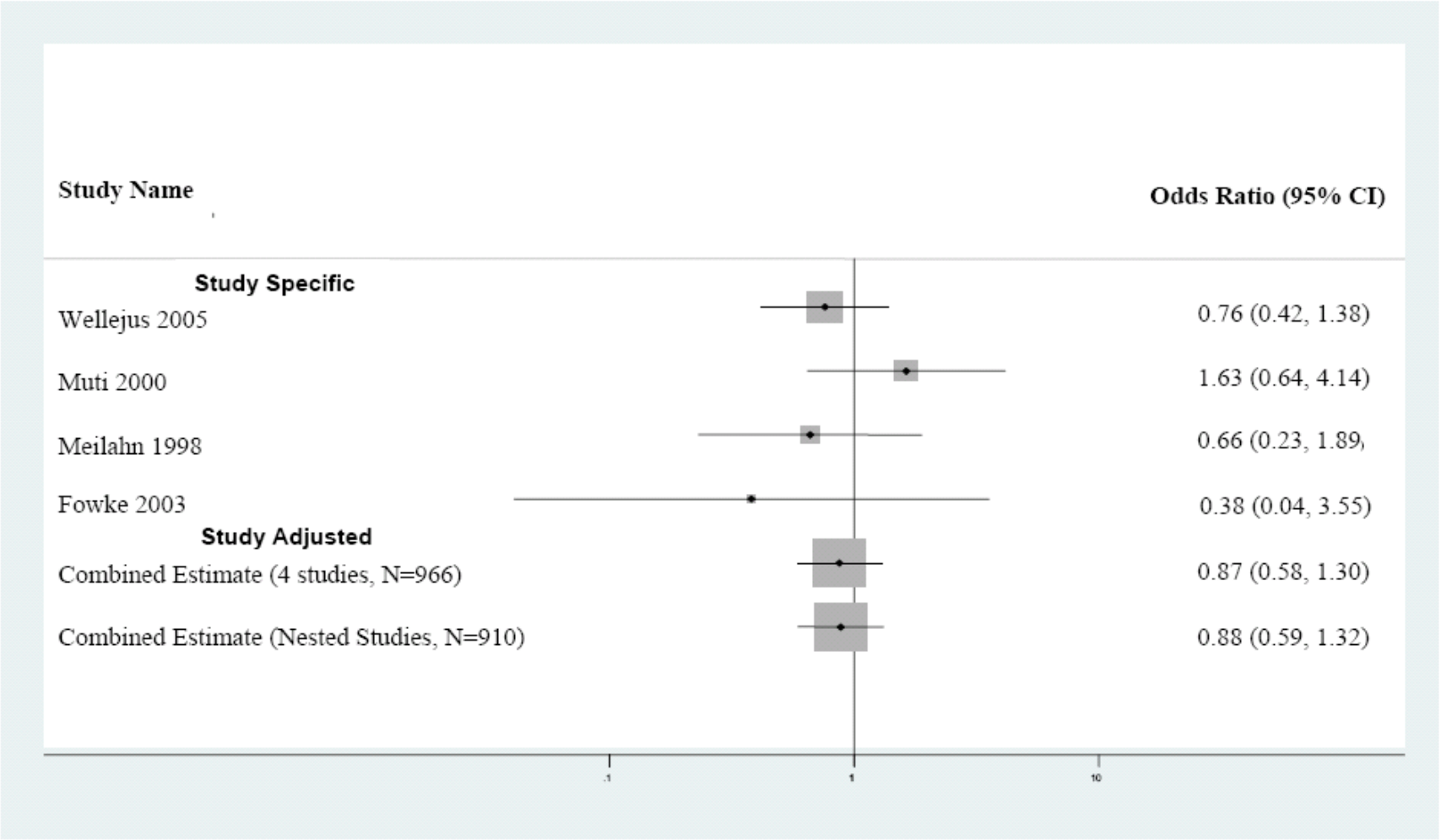

Figure 8 Odds Ratio Estimates: Association of the 2:16α-OHE ratio$_1$ (highest tertile vs. lowest tertile) and Breast Cancer†

†Unadjusted study specific odds ratio and 95% confidence limits shown refer to the highest tertile category of the 2:16α-OHE$_1$ ratio compared to the lowest (reference group): ≥ 2.46 vs. < 1.53. The common tertile categories were determined based on the distribution of the 2:16α-OHE$_1$ ratio among controls from all studies combined. Estimates generated using conditional logistic regression matched on 5 year age strata.
**Includes data from all four studies (N=966).
***Includes data only from the nested case-control studies (Wellejus et al., Meilahn et al. and Muti et al. (N=910)

Table 17 Descriptive Characteristics of the Combined Analysis Study Sample (N=966)

Variable	Cases (n=319)	Controls (n=647)	P-value[1]	P-value[2] (adjusted by study)
Study				
Fowke et al.	19	37		
Meilahn et al.	39	139	-	-
Muti et al.	71	272		
Wellejus et al.	190	199		
Age (years)*	57.7 ± 4.8	57.8 ± 5.1	0.90	0.60
Age at menopause	49.0 ± 4.8	48.6 ± 4.6	0.24	0.16
Ethnicity, N (%)				
Caucasian	300 (94.0)	610 (94.3)	0.88	-
Asian	19 (6.0)	37 (5.7)		
Family History of Breast Cancer, N (%)^	8 (2.5)	42 (6.5)	0.26	0.62
History of Benign Breast Disease, N (%)	70 (21.7)	96 (14.8)	0.012[3]	0.004
Body Mass Index (kg/m_2)*	26.3 ± 4.5 median: 26.0	26.1 ± 4.2 median: 25.0	0.80[4]	0.52
Waist-to-hip ratio **	0.81 ± 0.07	0.82 ± 0.07	0.15	0.15
Smoking				
Never	164 (51.4)	392 (60.6)		
Former	64 (20.1)	104 (16.1)		
Current	76 (23.8)	123 (19.0)		
Unknown	15 (4.7)	28 (4.3)	0.06	0.13
Alcohol Consumption				
Yes	235 (73.7)	349 (53.9)		
No	44 (13.8)	150 (23.2)		
Unknown	40 (12.5)	142 (21.9)	<0.001	0.42
Oral Contraceptive Use				
Never	202 (63.3)	479 (74.0)		
Former	114 (35.7)	161 (24.9)	0.02	0.58
Age at menarche > 13 years, N (%)	155 (48.6)	314 (48.5)	0.10	0.75
Nulliparous, N (%	50 (15.5)	86 (13.1)	0.28	0.35
Age at First Pregnancy*†	25.0 ± 4.7	25.2 ± 4.7	0.56	0.16
Estrogen Receptor, N (%)				
Positive	210 (65.8)	-	-	-
Negative	79 (24.8)			
Unknown	30 (9.4)			
Progesterone Receptor, N (%)				
Positive	66 (20.7)	-	-	-
Negative	64 (20.1)			
Unknown	189 (59.2)			

Note: Percentages may not sum to 100 due to missing values. *Mean ± SD or Median (Range)
**Waist to hip ratio data only available in Wellejus et al., Muti et al. and Fowke et al.
1=t-test p-value for continuous variables or chi-square p-value for categorical variables, unless otherwise noted
2=Wald test p-value adjusted for study, 3=Exact chi-square p-value, 4=Wilcoxon Rank Sum p-value
^=Information on family history of breast cancer missing in Wellejus et al.
†Among parous women

Table 18 Median Estrogen Metabolite Levels^ by Case/Control Status

Estrogen Metabolite	Cases	Controls	P-value†
(N=966)	**(N=319)**	**(N=319)**	
2-OHE_1	2.3 (0.20-97.0)	5.2 (0.14-115.4)	<0.0001
16α-OHE_1	1.6 (0.18-27.3)	2.7 (0.19-38.3)	<0.0001
2:16α-OHE_1	1.7 (0.04–6.9)	2.0 (0.21-13.9)	0.0001
(N=910)*	**(N=300)**	**(N=610)**	
2-OHE_1	2.3 (0.20-97.02)	5.2 (0.14-115.4)	<0.0001
16α-OHE_1	1.4 (0.18-27.3)	2.6 (0.19-38.3)	<0.0001
2:16α-OHE_1	1.8 (0.04-6.9)	2.0 (0.21-13.9)	0.0010

Urinary 2-OHE_1 and 16α-OHE_1 Metabolites (ng/ml) are standardized to urinary creatinine levels (mg/dl).
*Includes data from only the Caucasian nested case-control studies
^Median (Range)
† Wilcoxon rank sum p-value

Table 19 Association between Tertiles of Urinary Estrogen Metabolites (2-OHE_1, 16α-OHE_1 and 2:16α-OHE_1) and Postmenopausal Breast Cancer, (N=966)

Estrogen Metabolites[§]	Case/ control (319/647)	Unadjusted OR (95% CI)	Adjusted* OR (95% CI)	Adjusted** OR (95% CI) (by study only)	Adjusted*** OR (95% CI)
2-OHE_1					
< 2.99	184/213	1.00	1.00	1.00	1.00
2.99 - 8.49	83/221	0.44 (0.32, 0.61)	0.81 (0.51, 1.28)	0.87 (0.55, 1.36)	0.86 (0.54, 1.36)
≥ 8.50	52/213	0.28 (0.20, 0.41)	0.58 (0.34, 1.00)	0.69 (0.40, 1.19)	0.67 (0.38,1.18)
P-values[†]					
p-trend		<0.001	0.05	0.17	0.16
Study (Main Effect)		-	-	< 0.001	0.05
Interaction (2OHE*Study)		-	-	0.27	0.25
16α-OHE_1					
< 1.50	157/214	1.00	1.00	1.00	1.00
1.50 - 4.47	101/218	0.65 (0.48, 0.90)	1.48 (0.97, 2.28)	1.48 (0.97, 2.25)	1.46 (0.94, 2.26)
≥ 4.48	61/215	0.39 (0.28, 0.56)	1.16 (0.68, 1.96)	1.27 (0.76, 2.14)	1.19 (0.70, 2.03)
P-values[†]					
p-trend		<0.001	0.88	0.74	0.99
Study (Main Effect)		-	-	<0.001	0.03
Interaction (16OHE*Study)		-	-	0.62	0.42
2:16α-OHE_1					
< 1.53	126/209	1.00	1.00	1.00	1.00
1.53-2.45	122/223	0.91 (0.66, 1.24)	1.02 (0.73, 1.42)	1.03 (0.74, 1.44)	1.13 (0.80, 1.60)
≥ 2.46	71/215	0.56 (0.39, 0.79)	0.86 (0.59, 1.27)	0.87 (0.58, 1.29)	0.98 (0.66, 1.98)
P-values[†]					
p-trend		0.0009	0.45	0.47	0.92
Study (Main Effect)		-	-	<0.001	0.04
Interaction (2:16OHE*Study)		-	-	0.48	0.34

Note: Urinary 2-OHE_1 and 16α-OHE_1 metabolites (ng/ml) are standardized to urinary creatinine levels (mg/dl). Estimates generated using conditional logistic regression matched on 5 year age strata.
Abbreviations: OR=Odds Ratio, 95% CI=Confidence Intervals
§=Categories based on distribution of 2:16α-OHE_1 among all the controls
*Models adjusted for breast cancer risk factors including: family history of breast cancer (yes/no), history of benign breast disease (yes/no/unknown), age at first menstrual period (< 13/ ≥ 13/unknown), BMI (kg/m^2, continuous), oral contraceptive use (yes/no), smoking (yes/no/unknown) and pregnancy combined variable (never pregnant, age at first pregnancy <20, age at first pregnancy 20-29, age at first pregnancy ≥ 30 years).
**Models adjusted for study only.
***Models adjusted for study in addition to the known breast cancer risk factors mentioned above.
† P-values shown: (1) p-value for test of trend using the midpoint of each metabolite category, (2) Wald X^2 p-value for overall effect of study in models shown above and (3) Wald X^2 p-value for test for interaction between the metabolite and study from the model (not shown) including: the specific metabolite (categorical), study variable (categorical) and the metabolite by study interaction term.

Table 20 Association between Tertiles of Urinary Estrogen Metabolites (2-OHE_1, 16α-OHE_1 and 2:16α-OHE_1) and Postmenopausal Breast Cancer, (N=910, Nested case-control studies)

Estrogen Metabolites§	Case/ control (300/610)	Unadjusted OR (95% CI)	Adjusted* OR (95% CI)	Adjusted** OR (95% CI) (by study only)	Adjusted*** OR (95% CI)
2-OHE_1					
< 2.99	175/207	1.00	1.00	1.00	1.00
2.99 - 8.49	74/196	0.45 (0.32, 0.64)	1.02 (0.62, 1.68)	1.06 (0.65, 1.72)	1.05 (0.64, 1.74)
≥ 8.50	51/207	0.29 (0.20, 0.43)	0.82 (0.45, 1.48)	0.85 (0.47, 1.51)	0.82 (0.42, 1.50)
P-values†					
p-trend		<0.001	0.35	0.39	0.35
Study (Main Effect)		-	-	<0.001	0.47
Interaction (2OHE*Study)		-	-	0.62	0.58
16α-OHE_1					
< 1.50	156/213	1.00	1.00	1.00	1.00
1.50 – 4.47	89/196	0.65 (0.46, 0.90)	1.46 (0.94, 2.26)	1.50 (0.98, 2.30)	1.46 (0.94, 2.25)
≥ 4.48	55/201	0.38 (0.26, 0.55)	1.19 (0.69, 2.06)	1.31 (0.77, 2.23)	1.19 (0.69, 2.06)
P-values†					
p-trend		<0.0001	0.92	0.63	0.90
Study (Main Effect)		-	-	<0.001	0.53
Interaction (16OHE*Study)		-	-	0.41	0.35
2:16α-OHE_1					
< 1.53	112/180	1.00	1.00	1.00	1.00
1.53-2.45	118/220	0.87 (0.63, 1.21)	1.09 (0.76, 1.54)	1.00 (0.71, 1.41)	1.09 (0.77, 1.55)
≥ 2.46	70/210	0.55 (0.38, 0.79)	0.99 (0.66, 1.50)	0.88 (0.59, 1.32)	0.99 (0.65, 1.51)
P-values†					
p-trend		<0.001	0.98	0.52	0.96
Study (Main Effect)		-	-	<0.001	0.52
Interaction (2:16OHE*Study)		-	-	0.49	0.33

Note: Urinary 2-OHE_1 and 16α-OHE_1 Metabolites (ng/ml) are standardized to urinary creatinine levels (mg/dl). Estimates generated using conditional logistic regression matched on 5 year age strata.
Abbreviations: OR=Odds Ratio, 95% CI=Confidence Intervals
§=Categories based on distribution of 2:16α-OHE_1 among all the controls
*Models adjusted for breast cancer risk factors: family history of breast cancer (yes/no), history of benign breast disease (yes/no/unknown), age at first menstrual period (< 13/ ≥ 13/unknown), BMI (kg/m^2 continuous), oral contraceptive use (yes/no), smoking (yes/no/unknown) and pregnancy combined variable (never pregnant, age at first pregnancy <20, age at first pregnant 20-29, age at first pregnant ≥ 30 years).
**Models adjusted for study only.
***Models adjusted for study in addition to the known breast cancer risk factors mentioned above.
† P-values shown: (1) p-value for test of trend using the midpoint of each metabolite category, (2) Wald X^2 p-value for overall effect of study in models shown above and (3) Wald X^2 p-value for test for interaction between the metabolite and study from the model (not shown) including: the specific metabolite (categorical), study variable (categorical) and the metabolite by study interaction term.

Table 21 Relative Risk of Estrogen Receptor Status by Tertiles of Estrogen Metabolites (2-OHE_1, 16α-OHE_1 and 2:16α-OHE_1) as compared to controls, (N=910)*

		ER positive		ER negative		ER unknown	
Estrogen Metabolites	**Controls (N=610)**	**Cases (N=201)**	**RR (95% CI)**	**Cases (N=76)**	**RR (95% CI)**	**Cases (N=23)**	**RR (95% CI)**
2-OHE_1							
< 2.99	207	116	1.00	44	1.00	15	1.00
2.99 - 8.49	196	52	1.01 (0.61, 1.68)	14	0.36 (0.15, 0.85)	8	0.89 (0.27, 2.92)
≥ 8.50	207	33	0.64 (0.36, 1.12)	18	0.45 (0.20, 0.98)	0	n/a
16α-OHE_1							
< 1.50	213	104	1.00	41	1.00	11	1.00
1.50 – 4.47	196	61	1.29 (0.82, 2.01)	16	0.57 (0.28, 1.17)	12	2.17 (0.81, 5.85)
≥ 4.48	201	36	1.04 (0.59, 1.81)	19	0.68 (0.31, 1.51)		
2:16α-OHE_1							
< 1.53	180	75	1.00	29	1.00	8	1.00
1.53-2.45	220	80	0.92 (0.63, 1.35)	30	0.88 (0.51, 1.54)	8	0.88 (0.32, 2.42)
≥ 2.46	210	46	0.67 (0.43, 1.04)	17	0.59 (0.31, 1.12)	7	1.00 (0.34, 2.96)

Note: Urinary 2-OHE_1 and 16α-OHE_1 metabolites (ng/ml) are standardized to urinary creatinine levels (mg/dl).
Odds ratio estimates generated from multinomial models with controls as the comparison group. Estimates were generated using data from the Caucasian studies: Wellejus et al., Meilahn et al. and Muti et al.
*All multinomial models adjusted for study (categorical) and age using the following categories: ≤ 54, 55-59, 60-64, ≥ 65.

6.0 URINARY ESTROGEN METABOLITES AND BREAST CANCER FACTORS AMONG HEALTHY WOMEN: A COMBINED ANALYSIS

To be submitted for publication

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6.1 ABSTRACT

Objective: Most known breast cancer risk factors are thought to influence the carcinogenic process through cumulative estrogen exposure yet little is known about their potential effects on estrogen metabolites. Estrogen metabolism mainly occurs by two mutually exclusive pathways resulting in the formation of the 2-hydroxyestrone (2-OHE_1) and 16α-hydroxyestrone (16α-OHE_1) metabolites. A higher ratio (2:16α-OHE_1) favoring the 2-hydroxylation pathway is hypothesized to reduce breast cancer risk. We assessed the relationship between the urinary estrogen metabolites, 2-OHE_1, 16α-OHE_1, and their ratio 2:16α-OHE_1 with various lifestyle and behavioral risk factors by conducting a combined analysis of previously published studies.

Methods: This combined analysis included primary data from the control populations of five previously published studies yielding a study sample consisting of 544 premenopausal and 720 postmenopausal women. Urinary estrogen metabolite levels were measured using an ELISA assay. Univariate and multivariable linear regression analyses were performed to assess the relationships between each metabolite and the various factors among pre- and postmenopausal women separately. All models were adjusted for age and study. All statistical tests were two sided.

Results: Univariate and multivariable regression analyses among premenopausal women identified significant associations between the 2-OHE_1 and 2:16α-OHE_1 metabolites and body mass index (BMI) ($p \leq 0.05$). No significant associations were observed with 16α-OHE_1 and any of the factors among premenopausal women. Multivariable analyses among postmenopausal women revealed the following significant associations: age at first menstrual period (p-value=0.04), BMI (p=0.09) and alcohol consumption (p=<.01) with 2-OHE_1; age (p=-0.03), former smoking (p=0.04), alcohol consumption (p <.004) and history of benign breast disease

(p=0.01) with 16α-OHE_1; age (p=0.007), age at menopause (p=0.049), and history of benign breast disease (p=0.04) with the 2:16α-OHE_1 ratio.

Conclusions: These findings indicate potential relationships between various breast cancer risk factors and urinary estrogen metabolites.

6.2 INTRODUCTION

Various studies of estrogen metabolites have been conducted among populations of healthy women yet questions still remain with regard to potential modifying factors of estrogen metabolite levels. The normal range of urinary 2-OHE_1, 16α-OHE_1, and 2:16α-OHE_1 among healthy women remains unknown, in part, because of potential variation with ethnicity, menstrual cycle, and menopausal status, as well as individual characteristics (such as age and lifestyle factors) that also may contribute to intra-individual variability. The majority of previous studies have focused on dietary factors or physical activity (Lord 2002), and little is known about the relationship between other characteristics and urinary estrogen metabolite levels. Additionally, few studies have been conducted among healthy populations of premenopausal women and predictors of estrogen metabolites may vary by menopausal status.

Estrogen metabolites have been evaluated in relation to breast cancer due to their potential effects on the carcinogenic process. The 16α-OHE_1 metabolite is thought to have genotoxic and mitogenic effects (Telang 1992, Cavalieri 2006) while the 2-OHE_1 metabolite may act as either a weak estrogen or possibly an anti-estrogen (Schneider 1984). Moreover, the ratio of these specific metabolites (2:16α-OHE_1) has been used in research studies to evaluate the balance between the two estrogen metabolism pathways (Bradlow 1998), and may serve as a

potential biomarker of breast cancer. Many of the known breast cancer risk factors are related to a woman's cumulative lifetime exposure to estrogen; however, the underlying mechanism remains unclear. Understanding the association between known risk factors and the urinary estrogen metabolites 2-OHE_1 and 16α-OHE_1 among healthy populations of women may contribute insight into the underlying mechanism of estrogen exposure and estrogen metabolites, and may also offer an alternative strategy for prevention.

The aim of this analysis is to evaluate relationships between known breast cancer risk factors and urinary estrogen metabolites (2-OHE_1, 16α-OHE_1) and their ratio (2:16α-OHE_1), among healthy women using the control populations from five previously conducted research studies.

6.3 METHODS

6.3.1 Study design

The study population for this combined analysis is comprised of healthy controls who originally participated in studies of urinary estrogen metabolites and breast cancer. Table 6.1 provides a brief description of the participating studies with additional details provided in the original publications (Muti 2000, Meilahn 1998, Fowke 2003, Wellejus 2005, Ursin 1999).

6.3.2 Study Identification and Selection

Original research studies that evaluated the relationship between estrogen metabolites and breast cancer were identified by searching the National Library of Medicine and National Institutes of Health Pubmed database. The search strategy involved the following keyword search terms: (1) estrogen metabolites (n=2901 articles) and (2) estrogen metabolites with additional limits to females and English language (n=974 articles), (3) estrogen metabolites AND breast cancer (n=376 articles). Each of the 376 citations and abstracts were reviewed, and 12 articles were identified as having examined estrogen metabolites in relation to breast cancer. Reference lists from retrieved articles were also reviewed; no additional studies were identified. This search is current as of July 15, 2008.

Articles were considered eligible for potential inclusion in the overall combined analysis if they met the following pre-determined inclusion criteria: (1) an original research study, (2) measurement of 16α-OHE_1, 2-OHE_1, and/or the ratio of these two main estrogen metabolites, (4) urine as the sample source, (5) breast cancer assessed as the main outcome and (6) sample size of at least 50 subjects.

6.3.3 Selection results

Two studies by Ursin et al. were identified, one of which was an earlier pilot study (Ursin et al. 1997). The control population from the more inclusive study by Ursin et al., published in 1999 was included in this analysis. Eight studies were eligible for participation and fulfilled the above eligibility criteria (Fowke 2003, Kabat 1997, Ho 1998, Kabat 2006, Meilahn 1998, Muti 2000, Ursin 1999, Wellejus 2005). Investigators from the eligible studies were contacted and invited to

participate in this combined analysis. Multiple invitations were extended in an attempt to enlist the participation of all eligible studies. Five studies, three nested case-control studies (Muti et al., Meilahn et al., Wellejus et al.) and two case-control studies (Fowke et al., Ursin et al), agreed to participate.

6.3.4 Data Collection and Extraction

The participating studies utilized structured questionnaires to ascertain information on demographics, lifestyle and anthropometric factors, hormone use, reproductive history, and other known breast cancer factors. Study investigators were asked to submit their study-specific datafile along with a copy of the original questionnaire, a description of the variables and study methods, and an electronic datafile. For all premenopausal subjects in the study by Muti et al., urine samples were collected during the luteal phase of the menstrual cycle (20-24th day). Cases and controls in the original study by Meilahn et al. were matched on phase of menstrual cycle (follicular: within 15 days of the start of the last menstrual cycle, or luteal: more than 15 days). Menstrual phase at the time of urine collection was not available in Fowke et al. Definitions of menopausal status were provided by each study. In the study by Muti et al. women without menstrual bleeding for at least 12 months were considered postmenopausal. Wellejus et al. defined women as either known or probable postmenopausal. Known postmenopausal status included women with 1) no hysterectomy and no menstruation in the 12 months prior to study entry, or (2) a bilateral oophorectomy or (3) age at last menstruation lower than age at hysterectomy. Probable postmenopausal status included women with 1) menstruation during the 12 months prior to study entry and current use of HRT, or 2) hysterectomy with unilateral (or unknown) oophorectomy or 3) age at last menstruation same as age at hysterectomy. Women in

the study by Meilahn et al. were classified as postmenopausal if they reported (1) undergoing natural menopause, or (2) a hysterectomy with unilateral oophorectomy. Each datafile was checked for consistency and uniformity with previously published results. Discrepancies identified were resolved with the study investigators. Policies for data publication and authorship were reviewed and accepted by study investigators.

Completeness of Data

All variables requested were coded with a common format for this analysis. Information on waist-to-hip ratio and alcohol consumption was available in three studies (Wellejus, Fowke, Muti) and family history of breast cancer was not available in the Danish study (Wellejus). The datafile by Ursin et al. was missing the following variables: history of benign breast disease, alcohol consumption and smoking status (although current smokers were excluded from parent study). Otherwise, the proportion of missing values for each covariate was low with the exception of those noted above.

6.3.5 Laboratory measurement of main exposure

Spot urine samples were collected for Meilahn et al., Fowke et al, Ursin et al. and Wellejus et al. and an overnight spot urine sample was collected in the study by Muti et al. The use of a spot urine collection has been shown to be a reliable sample source for the measurement of urinary estrogen metabolites (Chen 1999) with no reported significant differences between spot urine samples or multiple urine collections over a 24 hour period (Westerlind 1999). Additionally, the potential affect of long term sample storage on metabolite levels was assessed in the original studies by Meilahn et al and Wellejus et al.; no effect on metabolite levels was reported. All participating studies measured urinary 2-OHE and 16α-OHE using a commercially available

competitive, solid-phase enzyme immunoassay kit (ESTRAMET) produced by Immunacare Corp (Bethlehem, PA, USA). With this assay kit, the binding of the monoclonal antibodies to estrogen metabolites (antigen) is captured directly on the solid phase. The urinary forms of 2-OHE and 16-OHE are found as glucuronide conjugates and require the removal of sugars before the monoclonal antibodies in the assay kit can detect the urinary metabolites. Studies by Fowke et al., Muti et al, and Wellejus et al. utilized the more recent ELISA kit (Bradlow 1998) whereas Meilahn et al. used the original assay developed by Klug (Klug 1994). The sensitivity of the modified kit is approximately 0.625 ng/ml for the 16α-OHE and 2-OHE metabolites (Bradlow 1998, Falk 2000). The reported mean within assay variability of this kit is approximately 4% while the mean between assay variability is about 10%. The components of the original kit are similar to those of the modified kit, with the exception of modifications that allow for an increased sensitivity level among postmenopausal women (Bradlow 1998, Falk 2000).

6.3.6 Statistical analysis

The 2-OHE_1 and 16α –OHE_1 metabolites (ng/ml) were divided by the urinary creatinine concentration (mg/dl) as a means of standardizing the metabolite values by total volume of urine. The 2:16α-OHE_1 variable is based on the ratio of these two individual estrogen metabolites. 2-OHE_1 and 16α-OHE_1 metabolite data was available on 98.7% of the study sample. The estrogen metabolite exposures (2-OHE_1, 16α-OHE_1 and the 2:16α-OHE_1 ratio) were non-normally distributed.

Additional exclusion criteria were implemented for the present analysis. Current users of oral contraceptives from Fowke et al. were excluded from this analysis (n=9) to be consistent with the original exclusion criteria from the other studies. Additionally, the analysis among

postmenopausal women was limited to women not currently on hormone replacement therapy (HRT) at the time of entry into the original studies due to noted differences in the 2:16α-OHE ratio by postmenopausal HRT status (Armamento-Villareal 2004, Wellejus 2005). Participants in the study by Wellejus et al. who were currently on HRT at cohort entry were excluded from these analyses (n=234 controls). All other participating studies excluded women currently on HRT as part of their exclusion criteria. Latina participants in the study by Ursin et al. were excluded due to the small number of women in this group (n=3). Lastly, information on estrogen metabolites was missing for 10 postmenopausal and 4 premenopausal women. After applying these additional exclusions, the present combined analysis includes 544 premenopausal and 720 postmenopausal women.

Linear regression analyses were used to assess the relationships between each of the metabolites (2-OHE_1, 16α-OHE_1 and 2:16α-$OHE_{1)}$ and the individual breast cancer factors in separate models adjusted for age and study. A natural log transformation was applied to the values of the 2-OHE_1, 16α-OHE_1 and the 2:16α-OHE_1 metabolites to improve normality. The following covariates were assessed: age (years), age at menopause (years), family history of breast cancer in a first degree relative (yes/no), history of benign breast disease (yes/no), body mass index (kg/m^2), waist-to-hip ratio, smoking (never/former/current/unknown), alcohol consumption (ever/never/unknown), age at first pregnancy and age at menarche (<13 years/≥ 13 years/unknown). These factors were chosen based on the characteristics of the Gail Model (Gail 1989) and due to their established associations with breast cancer. Separate analyses were performed by menopausal status. Due to noted differences in estrogen metabolites between Caucasian and Asian populations (Aldercruetz 1994, Sowers 2006), analyses were repeated among Caucasian women only. Additional adjustment for menstrual phase at urine collection

(follicular ≤ 14 days/luteal ≥ 15 days/ unknown) did not alter the results, and thus this variable was not included in the base models.

Variables with a p-value < 0.15 in the univariate analyses were considered for potential inclusion in the multivariable models. All significant variables from the univariate analyses were evaluated for inclusion in the final multivariable models using a backwards selection strategy. Variables with a p-value ≤ 0.10 were retained for inclusion in the final multivariable models. Normality of residuals was assessed for all final models using quantile normal plots of the residuals. Potentially influential observations were identified using Cook's distance.

All analyses were performed using SAS (Version 9.1, Cary, NC, USA) and STATA 10. Tests of significance were two-sided. Data were analyzed in Pittsburgh, PA and these datafiles did not include personal identifiers. Each study participating in this present analysis was approved by the Institutional Review Board or appropriate ethical committee at the respective institution, and participants provided informed consent.

6.4 RESULTS

The total sample of 1264 women included 544 premenopausal and 720 postmenopausal women, of which 13.2% and 5.1% of pre- and postmenopausal women were Asian, respectively. The mean age was approximately 42 years (range 34-56) for premenopausal women and 58 years (range 42-79) for postmenopausal women. Descriptive characteristics of the combined study sample by menopausal status are summarized in Table 23.

Significant differences in median metabolite levels by study were observed with each metabolite among pre- and postmenopausal women ($p<0.0001$) (Table 24). Overall, estrogen

metabolite levels were significantly lower among the Shanghai population as compared to the Caucasian populations.

Among premenopausal women, regression analyses adjusted for age and study indicated a significant inverse association between BMI and log 2-OHE_1 (p-value=0.026) (Table 25). In a subset of the premenopausal population with data available on waist to hip ratio (N=358), a significant inverse association was also observed with the waist to hip ratio (p=0.018) after adjustment for age and study (Table 25). Both BMI and WHR were also significantly associated with a decrease in log 2:16α-OHE_1 (p=0.0026 and p=0.027, respectively). In models adjusted for age, study and both BMI and WHR, WHR was significantly associated with a decrease in log 2-OHE_1 but not with 2:16α-OHE_1 (p-value=0.21). Age was positively associated with 2:16α-OHE_1 (p=0.05) but not with either of the individual metabolites (p > 0.10). No other factors were associated with 2-OHE_1 and 16α-OHE_1. Additionally, no significant associations were observed between any of the characteristics and log 16α-OHE_1 among premenopausal women.

Analyses restricted to Caucasian premenopausal women yielded results similar to those presented in Table 25. However, when analyses were performed in the Shanghai population, BMI was not significantly associated with log 2-OHE_1 among Asian premenopausal women (p=0.90) or with log 2:16α-OHE_1 (p=0.75) (data not shown). The mean BMI was significantly higher among Caucasian premenopausal women (mean ± SD: 24.7 ± 4.5) as compared to Asian premenopausal women (mean ± SD: 22.0 ± 2.9) (data not shown).

Separate univariate analyses adjusted for age and study among postmenopausal women revealed modest significant associations between log 2-OHE_1 and age at first menstrual period (p=0.069), age at first pregnancy among parous women (p=0.049), BMI (p=0.095), current smokers (p=0.085) and alcohol use (p=0.0004). History of benign breast disease (p=0.023),

former smoking status (p=0.09) and alcohol use (p=0.005) were significantly associated with 16α-OHE_1. Characteristics significantly associated with the log 2:16α-OHE in univariate regression models adjusted for age and study included: age at menopause (p=0.039), age at first pregnancy among parous women (p=0.0046), waist to hip ratio (p=0.042) and history of benign breast disease (p=0.048) (data not shown).

Table 26 summarizes the final multivariable models for 2-OHE_1, 16α-OHE_1 and 2:16α-OHE_1 among postmenopausal women. Age at first menstrual period (p=0.04) and BMI (p=0.09) remained inversely associated with log 2-OHE_1 while alcohol use remained positively associated with log 2-OHE_1 (p<.0001) in a multivariable model adjusted for age and study. Age at first pregnancy (combination variable) and smoking status did not remain statistically significant (p-value >.10) and were not included in the final multivariable model. In the multivariable 16α-OHE_1 model, independent associations were observed with age (p=0.03), former smoking status (p=0.04), alcohol use (p=0.004) and history of benign breast disease (p=0.01). Results from the multivariable analyses of log 2:16α-OHE_1 are also summarized in Table 26; age at menopause was the only characteristic that remained statistically significant at the p<.05 level. The multivariable analysis for 2:16α-OHE_1 was repeated among the subset of women with available WHR data. The overall findings were similar to those presented in Table 26 and WHR was not statistically significant in the multivariable models (data not shown).

Postmenopausal analyses were repeated among Caucasian women (excluding the Shanghai population) and the results were similar (data not shown) to those presented for the total combined population. Additionally, no significant associations were observed when the analyses were performed among only the Asian population (37 premenopausal and 73 postmenopausal women) (data not shown).

6.5 DISCUSSION

The primary objective of this analysis was to assess the relationship between various lifestyle and behavioral characteristics and urinary 2-OHE_1, 16α-OHE_1 and 2:16α-OHE_1 metabolites among healthy women. The results of this study suggest potential relationships between select factors and the 2-OHE_1, 16α-OHE_1 and 2:16α-OHE_1 metabolites and furthermore, that the particular factors associated with estrogen metabolite levels may vary among pre- and postmenopausal women.

Previous studies have evaluated the urinary estrogen metabolites, 2-hydroxyestrone and 16α-hydroxyestrone, in relation to diet and lifestyle factors, racial differences, hormone therapy, genetic polymorphisms and family history. The majority of studies have focused on dietary factors such as flaxseed consumption (Haggans 2000, McCann 2007, Sowers 2006), Brassica vegetable consumption (Fowke 2000), macronutrient intake (Fowke 2001), soya diet (Lu 2000), indole-3-carbinol supplementation (Michnovicz 1997) or physical activity (Pasagian-Macaulay 1996, Atkinson 2004, Bentz 2005, Matthews 2004). Some studies have assessed potential racial differences in estrogen metabolites, mainly between Caucasian and Asian populations (Aldercruetz 1994, Ursin 2001, Jernstrom 2003, Falk 2005, Sowers 2006) or between Caucasian and African American women (Sowers 2006, Taioli 1996), while others have evaluated potential variations in estrogen metabolites by family history of breast cancer (Ursin 2002) or postmenopausal hormone use (Alvarez-Vasquez 2002, Mueck 2001). However, questions still remain with regard to which breast cancer related factors may modify estrogen metabolite levels among both pre- and postmenopausal women as well as the corresponding magnitude.

Few studies have assessed the relationship between body composition and estrogen metabolites among healthy populations (Schneider 1983, Fishman 1975, Matthews 2004, Sowers

2006, Pasagian-Macaulay 1996) with results from most studies suggesting that adiposity, measured by higher BMI levels, is associated with a decrease in levels of 2-OHE_1 and subsequently the 2:16α-OHE ratio (Schneider 1983, Fishman 1975). Alternatively, a previous study among premenopausal women observed a modest increase in the 2:1 ratio with increases in weight, BMI and WHR (Pasagian-Macaulay 1996). Among premenopausal women in our study, we observed an inverse association between BMI and 2-OHE_1 and 2:16α-OHE_1 which is consistent with findings from some of the previous studies of BMI and/or weight (Sowers 2006). However, our finding is inconsistent with the observed protective effect of obesity on premenopausal breast cancer risk (Ursin 1995, Van den Brandt 2000). Considering the hypothesized protective effect of a higher 2:16α-OHE_1 ratio on breast cancer risk, one would expect increased BMI to be associated with increased 2-OHE_1 levels among premenopausal women, if the underlying mechanism is estrogen related.

A limited number of studies have evaluated the association between estrogen metabolites and smoking status (Michnovicz 1986, Sowers 2006, Jernstrom 2003) or alcohol consumption (Sowers 2006). In a study of premenopausal women, Jernstrom et al. reported no difference in estrogen metabolite levels by smoking status (Jernstrom 2003) while lower 2-OHE_1 and 16α-OHE_1 levels were observed among nonsmokers compared to smokers in a multiethnic population of premenopausal women (Sowers 2006). This is in contrast to our findings from our analyses of postmenopausal women in which we observed a reduction in 16α-OHE_1 among former smokers compared to nonsmokers; however, the difference between our results and those of Jernstrom et al. or Sowers et al. may in part be due to the difference in menopausal status of the study populations. Alcohol use was positively associated with both 16α-OHE_1 and 2-OHE_1 among postmenopausal women. Alcohol is metabolized in the liver and increased alcohol consumption

may lead to elevated estrogen levels (Gill 2000). Among premenopausal women, Sowers et al. (2006) reported a modest association between wine consumption and 2-OHE_1. However, higher mean levels of 2-OHE_1 were observed in the middle category and their findings do not support a dose response relationship (Sowers 2006). It remains unclear whether smoking status and/or alcohol consumption is related to estrogen metabolite levels and whether this relationship varies by menopausal status.

The overall prevalence of family history among both premenopausal and postmenopausal women in this study was fairly low and furthermore, family history was not significantly associated with either of the urinary estrogen metabolites or their ratio among pre- or postmenopausal women. To our knowledge, only one other study has evaluated estrogen metabolite levels by family history of breast cancer. Ursin et al. reported no difference in the 2:16α-OHE_1 ratio between premenopausal women with a positive family history of breast cancer (n=70) compared to women with a negative family history (n=27) (Ursin 2002). Whether or not urinary estrogen metabolites may be useful in predicting breast cancer risk among high risk women remains unclear. Further investigations among larger samples of high risk women with estrogen metabolites measured over time may better address this particular research question.

Interestingly, history of benign breast disease was negatively associated with 2:16α-OHE_1 and positively associated with 16α-OHE_1. To our knowledge, previous studies have not evaluated estrogen metabolite levels by history of benign breast disease. Explanations for this potential association are unknown as this has not been previously evaluated but may involve mechanisms related to cell proliferation in the breast tissue. The positive association between 16α-OHE_1 and history of benign breast disease may be biologically plausible given that results

from cell studies have suggested that 16α-OHE_1 may be associated with increased proliferation (Telang 2002). This finding warrants further investigation.

In this combined analysis, we were able to assess various factors in relation to urinary estrogen metabolites among healthy populations of both pre- and postmenopausal women. Primary level data from previously published studies of urinary estrogen metabolites and breast cancer were obtained and allowed us to not only create a larger study sample of healthy women and to create common categories of exposure, but this approach also provided information on both estrogen metabolite levels and a range of lifestyle and behavioral factors. This study also highlights the variability in estrogen metabolite levels in different populations of healthy women.

There are some limitations that should be considered when interpreting the results from this study. Estrogen metabolites may potentially be modified through dietary and lifestyle factors, such as physical activity (Matthews 2004, Bentz 2005, Campbell 2007), brassica foods (Fowke 2000) or caffeine consumption (Sowers 2006, Jernstrom), which have been shown to alter 2:16α-OHE levels. Information on these factors was not available in this combined study population. Additionally, Matthews et al. reported a significant interaction between BMI and physical activity (Matthews 2004) among North American and Chinese women. This same Shanghai population (Fowke 2001) was included in this combined analysis. Physical activity data was not requested from the Shanghai Study as this information was not available in the other participating studies. Thus, we were unable to account for possible interactions with levels of physical activity and this limitation should be considered when interpreting the observed significant association with BMI among premenopausal women. Additionally, we could not assess the potential influence of genetic polymorphisms which may alter the favored estrogen metabolism pathway. Furthermore, many of the categorical variables were based on crude

variable definitions. It is possible that additional details (ie. type of alcohol and duration, changes in body weight) would be more informative and would help clarify relationships between the studied factors and urinary estrogen metabolites.

In summary, this study furthers our understanding of the relationships between urinary estrogen metabolites and a range of factors among healthy women. Results of the multivariable analyses revealed different significant factors for premenopausal women than postmenopausal women suggesting that predictors of estrogen metabolites may vary by menopausal status. Future studies may consider longitudinal assessment of estrogen metabolite levels as well as more detailed information on potential predictors among both pre- and postmenopausal women.

6.6 TABLES AND FIGURES

Table 22 Characteristics of Participating Studies: Combined Analysis of Estrogen Metabolites

Study	Premenopausal Controls	Postmenopausal Controls	Study Population & Location	Age Range	Control Selection	Original Matching Criteria
Nested Case-Control						
Meilahn et al. (1998)	184	139	Guernsey III cohort, Guernsey Island, 1977-85	34-79 years	Randomly selected from cohort (those alive and free of breast cancer at end of cohort & with available urine)	Age ± 2 yrs, baseline exam ±1yr, menstrual phase 1:3 matching
Muti et al. (2000)	288	272	Hormones and Diet Etiology of Breast Cancer Study (ORDET), 1987-1992 Varese Province, Northern Italy	35-69 years	Randomly selected from cohort among those alive at time of diagnosis of matched case	Age ±5 years, time of blood draw, recruitment center , recruitment date ± 180 days 1:4 matching
Wellejus et al. (2005)	n/a	434 HRT+: 234 HRT-: 200	Greater Copenhagen or Aarhus areas 1993-2000	50 -65 years	Cancer free at exact age at diagnosis of case	Age at entry (6 month intervals), HRT status postmenopausal status (known/probably) 1:1 matching
Case-Control						
Ursin et al. (1999)	n/a	73	Los Angeles, Ca USA	53-70 years	Population based Neighborhood controls from the same area as the cases	Age ± 3 years, ethnicity, & neighborhood 1:1 matching *Matching not retained
Fowke et al. (2003)	72	37	Shanghai, China	34-63 years	Randomly selected from Shanghai population registry	Age ± 3 years and date of sample collection ± 30 days 1:1 matching

Table 23 Descriptive Characteristics of the Combined Study Sample by Menopausal Status, (N=1264)

Variable	Premenopausal (N=544)	Postmenopausal (N=720)
Study		
Fowke et al.	72	37
Ursin et al.	-	73
Meilahn et al.	184	139
Muti et al.	288	272
Wellejus et al.	-	199
Age (years)*	42.9 ± 4.9	58.2 ± 5.2
Age at menopause*	-	48.5 ± 5.1
Ethnicity, N (%)		
Caucasian	472 (86.8)	683 (94.9)
Asian	72 (13.2)	37 (5.1)
Family History of Breast Cancer, N (%)^	25 (4.6)	53 (10.2)
History of Benign Breast Disease, N (%)	121 (22.2)	96 (13.4)
Body Mass Index (kg/m_2)*	24.4 ± 4.4	25.9 ± 4.2
Waist-to-hip ratio *	0.78 ± 0.06	0.82 ± 0.07
Smoking		
Never	322 (59.4)	392 (54.4)
Former	72 (14.5)	104 (14.4)
Current	108 (19.9)	123 (17.1)
Unknown	33 (6.1)	101 (14.1)
Alcohol Consumption		
No	185 (34.0)	150 (23.4)
Yes	171 (31.4)	349 (54.5)
Unknown	188 (34.6)	142 (22.2)
Age at menarche > 13 years, N (%)	219 (40.3)	513 (71.3)
Nulliparous, N (%)	52 (9.6)	93 (12.9)
Age at First Pregnancy*†	25.3 ± 4.3	25.2 ± 4.7

Percentages may not sum to 100 due to missing values.
*Mean ± SD
Note: Waist to hip ratio data only available in Wellejus et al., Muti et al. and Fowke et al. History of alcohol consumption and smoking missing in the study by Ursin et al.
^=Information on family history of breast cancer missing in Wellejus et al.
†Among parous women

Table 24 Median Estrogen Metabolite Levels by Study and Menopausal Status

			PREMENOPAUSAL WOMEN (N=544)		
			Estrogen Metabolites*		
Study	**N^**		**2-OHE (ng/ml)**	**16α-OHE (ng/ml)**	**2:16α-OHE**
Muti et al.	288	Median 25th,75th Range	40.9 22.5, 74.7 2.5 - 351.8	16.9 8.9, 29.8 0.96 - 181.8	2.5 1.94, 3.2 0.42 - 7.6
Meilahn et al.	184	Median 25th,75th Range	17.5 11.4, 28.8 2.0 - 173.0	8.5 5.2, 14.0 1.2 - 91.0	2.1 1.5, 2.8 0.28 - 6.1
Fowke et al.	72	Median 25th,75th Range	6.9 4.4, 13.3 1.9 - 31.3	8.6 5.0, 13.3 1.6 - 40.2	0.85 0.59, 1.4 0.29 - 3.9
p-value†			<0.0001	<0.0001	<0.0001
			POSTMENOPAUSAL WOMEN (N=720)		
			Estrogen Metabolites*		
Study	**N^**		**2-OHE (ng/ml)**	**16α-OHE (ng/ml)**	**2:16α-OHE**
Wellejus et al.					
Overall	433	Median 25th,75th Range	2.8 1.4, 18.6 0.1 - 178.9	1.6 0.9, 8.7 0.2 - 118.4	1.8 1.2, 2.4 0.3 - 9.1
HRT +	233	Median 25th,75th Range	17.1 4.1, 29.9 0.3 – 178.9	7.8 2.8, 15.5 0.3 - 118.4	1.9 1.2, 2.8 0.3 - 9.1
HRT -	200	Median 25th,75th Range	1.5 1.1, 2.3 0.1 – 8.5	0.9 0.7, 1.3 0.2 - 8.2	1.6 1.2, 2.1 0.3 - 6.8
Muti et al.	282	Median 25th,75th Range	10.3 5.3, 17.8 0.7 - 115.4	3.9 2.1, 6.9 0.19 - 38.3	2.6 1.9, 3.3 0.38 - 13.9
Meilahn et al.	139	Median 25th,75th Range	7.1 5.0, 10.5 1.8 - 42.6	4.5 3.1, 6.7 0.9 - 15.1	1.7 1.2, 2.4 0.2 - 11.3
Fowke et al.	37	Median 25th,75th Range	3.7 3.2, 5.5 1.03 - 23.1	3.6 2.9, 4.7 1.5 - 30.2	0.95 0.7, 1.5 0.4 - 7.3
Ursin et al.	73	Median 25th,75th Range	6.5 4.4, 8.3 2.0 - 24.5	3.7 2.6, 4.7 0.90 - 10.3	1.8 1.4, 2.3 0.71 - 9.4
p-value†			<0.0001	<0.0001	<0.0001

*Urinary 2-OHE and 16α-OHE Metabolites (ng/ml) are standardized to urinary creatinine levels (mg/dl).
†= Wilcoxon rank sum p-value

Table 25 Results from separate linear regression models estimating the relationship between log (2-OHE_1), log(16α-OHE_1) and log(2:16α-OHE_1) and BMI among premenopausal women. Each model includes terms for age and separate intercepts for all studies

Terms in model	**log (2-OHE_1)**		**log (16α-OHE_1)**		**log (2:16α-OHE_1)**	
(N=540)	B (SE)	p-value	B (SE)	p-value	B (SE)	p-value
Intercept	4.05 (0.37)	<0.001	3.30 (0.36)	<0.001	0.75 (0.21)	< 0.001
Age	0.0011 (0.0078)	0.89	-0.0097 (0.0074)	0.19	0.011 (0.0044)	0.02
BMI	-0.018 (0.0081)	0.03	-0.0041 (0.0078)	0.60	-0.014 (0.0047)	0.003
Meilahn Study	-0.79 (0.082)	<0.001	-0.62 (0.079)	<0.001	-0.17 (0.047)	<0.001
Fowke Study	-1.67 (0.11)	<0.001	-0.68 (0.11)	<0.001	-1.00 (0.063)	<0.001
Terms in model	**log (2-OHE_1)**		**log (16α-OHE_1)**		**log (2:16α-OHE_1)**	
(N=356)*	B (SE)	p-value			B (SE)	p-value
Intercept	4.5 (0.46)	<0.001	3.95 (0.44)	<.0001	0.58 (0.25)	0.02
Age	-0.012 (0.0095)	0.23	-0.027 (0.009)	0.004	0.015 (0.0051)	0.003
BMI	-0.015 (0.010)	0.14	0.000041 (0.0096)	0.99	-0.015 (0.0054)	0.005
Fowke Study	-1.69 (0.12)	<0.001	-0.66 (0.11)	<.0001	-1.00 (0.061)	<.001
Intercept	5.51 (0.68)	<0.001	4.59 (0.65)	<0.001	0.92 (0.36)	0.01
Age	-0.0094 (0.0095)	0.32	-0.025 (0.009)	0.006	0.016 (0.0051)	0.002
BMI	-0.0072 (0.011)	0.51	0.0053 (0.01)	0.61	-0.012 (0.0058)	0.03
WHR	-1.62 (0.83)	0.05	-1.07 (0.79)	0.18	0.56 (0.44)	0.21
Fowke Study	-1.66 (0.12)	<0.001	-0.68(0.11)	<0.001	-0.99 (0.062)	<.001

Note: Metabolite outcomes are on the natural log scale. Urinary 2-OHE (ng/ml) standardized to urinary creatinine levels (mg/dl).

*WHR data available in Muti et al. and Fowke et al., but not in Meilahn et al.

Table 26 Results from separate multivariable linear regression models estimating the relationship between 2-OHE_1, 16α-OHE_1 and 2:16α-OHE_1 and various factors among postmenopausal women. Each model includes terms for age and separate intercepts for all five studies (N=720)

	log (2-OHE_1)	
Terms in model	B (SE)	p-value
Intercept	2.91 (0.37)	<0.001
Age	-0.0051 (0.0055)	0.36
First menstrual period	-0.035 (0.017)	0.04
BMI	-0.012 (0.0068)	0.09
Alcohol Yes	0.29 (0.086)	<0.001
Unknown	-0.39 (0.42)	0.35
Wellejus Study	-1.98 (0.076)	<.0001
Meilahn Study	0.23 (0.42)	0.58
Fowke Study	-0.70 (0.14)	<0.001
Ursin Study	0.087 (0.42)	0.84
	log (16α-OHE_1)	
Terms in model	B (SE)	p-value
Intercept	1.82 (0.31)	<0.001
Age	-0.011 (0.0052)	0.03
Former Smoker	-0.16 (0.079)	0.04
Current Smoker	0.092 (0.076)	0.22
Unknown	0.22 (0.15)	0.14
Alcohol Yes	0.24 (0.082)	0.004
Unknown	-0.35 (0.40)	0.38
History of benign breast disease		
Yes	0.20 (0.078)	0.01
Unknown	-0.19 (0.26)	0.45
Wellejus Study	-1.47 (0.076)	<0.001
Meilahn Study	0.59 (0.40)	0.14
Fowke Study	0.14 (0.13)	0.27
Ursin Study	0.44 (0.50)	0.38
	log (2:16α-OHE_1)	
	B (SE)	p-value
Intercept	0.90 (0.28)	0.001
Age	0.0074 (0.0041)	0.07
Age at menopause	-0.008 (0.004)	0.05
History of benign breast disease		
Yes	-0.10 (0.06)	0.09
Unknown	0.07 (0.20)	0.72
Wellejus Study	-0.47 (0.05)	<0.001
Meilahn Study	-0.43 (0.056)	<0.001
Fowke Study	-0.84 (0.094)	<0.001
Ursin Study	-0.47 (0.21)	0.03

Note: Metabolite outcomes are on the natural log scale. Urinary 2-OHE_1 and 16α-OHE_1 (ng/ml) are standardized to urinary creatinine levels (mg/dl).

7.0 GENERAL DISCUSSION

In 2008, an estimated 182,460 new invasive breast cancer cases were diagnosed among women in the United States (ACS 2008). While progress in early detection and breast cancer treatment has improved breast cancer survival, the incidence of this disease remains elevated. Advances in biomarker research have been made in relation to markers of breast cancer progression and treatment; however, biomarkers of breast cancer risk have yet to be identified. Prevention strategies have included the use of improved risk models among high risk women as well as the recommendation of lifestyle modifications for all women, such as reducing alcohol consumption, increasing exercise, and maintaining a healthy weight (Cummings 2009). However, despite these efforts, progress in breast cancer prevention has been limited in part because the underlying mechanisms leading to the development of breast cancer remain to be elucidated.

A large body of evidence implicates estrogen in the etiology of breast cancer yet various questions remain regarding the role of estrogen metabolites in carcinogenesis. Estrogen metabolites, and in particular 2:16α-OHE_1, have been evaluated in relation to breast cancer risk among pre and postmenopausal women with the anticipation that this ratio of metabolites may serve as a potential predictor of breast cancer risk. However, the findings from studies which evaluated the association between estrogen metabolites and breast cancer have been inconclusive. Among premenopausal women in particular, results from previous studies have suggested a protective association with higher 2:16α-OHE_1 levels but these findings have mostly

been based on studies of relatively small sample sizes. The intent of this combined analysis was to create a larger sample size of pre- and postmenopausal women in an effort to clarify the association between estrogen metabolites (2-OHE_1, 16α-OHE_1, and 2: 16α-OHE_1) and pre-and postmenopausal breast cancer, to identify potential predictors of estrogen metabolites among healthy women, and to increase our overall understanding of urinary 2-OHE_1 and 16α-OHE_1 metabolites.

7.1 ARTICLE 1: ESTROGEN METABOLITES AND BREAST CANCER AMONG PREMENOPAUSAL WOMEN

We utilized a combined analysis approach to evaluate the association between 2-OHE_1, 16α-OHE_1 and 2:16α-OHE_1 and breast cancer among 731 premenopausal women (183 cases/548 controls). Primary data from previously published studies were re-evaluated in this larger combined sample using common estrogen metabolite cutpoints. Although estrogen metabolites have been investigated in relation to breast cancer, only six studies have assessed this relationship among premenopausal studies (Fowke 2003, Kabat 1997, Kabat 2006, Ho 1998, Meilahn 1998, Muti 2000) with sample sizes ranging from 19 to 105 cases and 12 to 264 controls. Thus, we investigated whether higher levels of 2:16α-OHE_1 would be associated with a reduction in breast cancer risk among premenopausal women using a larger combined sample of premenopausal women.

The unadjusted median 2:16α-OHE_1 values were significantly lower among cases (1.8) as compared to controls (2.2), suggesting a potential difference by case/control status. However, the apparent reduction in breast cancer risk in unadjusted models ($OR_{\text{Tertile 3 vs. Tertile 1}}$=0.51, 95% CI:

0.33, 0.78) was attenuated and no longer statistically significant when models included adjustment for study ($OR_{\text{Tertile 3 vs. Tertile 1}}$=0.81, 95% CI: 0.49, 1.32). The original studies by Meilahn et al. and Muti et al. suggested protective effects of estrogen metabolites but did not reach statistical significance. The results of this combined analysis may be due to insufficient power to detect a small effect or may reflect the true association between estrogen metabolites and breast cancer among premenopausal women. In unadjusted analyses, we had sufficient approximate post hoc power to detect an OR=0.6; however, we did not have sufficient power to detect a smaller effect size (OR=0.80) in study adjusted models. Although results from study adjusted models did not reach statistical significance, the direction and magnitude of effect were suggestive of a modest reduction in breast cancer risk with higher levels of 2:16α-OHE_1. Furthermore, Kabat et al. (2006) reported a significant reduction in breast cancer risk with higher 2:16α-OHE_1 levels in a case-control study which included 105 premenopausal invasive cases (data not included in this combined analysis), which is smaller than our combined sample of premenopausal cases (n=183).

There was no statistical evidence of interaction by study; however, study appears to be an important confounding factor. This suggests that there are potential differences across the studies which may be important to consider such as differences in study design, laboratory variability and the distribution of the estrogen metabolites among various ethnic populations, in particular Caucasian and Asian women. Although all studies used a commercially available kit to measure the estrogen metabolites, the possibility of inter- and intra-laboratory variability cannot be dismissed. We adjusted the conditional logistic regression models by study to account for these potential differences as well as other unknown sources of variability.

Although adjustment for various covariates did not alter the direction, magnitude or statistical significance of the observed effects, the estimates were attenuated and no longer statistically significant after adjustment for study. This was likely driven by differences in the Fowke et al. study. The indicator variable for the Fowke et al. study (compared to Muti et al.) was statistically significant, and is essentially a proxy for race and study design. The study by Fowke et al. utilized a case-control design and was conducted among an Asian population, whereas the studies by Muti et al. and Meilahn et al. were nested case-control studies conducted within Caucasian populations. The exposure measure differs within these two study designs in that estrogen metabolites were measured either before or after breast cancer diagnosis depending on the particular study design, and it remains unclear whether estrogen metabolite levels are affected by disease status. Additionally, metabolite levels have been shown to vary by race (Aldercreutz 1994, Jernstrom 2003, Ursin 2001, Taioli 1996, Matthews 2004); however, research in this area is relatively limited, particularly among premenopausal women. The observed differences in the overall distribution of estrogen metabolites among Asian women compared to Caucasian women may be explained by differences in diet, lifestyle factors as well as genetic polymorphisms, all of which have been suggested to differ by race and to alter estrogen metabolite levels.

We attempted to evaluate the relationship between estrogen metabolites and breast cancer by various factors (including hormone receptor status, BMI, and smoking status); however, no significant associations were detected. Although our combined sample size was larger than previous individual studies, our interpretation of these additional analyses was limited by the small number of cases and controls in the various subgroups. Larger studies of premenopausal

women, as well as studies with more complete ER and PR information, may better address this research question.

Adjustment for menstrual phase at the time of urine collection (luteal vs. follicular) did not alter the results in this combined analysis. Whether metabolite levels vary during different phases of the menstrual cycle is not fully understood. The limited number of studies which have evaluated the association between estrogen metabolites and breast cancer among premenopausal women may partly be due to the measurement issues surrounding the timing of urine collection during the menstrual cycle.

Although we did not observe a significant association in this study, our results suggest a reduction in breast cancer risk with higher levels of the 2:16 ratio among premenopausal women. Furthermore, this combined analysis highlights the limited research that has evaluated the association between estrogen metabolites and breast cancer among premenopausal women and also emphasizes the variability in estrogen metabolite levels across different populations.

7.2 ARTICLE 2: ESTROGEN METABOLITES AND BREAST CANCER AMONG POSTMENOPAUSAL WOMEN

Estrogen metabolites were first considered as a potential component in breast carcinogenesis in the early 1970's, yet since then, only 12 studies have been conducted in this area, including 5 case-control (Fowke 2003, Ursin 1999, Ho 1998, Kabat 1997, Kabat 2006) and 7 nested case-control or case-cohort studies (Meilahn 1998, Modugno 2006, Muti 2000, Cauley 2003, Modugno 2006, Wellejus 2005, Eliassen 2008). This number is surprisingly small considering the importance of estrogen in breast cancer development. Possible explanations for the relative

dearth of studies in this research area include difficulties in measuring estrogen metabolite levels, intra-individual variation, and potential concerns with establishing temporal inference in case control studies.

In this combined analysis among postmenopausal women, we did not observe a significant association between either 2:16α-OHE_1 or the individual metabolites and breast cancer. Our null findings are consistent with those from individual studies among postmenopausal women not currently on HRT, particularly those of nested case-control or case-cohort studies (Meilahn 1998, Muti 2000, Cauley 2003, Wellejus 2005, Eliassen 2008). In addition, findings from previous nested case-control studies have reported no association between 2:16α-OHE_1 and breast cancer irrespective of the type of biological specimen (urine, serum, plasma).

Our analyses investigating potential differences in the association between estrogen metabolites and breast cancer by hormone receptor status, BMI, and smoking did not reveal heterogeneity by these factors. However, this combined analysis highlights the variation in the distribution of estrogen metabolites both within and across ethnic populations. In our analyses, we utilized common cutpoints of the estrogen metabolites based on the distribution in the control population. Median levels in both the Danish and Shanghai populations were mostly distributed among the lowest tertile. Lower estrogen metabolite levels among the Shanghai population are not unexpected as lower levels of circulating estrogen and estrogen metabolites have been reported among Asian populations (Alderceutz 1994, Sowers 2006). However, the observed variation within Caucasian populations underscores differences within similar ethnic populations. Although all studies measured urinary estrogen metabolites using the commercially available kit (ESTRAMET), it is important to note that the possibility for inter- and intra-

laboratory variation cannot be excluded. Final models were adjusted for the main effect of study in an attempt to account for this potential laboratory variation as well as other unknown sources of variation not accounted for by the known breast cancer factors.

In addition to differences in the distribution of estrogen metabolites by study, there appeared to be some variation in the study specific estimates; although the Chi-Square test of heterogeneity was not statistically significant. Study specific odds ratio estimates for the association of the 2:16α-OHE_1 (Tertile 3: $\geq$ 2.46 vs. Tertile 1: < 1.53) and postmenopausal breast cancer were below one for all studies (although near 1) with the exception of the study by Muti et al. The study specific estimates for Muti et al. suggest an increase in postmenopausal breast cancer risk with higher 2:1616α-OHE_1 levels, which is in contrast to our hypothesis. The original findings by Muti et al. also suggest an increase risk although the magnitude of the effect is lower, most likely due to the use of quintile cutpoints rather than the tertile cutpoints used in this combined analysis. Within the same study by Muti et al. study specific estimates among premenopausal women suggested a protective effect with higher levels of the 2:16 ratio and this finding is consistent with the proposed hypothesis. Potential explanations for this observed difference by menopausal status remain unclear but may include changes in breast cancer risk factors during the time between exposure measurement at baseline and breast cancer diagnosis (as was discussed in the original publication by Muti et al.). The authors mention the possibility of weight gain or changes in breast cancer factors such as use of hormone therapy after urine collection as additional possible explanations for the observed increase in risk, as well as the potential for increased laboratory variability due to the lower estrogen metabolite levels observed among postmenopausal women. However, the potential for laboratory variability is not specific only to the study by Muti et al., and thus is not a likely explanation. Despite these proposed

explanations, it remains unclear why the Muti et al. study specific estimates would suggest an increase in breast cancer risk for postmenopausal women but a protective association among premenopausal women.

In summary, the results from this combined analysis indicate no relationship between estrogen metabolites measured at one time point and postmenopausal breast cancer. Whether estrogen metabolites measured at one time point reflect a woman's typical level or whether they differ over the duration of many years, remains unknown. Among a subgroup of women within a nested case-control study (Nurse's Health Study), baseline estrogen metabolite levels measured in serum were fairly correlated with levels measured three years later (correlation coefficient = 0.73 for 2:16α-OHE_1) (Eliassen 2008). With the exception of this reproducibility study conducted by Eliassen et al., studies have not assessed the reproducibility of estrogen metabolites over a long duration. Future studies examining the role of estrogen metabolites in relation to postmenopausal breast cancer should consider estrogen metabolite levels measured at multiple time points. Furthermore, methodological concerns regarding the use of one measurement of estrogen metabolite levels vs. multiple measurements as well as sources of intra-individual variability should also be considered prior to conducting future studies.

7.3 ARTICLE 3: URINARY ESTROGEN METABOLITES AND BREAST CANCER RELATED FACTORS AMONG HEALTHY WOMEN

Several studies have evaluated estrogen metabolites among populations of healthy women with the majority of studies having focused on dietary factors or physical activity (Lord 2002). Variation in estrogen metabolite levels by characteristics such as age, race, obesity, family

history of breast cancer, smoking status, and alcohol use remains largely unknown. Findings from previous studies which have investigated these potential associations have been inconsistent. In this combined analysis, we utilized the control populations from five previous studies of estrogen metabolites and breast cancer in an attempt to identify potential associations between various breast cancer related characteristics and levels of 2-OHE_1, 16α-OHE_1 and 2:16α-OHE_1.

Analyses among premenopausal women identified an inverse association between BMI and 2-OHE_1 and 2:16α-OHE_1 whereas among postmenopausal women a different set of characteristics remained statistically significant in multivariable regression analyses. Among postmenopausal women, age was positively associated with 2:16α-OHE_1 while a negative association was observed between 2:16α-OHE_1 and age at menopause and history of benign breast disease. The association with history of benign breast disease is interesting considering that 16α-OHE_1 has been suggested to increase proliferation in cell studies (Telang 1992) and it is possible that the observed association may be related to mechanisms of cell proliferation in breast tissue. Furthermore, history of benign breast disease was positively associated with 16α-OHE_1 which supports this potential explanation. To our knowledge this association with history of benign breast disease has not been evaluated in previous studies. Our findings are based on cross-sectional associations and warrant further investigation.

Our results suggest that estrogen metabolite levels among pre- and postmenopausal women may be associated with different factors. This is not unexpected as levels of circulating estrogens and estrogen metabolites are lower among postmenopausal women compared to premenopausal women. In addition, the main source of circulating estrogens varies by menopausal status. Although questions still remain with regard to important predictors of

estrogen metabolites, our findings contribute to the existing body of knowledge regarding urinary estrogen metabolites and warrant further investigation.

7.4 SUMMARY

Estrogen plays an important role in the development of breast cancer. Previous studies of circulating estrogens have reported an increase in breast cancer risk with higher circulating estrogen levels among postmenopausal women (EHBCCG 2002, Eliasssen 2006, Cauley 1999, Zeleniuch-Jacquotte (1995), Missmer 2004, Helzlsouer 1994, Toniolo 1995, Berrino 1996, Dorgan 1996, Thomas 1997). Although estrogen is an integral factor for reproductive development and is beneficial for brain and cardiovascular function, estrogen may also stimulate cell proliferation and thus, indirectly influence carcinogenesis. Among premenopausal women the main circulating estrogen is estradiol, secreted by the ovaries, while among postmenopausal women the main source of circulating estrogen is estrone, which results from the aromatization of androgens to estrogens. Although the ovarian production of estrogen is higher among premenopausal women, the incidence of breast cancer is highest among postmenopausal women. Considering the estrogen hypothesis, the higher incidence among postmenopausal women may be considered counter intuitive. Potential explanations include increased aromatase activity in breast tissue (Jefcoate 2000) or the role of non-estrogen related pathways.

The metabolism of estrogen has been suggested as a possible mechanism of carcinogenesis due to the genotoxic and mutagenic effects of specific estrogen metabolites. In addition to the observed differences in circulating estrogen levels among pre- and postmenopausal women, estrogen metabolite levels also differ by menopausal status. Estrogen

metabolites are generally higher among premenopausal women compared to postmenopausal women. The 2-OHE_1 and 16α-OHE_1 metabolites, and their ratio, have been evaluated in relation to pre-and postmenopausal breast cancer risk due to their differing biological properties. The results of this combined analysis suggested a protective association with higher levels of 2:16α-OHE_1 among premenopausal women, albeit this finding was not statistically significant. However, among postmenopausal women, our results do not support a reduction in breast cancer with higher 2:1616α-OHE_1 levels.

There are potential explanations for the observed differences in the association between 2:16α-OHE_1 and breast cancer by menopausal status. These may include the observed lower levels of estrogen metabolites among postmenopausal women, differences in the definition of menopause by study, whether women underwent natural or surgical menopause as well as the primary source of estrogen (ovarian vs. nonovarian). Despite the potential differences in definitions of menopausal status, the original findings from the studies included in our combined analyses did not support a reduction in postmenopausal breast cancer risk with higher 2:16α-OHE_1 levels, and thus differences in definitions of menopausal status is an unlikely explanation. Furthermore, the null findings from our combined analysis among postmenopausal women are consistent with results from other nested case-control studies of postmenopausal women which measured metabolites in serum (Cauley 1999) or plasma (Eliassen 2008). Thus, it is possible that the 2:16α-OHE_1 is not associated with breast cancer among postmenopausal women.

Predictors of estrogen metabolite levels may also vary by menopausal status. The results from the combined analysis among the control populations suggest different significant associations among pre- and postmenopausal women and highlight the importance of assessing estrogen metabolites separately by menopausal status. Although the findings from the combined

analysis among the control population were based on cross-sectional associations, our results contribute to the overall literature surrounding estrogen metabolites among healthy populations of women and suggest that future studies evaluate the association between estrogen metabolites and factors such as BMI, smoking status, alcohol consumption and history of benign breast disease using more detailed exposure measures among populations of both pre- and postmenopausal women. To date, the majority of studies have focused on dietary and nutritional factors and the few studies which have evaluated a range of breast cancer factors in relation to estrogen metabolite levels have been among premenopausal women (Jernstrom 2003, Sowers 2006). Evaluating estrogen metabolites among populations of healthy women may give insight into the normal function of estrogen metabolites, the underlying mechanisms for the potential association between estrogen metabolites and breast cancer, and furthermore, we may gain insight into the mechanisms by which specific factors affect a woman's cumulative estrogen exposure, and subsequently breast cancer risk.

Few of the known breast cancer risk factors can be potentially modified and these include lifestyle factors such as physical activity, weight management and alcohol consumption. Estrogen metabolite levels may potentially be modified through dietary intervention or supplementation (Lord 2002), and the possibility of altering the favored metabolic pathway may offer an alternative means of prevention. If the observed associations in our combined analysis among the control populations are in fact true associations, and additional studies confirm our findings with more detailed measures of exposure, altering factors such as BMI or alcohol consumption may offer an alternative method of improving estrogen metabolism and potentially reducing breast cancer risk. It is important to note, however, that to our knowledge no studies

have evaluated whether modifications in estrogen metabolism directly leads to changes in breast cancer risk.

In summary, the use of a combined analysis approach offered numerous benefits including the use of existing data to create a larger sample size and to increase the overall power to detect an association. This approach also provided an opportunity to synthesize the existing literature as the majority of eligible pre- and postmenopausal studies participated in this combined analysis and thus, we were able to identify gaps and to suggest potential future directions in this research area. Furthermore, the results from this combined analysis highlight the variability in estrogen metabolite levels, the observed differences by menopausal status, and also emphasize the importance of addressing methodological issues pertaining to the measurement of estrogen metabolites. Additional opportunities for research are discussed in the next section.

7.5 FUTURE DIRECTIONS

Previous studies have investigated the association between estrogen metabolites and breast cancer using estrogen metabolite levels measured at one time point. Although the results from this combined analysis do not support an association between higher levels of the 2:16α-OHE_1 and breast cancer, our null findings, particularly those observed in the analysis among postmenopausal women, suggest that future studies attempt to address this research question from an alternative perspective. Although the results from the analysis among premenopausal women suggest a reduction in breast cancer risk with higher levels of 2:16α-OHE_1, it remains

unknown whether estrogen metabolites measured at one time point is the most appropriate measure of usual levels.

As mentioned in the individual articles, future studies should consider variation in estrogen metabolite levels over time. Intra-individual variability during different periods of life is one area of research which may help clarify the function of estrogen metabolites both in healthy populations of women as well as among breast cancer patients. In general, few studies have addressed the methodological concerns with measuring estrogen metabolites, and furthermore, the limited studies which have assessed the reproducibility or validity of the measurements, have focused on short time periods. Thus, numerous methodological questions remain to be answered.

Additional opportunities for future research include understanding the distribution of estrogen metabolites among different ethnic populations. As shown in our analysis, estrogen metabolite levels can vary between Caucasian and Asian populations, and previous studies have also reported differences between Caucasian and African American women. Overall, very few studies have included multi-ethnic populations in their evaluation of estrogen metabolites. This is an important area for future research in consideration of the racial differences in breast cancer incidence and mortality as well as the observed differences in metabolite levels by race.

Future studies should also investigate the role of additional estrogen metabolites. The 4-hydroxyestrone (4-OHE) metabolite has been evaluated in relation to breast cancer. Both 4-OHE and 2-OHE_1 can undergo additional redox cycling leading to the formation of estrogen by-products which may then react with DNA, resulting in the formation of either stable or depurinating adducts. It is hypothesized that estrogen quinone metabolites may serve as

endogenous chemical carcinogens, and ultimately lead to cancer initiation. However, limited research has been conducted in this area, especially in regards to breast cancer.

An additional important consideration is the influence of dietary macronutrients on estrogen metabolites as some research suggests that estrogen metabolites may potentially be modified through dietary intervention (Lord 2002). In our combined analysis, we were unable to assess the influence of dietary factors. Future studies aimed at evaluating the relationship between estrogen metabolites and breast cancer should account for the role of lifestyle factors, such as dietary intake and physical activity, on estrogen metabolite levels.

Lastly, the relationship between estrogen metabolites and breast cancer may vary according to risk of breast cancer. Limited research has been conducted among women considered at high risk, including women with a strong family history of breast cancer or women with a history of benign breast disease. More research, particularly among subgroups, is needed to better understand the association between estrogen metabolites and breast cancer.

7.6 PUBLIC HEALTH SIGNIFICANCE

Among women in the United States, breast cancer is the most common cancer diagnosed and is the second leading cause of cancer death (ACS 2008). A key factor in breast carcinogenesis is cumulative estrogen exposure; however the mechanism by which estrogen affects breast cancer remains unclear. Various risk factors have been identified, including reproductive, lifestyle, and hormonal factors, yet many of these are not potentially modifiable. Estrogen metabolites, however, may potentially be modified through dietary intervention or supplementation and modification of estrogen metabolite levels may be an alternative mode of prevention. Additionally,

the utility of the 2:16 ratio as a potential biomarker may offer a means of identifying women at increased risk and for whom prevention strategies may be useful.

This research enhances our knowledge of estrogen metabolites and makes a significant contribution to public health. Although we did not observe significant associations between metabolite levels measured at one time point and breast cancer, our findings among premenopausal women suggest a reduction in breast cancer with higher levels of the 2:16α-OHE_1, which is consistent with our hypothesis. Furthermore, we were able to identify important areas of future research in relation to breast cancer and in relation to future studies of healthy populations. In summary, the results of our combined analysis confirm previous findings and suggest that alternative approaches be employed in future studies aimed at addressing this research question. Whether estrogen metabolite levels may serve as potential biomarkers of breast cancer remains to be determined. Nonetheless, our findings have contributed to the overall body of knowledge pertaining to estrogen metabolites and breast cancer.

APPENDIX A: OVERALL METHODS OF THE COMBINED ANALYSIS

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A.1 SUMMARY OF DATA COLLECTION

Table 27 Summary of Variables Requested and Received from Investigators

No	Variable	Possible categories	Fowke	Meilahn	Muti	Ursin	Wellejus
1	ID		√	√	√	√	√
2	Case/control status Match set	Control/Case	√	√	√	√	√
3	16α -OHE1	Continuous variable	√	√	√	√	√
4	2-OHE1	Continuous variable	√	√	√	√	√
5	2-OHE1/16α -OHE1 ratio	Continuous variable	√	√	√	√	√
6	Age (years)	Continuous variable	√	√	√	√	√
7	Ethnicity	Caucasians African Americans Latino Asians Other/specify	√	√	√	√	√
8	Education Levels		√	√	√	√	√
9	Socioeconomic Status		√	---	---	√	---
10	Height	Continuous variable	√	√	√	(BMI)	√
11	Weight	Continuous variable	√ (BMI)	√	√	√ (BMI)	√
12	Waist to hip ratio	Continuous variable	√	----	√	---	√
13	Smoking	Never Former Current smoker No info	√	√	√	?	√
14	Alcohol consumption	Never , former, current	√	---	√	√	√
15	Menopausal Status	Pre Peri Post Unknown	√	√	√	All post	All post

Table 27 continued.

No	Variable	Possible categories	Fowke	Meilahn	Muti	Ursin	Wellejus
16	Age at menopause (years)	Continuous variable	√	√	√	√	√
17	Hormone Replacement Therapy	Never Former use Current use	√	√	√	√	√
18	Duration of Hormone Therapy Use		---	√	√	√	√
19	Use of Oral Contraceptives	Never Former use Current use	√	√	√	--	√
20	Duration of Oral Contraceptive Use (years)		---	√	√	---	√
21	Family history of breast cancer in first degree relatives	Yes No	√	√	√	√	---
22	History of benign breast disease	Yes No	---	√	√	--	√
23	Reproductive Variables related to breast cancer		√	√	√	√	√
24	Phase of menstrual cycle at time of urine collection or Pre/Post Treatment		--- √	√ ---	√ ---	N/A ---	N/A
25	Tumor Type	Invasive Breast Cancer In situ Breast Cancer	√	√	√	√	√
26	Tumor stage		√	---	√	---	√
27	Tumor histology		----	√	√	---	√
28	Estrogen Receptor Status		√	√	√	√	√
29	Progesterone Receptor Status		√	---	√	√	√

Table 28 Characteristics of Nested Case-Control Studies Participating in the Combined Analysis of Estrogen Metabolites

Authors Year	Design / Study Features	Cases/ Controls N	Matching	Matching Criteria	Population	Case Selection	Control Selection	Exclusion Criteria
Meilahn et al. 1998	Nested Case-control Study period: 1977-85	Post: 42/139 Pre: 60/184	1:3	- age ± 2 yrs - baseline exam ±1yr - menopausal status (pre, 0-2 or 3+ post) - menstrual phase (follicular, within 15 days of start of last cycle, luteal, more than 15 days)	- age ≥ 35 yrs - residents of Guernsey Island - 31% of age eligible women volunteered to participate	Primary clinically diagnosed breast cancer at least 6 months after baseline	Random controls from cohort – alive and breast cancer free at end of cohort, & with urine available	At baseline: - irregular cycles in prior 6 months -< 60 yrs with prior hysterectomy -used OC - HRT - history of oopherectomy - prior diagnosis of cancer (other than non-melanoma skin cancer)
Muti et al. 2000	Nested Case-control Study period: 1987-1992	Post: 71/274 Pre: 67/264	1:4	- age ±5 years - menopausal status - time of blood draw - recruitment center (two centers 25 km apart) - recruitment date ± 180 days	- Ages 35 -69 -residents of Varese province -volunteers recruited via media, tv, radio and local groups -total recruited represents 7% of eligible population	Linkage with local cancer registry (Lombardy Cancer Registry)	Random controls from cohort alive at time of diagnosis of matched case	-history of cancer -history of bilateral oopherectomy - pregnant or breastfeeding - vhronic or acute liver disease - HRT within 3 months prior to recruitment
Wellejus et al. 2005	Nested C/C Study Period: 1993-2000	Post: 426/426	1:1	- age at entry into cohort (6 month intervals) - certainty of postmenopausal status (known/probably), use of HRT at study entry (current/ former/never)	-Age 50-64 -Danish women living in the greater Copenhagen or Aarhus areas - participants visited one of two study centers	Linkage with the Danish Cancer Registry and the Danish Breast Cancer Cooperative Group Registry	Cancer free at the exact age at the diagnosis of the case	- prior history of cancer - missing lifestyle survey -premenopausal (at least one menstruation period and no HRT), no lifetime history of menstruation, no info on HRT use

Table 29 Characteristics of Case-Control Studies Participating in the Combined Analysis of Estrogen Metabolites

Authors Year	Design / Study Features	Cases/ Controls N	Matching	Matching Criteria	Population	Case Selection	Control Selection	Exclusion Criteria
Ursin et al. 1999	Case-control	Post: 66/76	1:1	- age ± 3 years - ethnicity (latina/non latina) - neighborhood of residence *did not retain matching from original study	English speaking residents of LA country	Identified through LA County Cancer Surveillance Program (SEER Registry) Incident cancer localized to the breast – no distant metastasis) No in situ cases Diagnosed 1987-1989, or in 1992 *prevalent cases	Population based controls Neighborhood controls from housing units in the are where the case lived at the time of diagnosis	-Medications in prior 6 months (cimetidine, thyroid supplements, estrogen or progesterone, tamoxifen or omega 3 fatty acid supplements) -cancer chemotherapy - no general anesthesia in the past 3 months -weight < 80 lbs or > 200 lbs -smoker (within past three years)
Fowke et al. 2006	Case-control	Post: 37/37 Pre: 63/63	1:1	- age ± 3 years - menopausal status - date of sample collection ± 30 days	- Age 25-65 Shanghai Breast Cancer Study - First 110 cases and controls to participate in substudy were included in the estrogen metabolite study	Incident cases identified from registries (hospital or tumor) -rapid case ascertainment system	Randomly selected from a complete registry of the Shanghai population	Prior history of cancer

APPENDIX B: URINARY ESTROGEN METABOLITES AND PREMENOPAUSAL BREAST CANCER

B.1 ADDITIONAL ANALYSES

Table 30 Main Effect of Study on Premenopausal Breast Cancer (N=731)

Study	Cases/Controls[†]	Adjusted* OR (95% CI) (by study only)	Adjusted** OR (95% CI)
2-OHE_1			
Muti et al.	71/292	1.00	1.00
Meilahn et al.	55/184	1.13 (0.71, 1.82)	1.07 (0.62, 1.84)
Fowke et al.	57/72	2.95 (1.69, 5.16)	3.21 (1.69, 6.09)
16α-OHE_1			
Muti et al.	71/292	1.00	1.00
Meilahn et al.	55/184	1.28 (0.81, 2.00)	1.30 (0.78, 2.17)
Fowke et al.	57/72	3.52 (2.17, 5.71)	4.16 (2.45, 7.07)
2:16α-OHE_1			
Muti et al.	71/292	1.00	1.00
Meilahn et al.	55/184	1.16 (0.75, 1.79)	1.08 (0.65, 1.81)
Fowke et al.	57/72	2.88 (1.71, 4.86)	3.02 (1.66, 5.50)

Note: This table corresponds to Table 4 and includes details on the main effects of study on the breast cancer models. Estimates generated using conditional logistic regression matched on 5 year age strata. Information on 2-OHE_1 and 16α-OHE_1 missing for 5 controls. Abbreviations: OR=Odds Ratio, 95% CI=Confidence Intervals

*Models adjusted for study only.

**Each model is adjusted for the specific estrogen metabolite (categorical variable) and known breast cancer risk factors including: family history of breast cancer (yes/no), history of benign breast disease (yes/no/unknown), age at first menstrual period (continuous), body mass index (kg/m^2, continuous), oral contraceptive use (yes/no), smoking (yes/no/unknown) and pregnancy combined variable (never pregnant, age at first pregnancy <20, age at first pregnant 20-29, age at first pregnant ≥ 30 years), and study.

Table 31 Main Effect of Study on Premenopausal Breast Cancer among Caucasian Women (N=602)

Study	Cases/Controls†	Adjusted* OR (95% CI) (by study only)	Adjusted** OR (95% CI)
2-OHE_1			
Muti et al.	71/292	1.00	1.00
Meilahn et al.	55/184	1.21 (0.75, 1.95)	1.12 (0.64, 1.95)
16α-OHE_1			
Muti et al.	71/292	1.00	1.00
Meilahn et al.	55/184	1.36 (0.86, 2.17)	1.43 (0.84, 2.41)
2:16α-OHE_1			
Muti et al.	71/292	1.00	1.00
Meilahn et al.	55/184	1.19 (0.76, 1.85)	1.10 (0.65, 1.85)

Note: This table corresponds to Table 4.5 and includes details on the main effects of study on the breast cancer models. Estimates generated using conditional logistic regression matched on 5 year age strata. Information on 2-OHE_1 and 16α-OHE_1 missing for 4 controls. Abbreviations: OR=Odds Ratio, 95% CI=Confidence Intervals
*Models adjusted for study only.
**Each model is adjusted for the specific estrogen metabolite (categorical variable) and known breast cancer risk factors including: family history of breast cancer (yes/no), history of benign breast disease (yes/no/unknown), age at first menstrual period (continuous), body mass index (kg/m^2, continuous), oral contraceptive use (yes/no), smoking (yes/no/unknown) and pregnancy combined variable (never pregnant, age at first pregnancy <20, age at first pregnant 20-29, age at first pregnant ≥ 30 years), and study.

Table 32 Tertiles of 2-OHE_1, 16α-OHE_1, and 2:16α-OHE_1 and their Association with Breast Cancer among Premenopausal Women by Subgroups of Smoking Status (N=602) (Caucasian Women Only)

Estrogen Metabolites	Current Smokers (N=133)			Non-Current Smokers (N=430)	
	Cases/Controls (24/109)	OR (95% CI)		Cases/Controls (89/341)	OR (95% CI)
2-OHE_1					
< 15.33	10/30	1.00		14/77	1.00
15.33-38.75	10/32	1.26 (0.41, 3.84)		37/133	1.52 (0.77, 3.00)
≥ 38.76	4/46	0.28 (0.07, 1.11)		38/128	1.62 (0.79, 3.32)
16α-OHE_1					
< 7.97	9/35	1.00		18/96	1.00
7.97-17.20	9/28	1.35 (0.44, 4.12)		29/120	1.29 (0.68, 2.47)
≥ 17.21	6/45	0.61 (0.17, 2.12)		42/122	1.87 (0.99, 3.54)
2:16α-OHE_1					
< 1.76	7/28	1.00		27/78	1.00
1.76-2.66	7/41	0.66 (0.20, 2.19)		34/130	0.71 (0.39, 1.28)
≥ 2.76	10/39	0.98 (0.31, 3.06)		28/130	0.57 (0.31, 1.06)

Abbreviations: OR=Odds Ratio, 95% CI=Confidence Intervals
Note: Urinary 2-OHE_1 and 16α-OHE_1 metabolites (ng/ml) are standardized to urinary creatinine levels (mg/dl). OR estimates generated using conditional logistic regression matched on 5 year age strata, adjusted for study

Table 33 Tertiles of 2-OHE_1, 16α-OHE_1, and 2:16α-OHE_1 and their Association with Breast Cancer among Premenopausal Women by Subgroups of Body Mass Index (N=602) (Caucasian Women Only)

Estrogen Metabolites	BMI < 25 kg/m² (N=377)			BMI ≥ 25 kg/m² (N=222)	
	Cases/Controls (87/290)	OR (95% CI)		Cases/Controls (39/183)	OR (95% CI)
2-OHE_1					
< 15.33	26/70	1.00		7/52	1.00
15.33-38.75	30/110	0.80 (0.43, 1.49)		20/60	2.28 (0.87, 5.95)
≥ 38.76	31/106	0.92 (0.47, 1.81)		12/71	1.18 (0.40, 3.47)
16α-OHE_1					
< 7.97	23/89	1.00		10/57	1.00
7.97-17.20	32/95	1.34 (0.72, 2.47)		11/57	1.09 (0.42, 2.81)
≥ 17.21	32/102	1.43 (0.75, 2.75)		18/69	1.46 (0.60, 3.55)
2:16α-OHE_1					
< 1.76	26/67	1.00		12/50	1.00
1.76-2.66	32/114	0.69 (0.37, 1.26)		15/65	1.00 (0.42, 2.39)
≥ 2.76	29/105	0.71 (0.38, 1.34)		12/68	0.70 (0.28, 1.73)

Abbreviations: OR=Odds Ratio, 95% CI=Confidence Intervals

Note: Urinary 2-OHE_1 and 16α-OHE_1 metabolites (ng/ml) are standardized to urinary creatinine levels (mg/dl). Data on 2-OHE_1 and 16α-OHE_1 missing for 4 controls. OR estimates generated using conditional logistic regression matched on 5 year age strata, adjusted for study.

Table 34 Relative Risk of Progesterone Receptor Status by Tertiles of Estrogen Metabolites (2-OHE_1, 16α-OHE_1 and 2:16α-OHE_1) as compared to controls among Caucasian Women (N=602)*

		PR positive		PR negative		PR unknown	
Estrogen Metabolites	**Controls (N=476)**	**Cases (N=50)**	**RR (95% CI)**	**Cases (N=16)**	**RR (95% CI)**	**Cases (N=60)**	**RR (95% CI)**
2-OHE_1							
< 15.33	122	6	1.00	3	1.00	24	1.00
15.33-38.75	171	19	1.30 (0.48, 3.52)	7	1.00 (0.24, 4.09)	24	1.08 (0.56, 2.08)
≥ 38.76	179	25	1.03 (0.39, 2.71)	6	0.52 (0.12, 2.20)	12	1.36 (0.59, 3.14)
16α-OHE_1							
< 7.97	147	9	1.00	2	1.00	22	1.00
7.97-17.20	153	17	1.42 (0.59, 3.43)	6	2.44 (0.47, 12.72)	20	1.10 (0.55, 2.19)
≥ 17.21	172	24	1.19 (0.52, 2.75)	8	2.00 (0.40, 9.84)	18	2.03 (0.95, 4.32)
2:16α-OHE_1							
< 1.76	117	7	1.00	4	1.00	22	1.00
1.76-2.66	179	21	0.72 (0.32, 1.62)	5	0.55 (0.14, 2.18)	24	0.96 (0.50, 1.87)
≥ 2.76	176	22	0.71 (0.31, 1.59)	7	0.65 (0.18, 2.43)	14	0.74 (0.35, 1.59)

Note: Urinary 2-OHE_1 and 16α-OHE_1 metabolites (ng/ml) are standardized to urinary creatinine levels (mg/dl). Data on 2-OHE_1 and 16α-OHE_1 are missing for 4 controls. Odds ratio estimates generated from multinomial models with controls as the comparison group. Estimates were generated using data from the Caucasian studies: Meilahn et al. and Muti et al.
*All multinomial models adjusted for study (categorical) and age using the following categories: ≤ 39, 40-44, ≥ 45.

Table 35 Estrogen and Progesterone Receptor Status by Age (N=183 premenopausal cases)

Age	Estrogen Receptor N (%)				Progesterone Receptor N (%)		
	Positive	**Negative**	**Unknown**		**Positive**	**Negative**	**Unknown**
<35	2 (2.8)	0 (0)	0 (0)		2 (2.5)	0 (0)	0 (0)
35-39	12 (16.9)	12 (28.6)	33 (47.1)		17 (21.3)	6 (20.7)	34 (46.0)
40-44	17 (23.9)	9 (21.4)	26 (37.1)		19 (23.7)	7 (24.1)	26 (35.1)
45-49	28 (39.5)	20 (47.6)	9 (12.9)		34 (42.5)	12 (41.4)	11 (14.8)
50-54	12 (16.9)	1 (2.4)	2 (2.9)		8 (10.0)	4 (13.8)	3 (4.1)
Total	71	42	70		80	29	74

Table 36 Median Estrogen Metabolite Levels, by Study

	Study Cases/Controls Median (range)			
Estrogen* Metabolite	**Fowke 73/72**	**Fowke** 57/72**	**Meilahn 55/184**	**Muti 73/288**
2-OHE_1	6.9 (0.5-49.6)/ 6.9 (1.9-31.3)	5.9 (0.5, 24.0)/ 6.9 (1.9-31.3)	17.9 (4.0-85.7)/ 17.5 (2.0-173.2)	37.9 (7.1-309.9)/ 40.9 (2.5-351.8)
16-OHE_1	8.0 (2.1-29.3)/ 8.6 (1.6-40.2)	7.7 (2.4, 29.3)/ 8.6 (1.6-40.2)	9.5 (2.3-44.7)/ 8.5 (1.2-91.0)	17.5 (2.2-145.5)/ 16.9 (0.96-181.8)
2:16α-OHE_1	0.94 (0.03/6/5)/ 0.85 (0.3-3.9)	0.88 (0.03, 6.5)/ 0.85 (0.3-3.9)	2.1 (0.42-4.3)/ 2.1 (0.3-6.1)	2.5 (0.95-5.55)/ 2.5 (0.43-7.6)

*Median metabolite values (range), **Excludes cases with post-treatment urine collection (N=14)

Note: Urinary 2-OHE_1 and 16α-OHE_1 metabolites (ng/ml) are standardized to urinary creatinine levels (mg/dl). Data on 2-OHE_1 and 16α-OHE_1 missing for 5 subjects.

Table 37 Descriptive Characteristics of Cases by Estrogen Receptor Status

Variable	Estrogen Receptor Positive (N=71) N (%)	Estrogen Receptor Negative (N=42) N (%)	Estrogen Receptor Unknown (N=70) N (%)
Study			
Muti et al.	39 (54.9)	28 (66.7)	4 (5.7)
Meilahn et al.	0 (0)	3 (7.1)	52 (74.3)
Fowke et al.	32 (45.1)	11 (26.2)	14 (20.0)
Age (years)*	44.8 ± 5.2	43 ± 4.1	40.8 ± 4.2
Family History of Breast Cancer, N (%)	3 (4.2)	1 (2.4)	7 (10.0)
History of Benign Breast Disease, N (%)	25 (35.2)	11 (26.2)	10 (14.3)
Body Mass Index (kg/m_2)*	23.9 ± 3.8	23.4 ± 2.9	23.9 ± 3.5
Smoking			
Never	61 (85.9)	30 (71.4)	33 (47.1)
Former	4 (5.6)	4 (9.5)	9 (12.9)
Current	6 (8.5)	8 (19.1)	11 (15.7)
Unknown	0 (0)	0 (0)	17 (24.3)
Alcohol Consumption			
Yes	24 (33.8)	16 (38.1)	5 (7.1)
No	47 (66.2)	23 (54.8)	13 (18.6)
Unknown	0 (0)	3 (7.1)	52 (74.3)
Oral Contraceptive Use			
Yes	19 (27.1)	15 (35.7)	41 (58.6)
No	51 (72.9)	27 (64.3)	29 (41.4)
Age at menarche > 13 years, N (%)	34 (47.9)	19 (46.3)	32 (45.7)
Nulliparous, N (%)	6 (8.6)	3 (7.1)	8 (11.4)
Age at First Pregnancy*	27.2 ± 4.0	25 ± 3.8	25.0 ± 9.2

Note: Percentages may not sum to 100 due to missing values. *Mean ± SD or Median (Range).
†Among parous women

Table 38 Descriptive Characteristics of Cases by Progesterone Receptor Status

Variable	**Progesterone Receptor Positive (N=80)** N (%)	**Progesterone Receptor Negative** (n=29) N (%)	**Progesterone Receptor Unknown** (n=74) N (%)
Study			
Muti et al.	50 (62.5)	16 (55.2)	5 (6.8)
Meilahn et al.	-	-	55 (74.3)
Fowke et al.	30 (37.5)	13 (44.8)	14 (18.9)
Age (years)*	44.0 ± 5.1	44.2 ± 4.2	41.0 ± 4.4
Family History of Breast Cancer, N (%)	2 (2.5)	1 (3.5)	8 (10.8)
History of Benign Breast Disease, N (%)	28 (35.0)	7 (24.1)	11 (14.9)
Body Mass Index (kg/m_2)*	24.0 ± 3.6	23.0 ± 3.1	24.0 ± 3.5
Smoking			
Never	65 (81.3)	24 (82.8)	35 (47.3)
Former	7 (8.8)	1 (3.5)	9 (12.1)
Current	8 (10.0)	4 (13.8)	13 (17.6)
Unknown	0 (0)	0 (0)	17 (23.0
Alcohol Consumption			
Yes	30 (37.5)	9 (31.0)	13 (17.6)
No	50 (62.5)	20 (69.0)	6 (8.1)
Unknown	0 (0)	0 (0)	55 (74.3)
Oral Contraceptive Use			
Yes	22 (27.9)	8 (27.6)	29 (39.2)
No	57 (72.2)	21 (72.4)	45 (60.8)
Age at menarche > 13 years, N (%)	38 (48.1)	12 (41.4)	35 (47.3)
Nulliparous, N (%)	5 (6.3)	4 (13.8)	53 (9.7)
Age at First Pregnancy*	26.8 ± 4.2	26.3 ± 3.3	25.3 ± 4.2

Note: Percentages may not sum to 100 due to missing values. *Mean ± SD or Median (Range).
†Among parous women

Table 39 Median Estrogen Metabolite Levels by Menstrual Phase at the Time of Urine Collection

Estrogen Metabolites	**Menstrual Cycle***		
	Follicular, ≤ 14 days case/control 26/81	**Luteal , ≥ 15 days** case/control 100/391	**Unknown** case/control 57/71
2-OHE_1	13.4/14.8	33.9/30.7	5.9/6.9
16α-OHE_1	9.10/7.6	16.2/14.4	7.7/8.7
2:16α-OHE_1	2.09/2.07	2.3/2.4	0.89/0.84

Note: Urinary estrogen metabolites (ng/ml) divided by urinary creatinine (mg/dl).
*Menstrual phase determined based on an average cycle length of 28 days.

B.2 LOWESS SMOOTHER PLOTS FOR EACH ESTROGEN METABOLITE, LOGIT SCALE

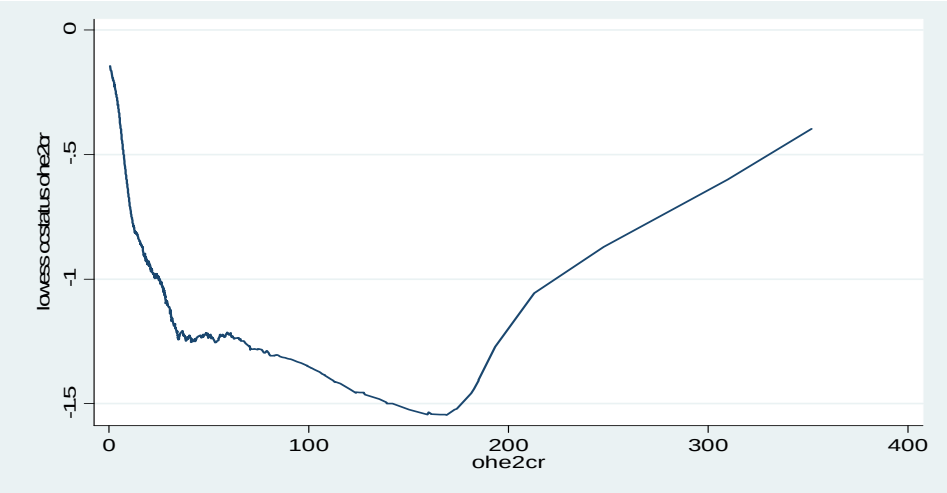


Figure 9 Lowess Smoother Plot for 2-OHE$_1$, Logit Scale (Premenopausal Studies)

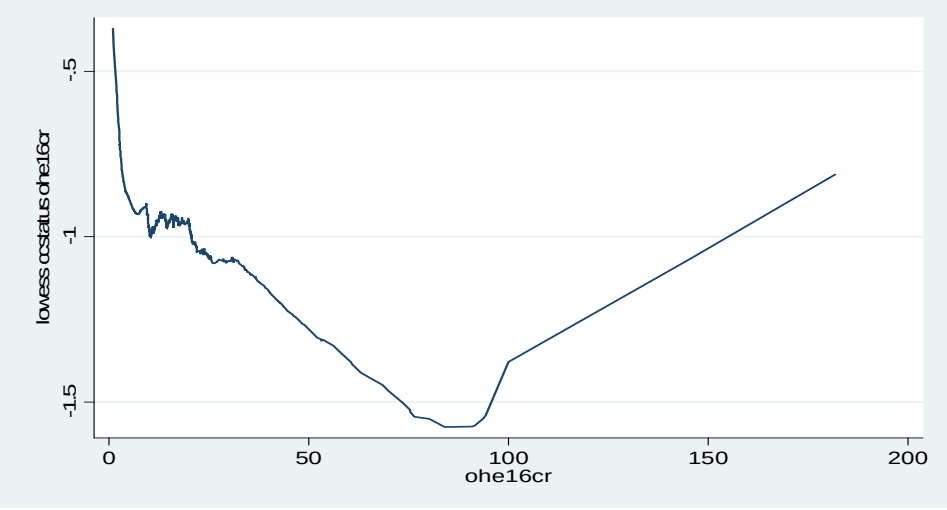


Figure 10 Lowess smoother plot for 16α-OHE$_1$, Logit Scale (Premenopausal Studies)

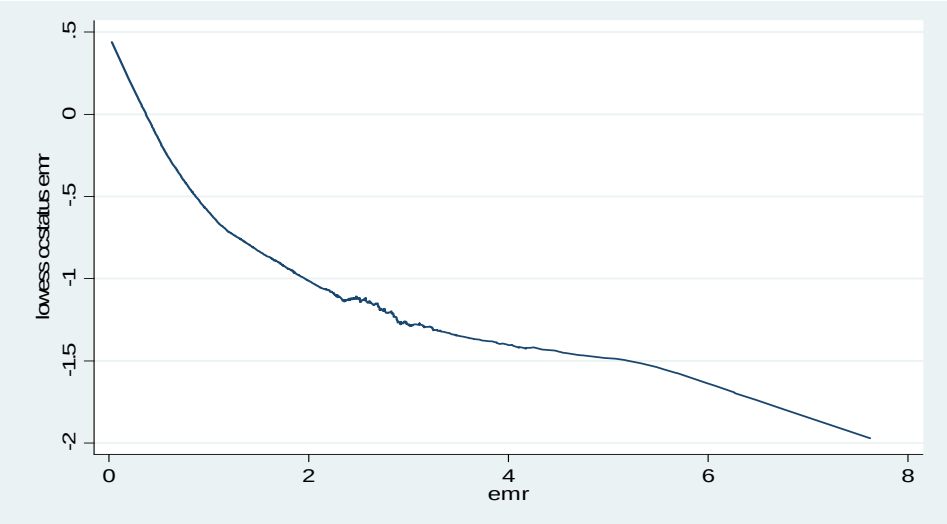


Figure 11 Lowess Smoother Plot for 2:16α-OHE_1, Logit Scale (Premenopausal Studies)

B.3 LOWESS SMOOTHER PLOTS FOR EACH ESTROGEN METABOLITE BY STUDY (LOGIT SCALE)

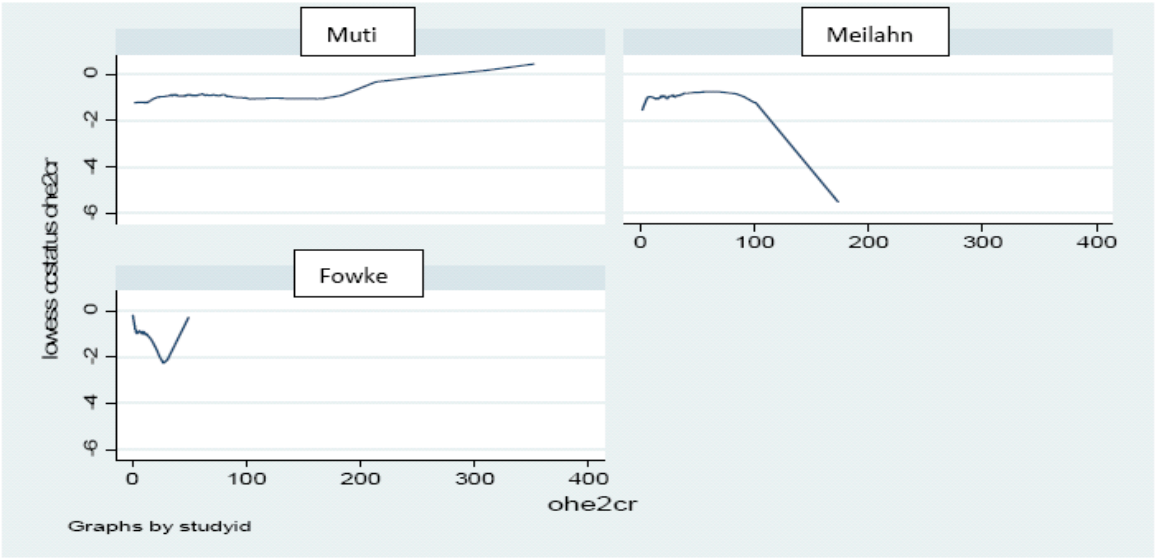


Figure 12 Lowess Smoother for 2-OHE_1 by Study, Logit Scale (Premenopausal Studies)

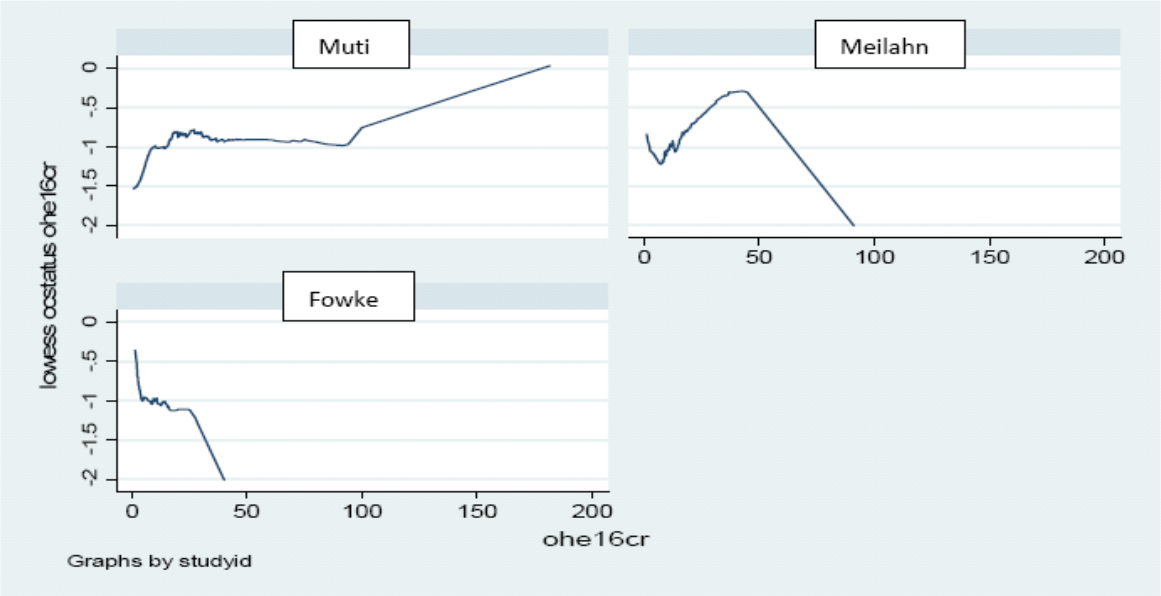


Figure 13 Lowess Smoother for 16α-OHE$_1$ by Study, Logit Scale (Premenopausal Studies)

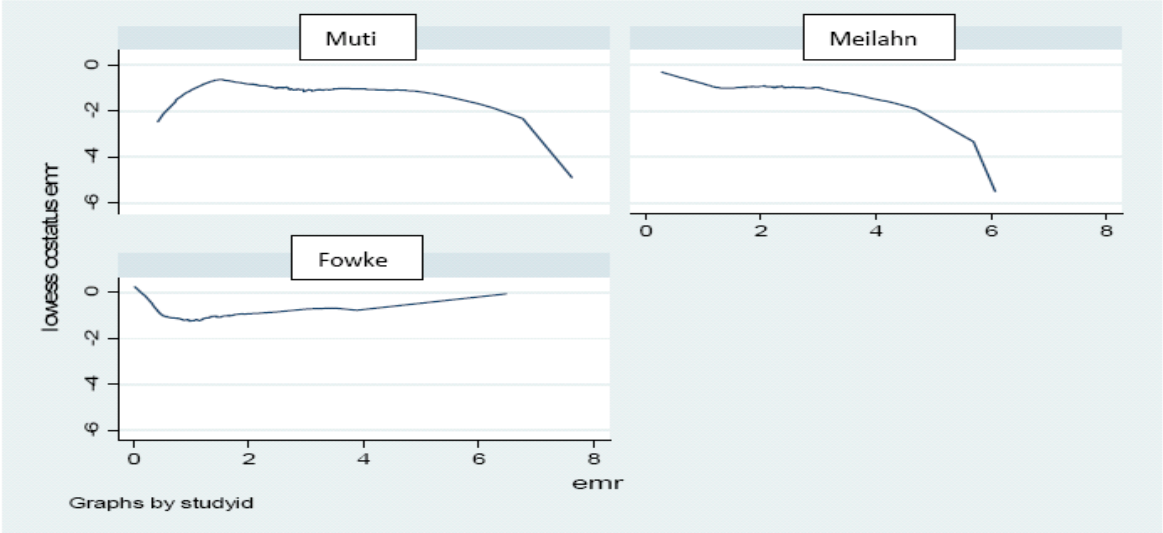
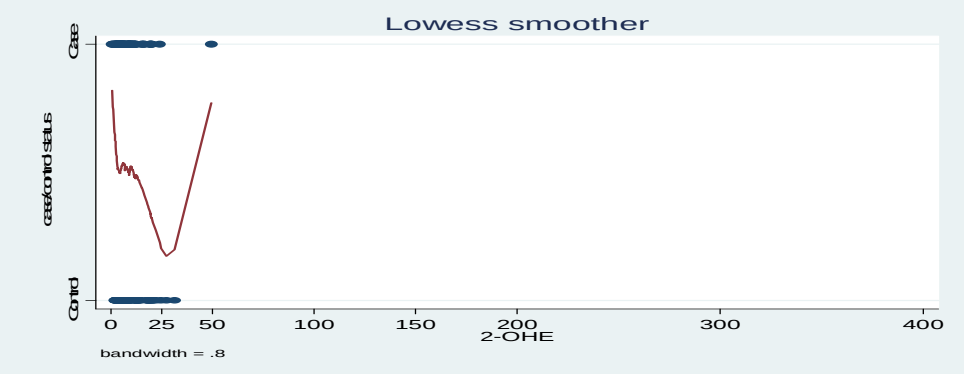


Figure 14 Lowess Smoother for 2:16α-OHE$_1$ by Study, Logit Scale (Premenopausal Studies)

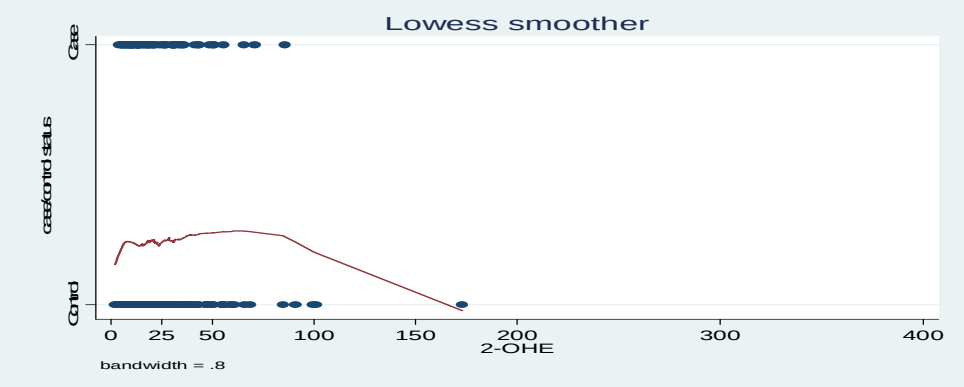
B.4 LOWESS SMOOTHER PLOTS FOR EACH ESTROGEN METABOLITE BY STUDY (P SCALE)

2-OHE$_1$

Fowke et al.



Meilahn et al.



Muti et al.

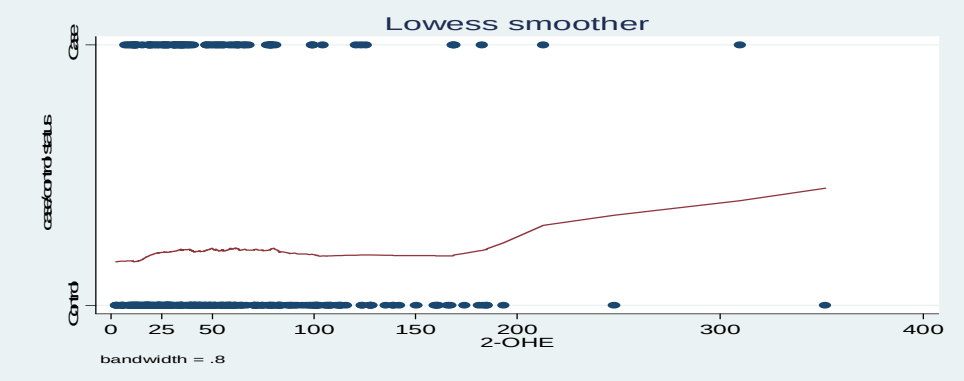
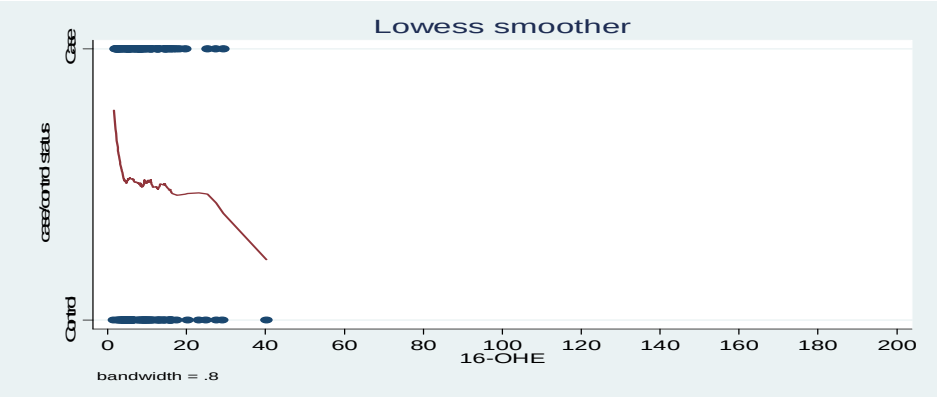


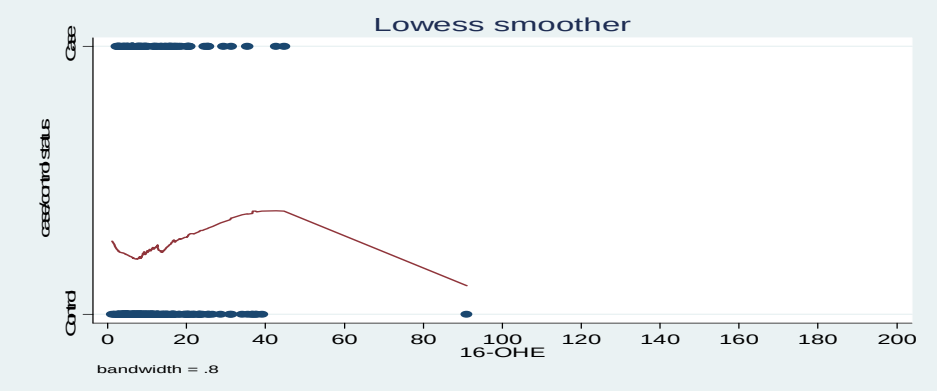
Figure 15 Lowess Smoother Plots for 2-OHE$_1$ by Study, P Scale (Premenopausal Studies)

16α-OHE$_1$ (ng/ml per mg/dl creatinine)

Fowke et al.



Meilahn et al.



Muti et al.

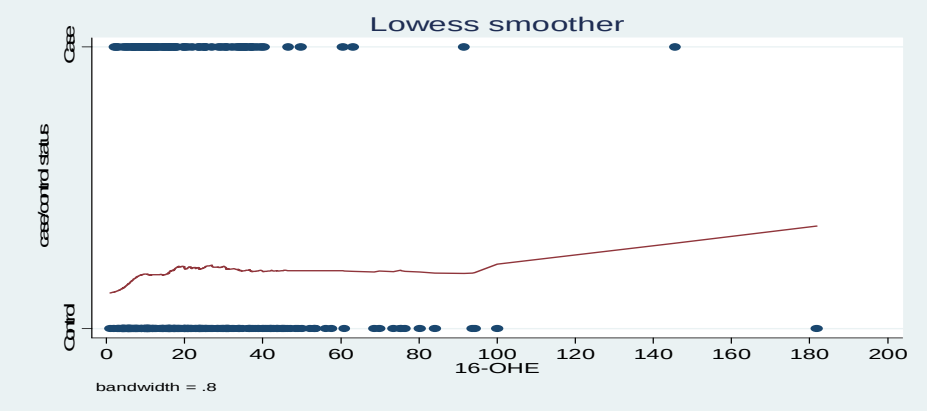
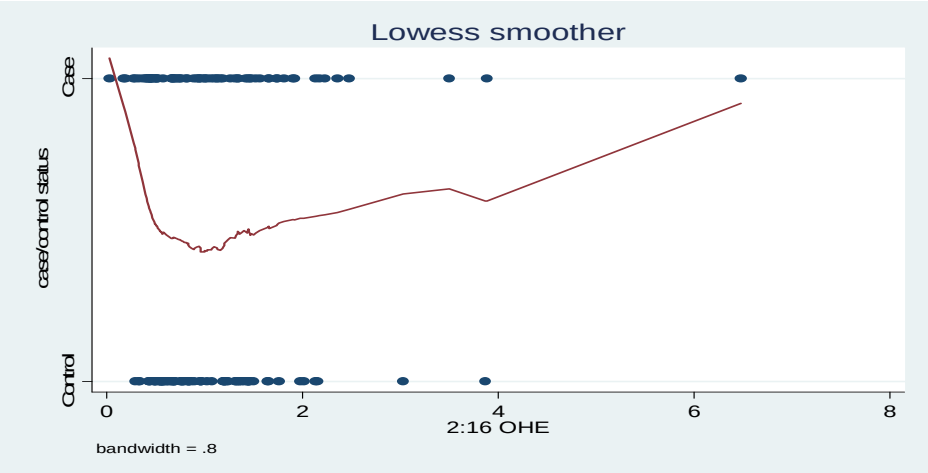


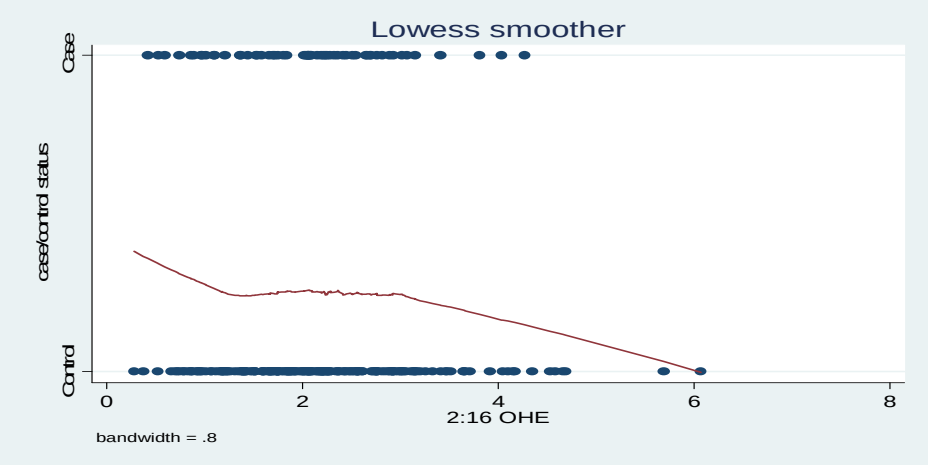
Figure 16 Lowess Smoother Plots for 16α-OHE$_1$ by Study, P Scale (Premenopausal Studies)

2:16α-OHE$_1$

Fowke et al.



Meilahn et al.



Muti et al.

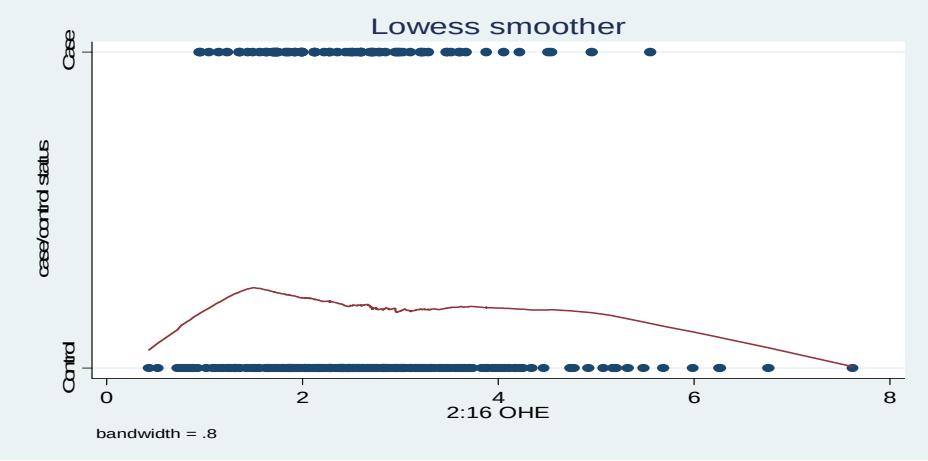


Figure 17 Lowess Smoother Plots for 2:16α-OHE$_1$ by Study, P Scale (Premenopausal Studies)

APPENDIX C: URINARY ESTROGEN METABOLITES AND POSTMENOPAUSAL BREAST CANCER

C.1 ADDITIONAL ANALYSES

Table 40 Tertiles of 2-OHE_1, 16α-OHE_1, and 2:16α-OHE_1 and their Association with Breast Cancer among Postmenopausal Women by Subgroups of Body Mass Index (N=910)

Estrogen Metabolites	BMI < 25 kg/m^2 (N=336)			BMI ≥ 25 kg/m^2 (N=572)	
	Cases/Control (113/223)	OR (95% CI)		Cases/Controls (186/386)	OR (95% CI)
2-OHE_1					
< 2.99	63/78	1.00		112/129	1.00
2.99 - 8.49	25/71	1.31 (0.60, 2.88)		49/124	0.97 (0.51, 1.83)
≥ 8.50	25/74	1.39 (0.53, 3.66)		25/133	0.60 (0.28, 1.28)
16α-OHE_1					
< 1.50	57/82	1.00		99/131	1.00
1.50 – 4.47	34/70	1.86 (0.93, 3.72)		55/125	1.39 (0.80, 2.42)
≥ 4.48	22/71	1.40 (0.59, 3.34)		32/130	1.10 (0.55, 2.22)
2:16α-OHE_1					
< 1.53	37/59	1.00		75/121	1.00
1.53-2.45	46/83	1.04 (0.58, 1.85)		72/137	0.99 (0.64, 1.53)
≥ 2.46	30/81	1.07 (0.53, 2.17)		39/128	0.80 (0.48, 1.33)

Abbreviations: OR=Odds Ratio, 95% CI=Confidence Intervals

Note: Urinary 2-OHE_1 and 16α-OHE_1 metabolites (ng/ml) are standardized to urinary creatinine levels (mg/dl). OR estimates generated using conditional logistic regression matched on 5 year age strata, adjusted for study.

Table 41 Tertiles of 2-OHE_1, 16α-OHE_1, and 2:16α-OHE_1 and their Association with Breast Cancer among Postmenopausal Women by Subgroups of Smoking Status (N=910)

Estrogen Metabolites	Current Smokers (N=)			Non-Current Smokers (N=685)	
	Cases/Controls (75/123)	OR (95% CI)		Cases/Controls (216/469)	OR (95% CI)
2-OHE_1					
< 2.99	48/64	1.00		127/143	1.00
2.99 - 8.49	18/35	1.40 (0.60, 3.23)		49/149	1.00 (0.54, 1.84)
≥ 8.50	9/24	1.22 (0.27, 5.50)		40/177	0.79 (0.40, 1.55)
16α-OHE_1					
< 1.50	39/61	1.00		117/152	1.00
1.50 – 4.47	25/36	2.06 (0.96, 4.43)		59/153	1.36 (0.80, 2.31)
≥ 4.48	11/26	3.17 (0.84, 12.03)		40/164	1.12 (0.60, 2.08)
2:16α-OHE_1					
< 1.53	33/44	1.00		76/123	1.00
1.53-2.45	25/45	0.77 (0.38, 1.56)		88/171	1.04 (0.69, 1.57)
≥ 2.46	17/34	0.75 (0.32, 1.74)		52/175	0.90 (0.55, 1.45)

Abbreviations: OR=Odds Ratio, 95% CI=Confidence Intervals

Note: Urinary 2-OHE_1 and 16α-OHE_1 metabolites (ng/ml) are standardized to urinary creatinine levels (mg/dl). OR estimates generated using conditional logistic regression matched on 5 year age strata, adjusted for study.

Table 42 Relative Risk of Progesterone Receptor Status by Tertiles of Estrogen Metabolites (2-OHE_1, 16α-OHE_1 and 2:16α-OHE_1) as compared to controls among Caucasian Women, (N=910)*

		PR positive		PR negative		PR unknown	
Estrogen Metabolites	**Controls (N=610)**	**Cases (N=59)**	**RR (95% CI)**	**Cases (N=58)**	**RR (95% CI)**	**Cases (N=183)**	**RR (95% CI)**
2-OHE_1							
< 2.99	207	19	1.00	29	1.00	127	
2.99 - 8.49	196	18	2.33 (1.01, 5.36)	14	1.51 (0.66, 3.47)	42	0.47 (0.27, 0.82)
≥ 8.50	207	22	2.83 (1.18, 6.80)	15	1.79 (0.70, 4.60)	14	0.15 (0.07, 0.30)
16α-OHE_1							
< 1.50	213	18	1.00	29	1.00	109	1.00
1.50 – 4.47	196	24	2.51 (1.20, 5.24)	14	1.18 (0.55, 2.52)	51	0.88 (0.55, 1.40)
≥ 4.48	201	17	2.22 (0.92, 5.36)	15	1.75 (0.70, 4.39)	23	0.49 (0.27, 0.91)
2:16α-OHE_1							
< 1.53	180	13	1.00	19	1.00	80	1.00
1.53-2.45	220	22	1.46 (0.71, 3.00)	20	0.93 (0.47, 1.81)	76	0.80 (0.55, 1.18)
≥ 2.46	210	24	1.73 (0.84, 3.55)	19	1.16 (0.58, 2.33)	27	0.36 (0.22, 0.60)

Note: Urinary 2-OHE_1 and 16α-OHE_1 metabolites (ng/ml) are standardized to urinary creatinine levels (mg/dl).
Odds ratio estimates generated from multinomial models with controls as the comparison group. Estimates were generated using data from the Caucasian studies: Wellejus et al., Meilahn et al. and Muti et al.
*All multinomial models adjusted for study (categorical) and age using the following categories: ≤ 54, 55-59, 60-64, ≥ 65.

Table 43 Median Estrogen Metabolite Levels, by Study

		Study Cases/Controls Median (Range)			
Estrogen* Metabolite	**Fowke** (19/37)	**Meilahn** (39/139)	**Muti** (71/282)	**Wellejus** (193/199)	**Ursin** (66/76)
2-OHE_1	3.0 (0.5-15.8)/ 3.7 (1.0-12.3)	6.4 (1.1-16.3)/ 7.1 (5.0, 10.5)	9.0 (1.3-97.0)/ 10.3 (0.7-115.4)	1.5 (0.2-22.6)/ 1.5 (0.1-8.5)	7.0 (1.5-30.5)/ 6.5 (2.0-24.5)
16α-OHE_1	4.0 (1.1-8.5)/ 3.6 (1.5-30.20	4.4 (0.75-13.6)/ 4.5 (0.9-15.1)	4.1 (0.7-27.3)/ 3.9 (0.19-38.3)	0.99 (0.2-6.2)/ 0.94 (0.2-8.2)	3.7 (1.1-20.0)/ 3.7 (0.9-10.3)
2:16α-OHE_1	0.87 (0.16-3.2)/ 0.95 (0.4-7.3)	1.6 (0.2-4.6)/ 1.7 (0.2-11.3)	2.6 (0.6-6.9)/ 2.6 (0.4-13.9)	1.6 (0.04-5.7)/ 1.6 (0.3-6.8)	1.8 (0.4-4.8)/ 1.7 (0.7-9.4)

Note: Urinary 2-OHE_1 and 16α-OHE_1 metabolites (ng/ml) are standardized to urinary creatinine levels (mg/dl).
Estrogen metabolite data missing for 13 subjects.
*Median metabolite values (range)

Table 44 Median Estrogen Metabolite Levels by 5 year groups

Estrogen Metabolite	Age Categories (years)						
	< 50 (N=30)	**50-54** (N=231)	**55-59** (N=414)	**60-64** (N=333)	**65-69** (N=88)	**70-74** (N=7)	**≥ 75** (N=5)
2-OHE$_1$	7.0	3.4	4.1	3.7	7.3	7.4	12.9
16α-OHE$_1$	4.8	2.0	2.3	2.3	4.0	4.2	6.0
2:16α-OHE$_1$	1.8	1.8	1.9	1.8	2.1	1.6	1.7

Note: Urinary 2-OHE$_1$ and 16α-OHE$_1$ metabolites (ng/ml) are standardized to urinary creatinine levels (mg/dl).

Table 45 Estrogen and Progesterone Receptor Status by Age (n=300 postmenopausal cases)

Age	Estrogen Receptor N (%)				Progesterone Receptor N (%)		
	Positive	**Negative**	**Unknown**		**Positive**	**Negative**	**Unknown**
<50	2	3	0		1	2	2
50-55	44	24	5		9	19	45
55-60	88	25	9		22	19	81
60-65	55	23	6		21	17	46
65-70	11	0	2		6	1	6
70-75	0	0	1		0	0	1
≥ 75	1	1	0		0	0	2
Total	201	76	23		59	58	183

C.2 LOWESS SMOOTHER PLOTS FOR EACH ESTROGEN METABOLITE, LOGIT SCALE: including all postmenopausal studies

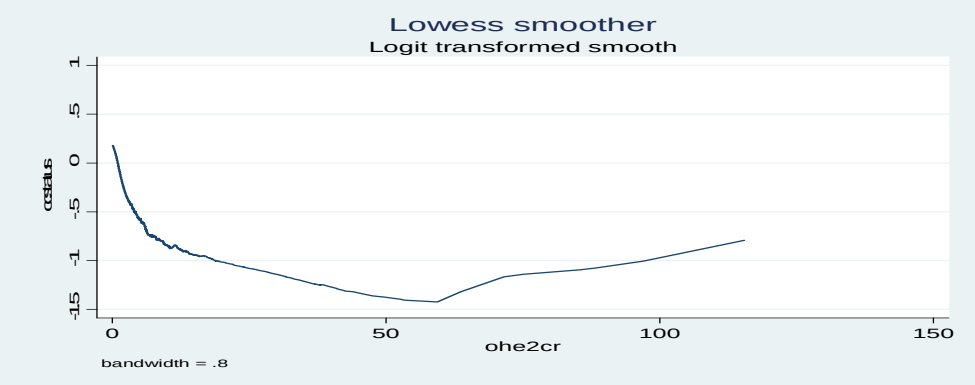


Figure 18 Lowess Smoother Plot for 2-OHE$_1$, Logit Scale (All Postmenopausal Studies)

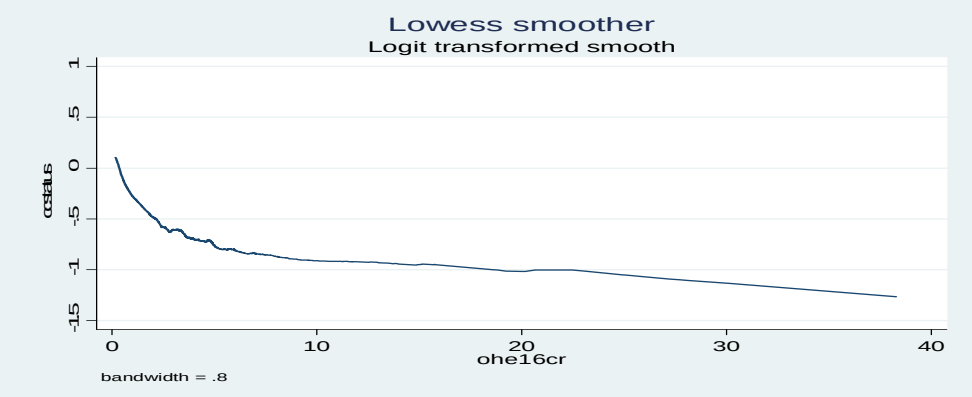


Figure 19 Lowess Smoother Plot for 16α-OHE$_1$, Logit Scale (All Postmenopausal Studies)

Figure 20 Lowess Smoother Plot for 2:16α-OHE$_1$, Logit Scale (All Postmenopausal Studies)

C.3 LOWESS SMOOTHER PLOTS FOR EACH ESTROGEN METABOLITE, LOGIT SCALE: INCLUDING 3 NESTED CASE CONTROL STUDIES

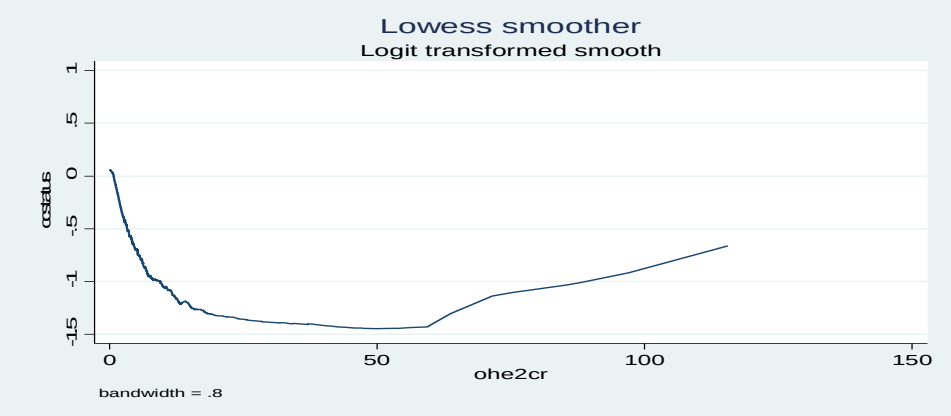


Figure 21 Lowess Smoother Plot for 2-OHE$_1$, Logit Scale (only nested case-control studies)

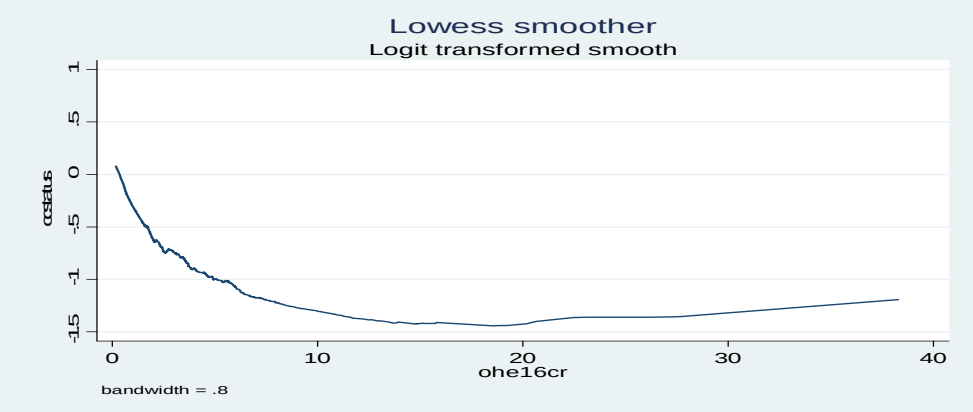


Figure 22 Lowess Smoother Plot for 16α-OHE$_1$, Logit Scale (only nested case-control studies)

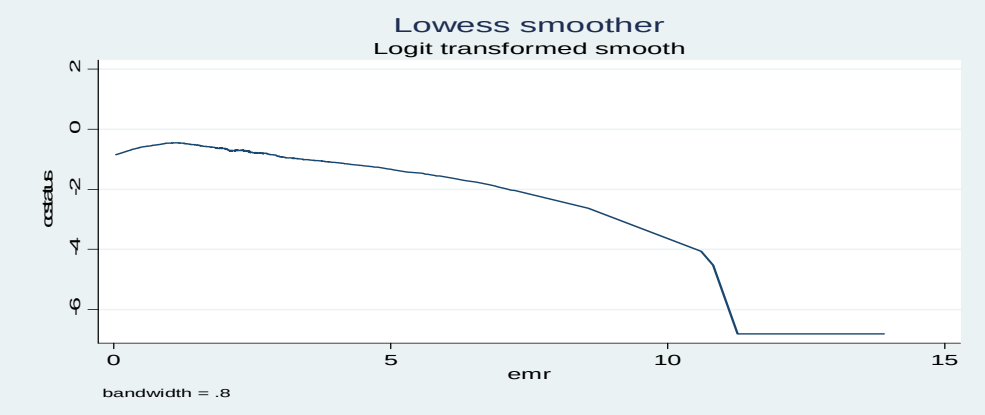


Figure 23 Lowess Smoother Plot for 2:16α-OHE$_1$, Logit Scale (only nested case-control studies)

C.4 LOWESS SMOOTHER PLOTS FOR EACH ESTROGEN METABOLITE BY STUDY, LOGIT SCALE

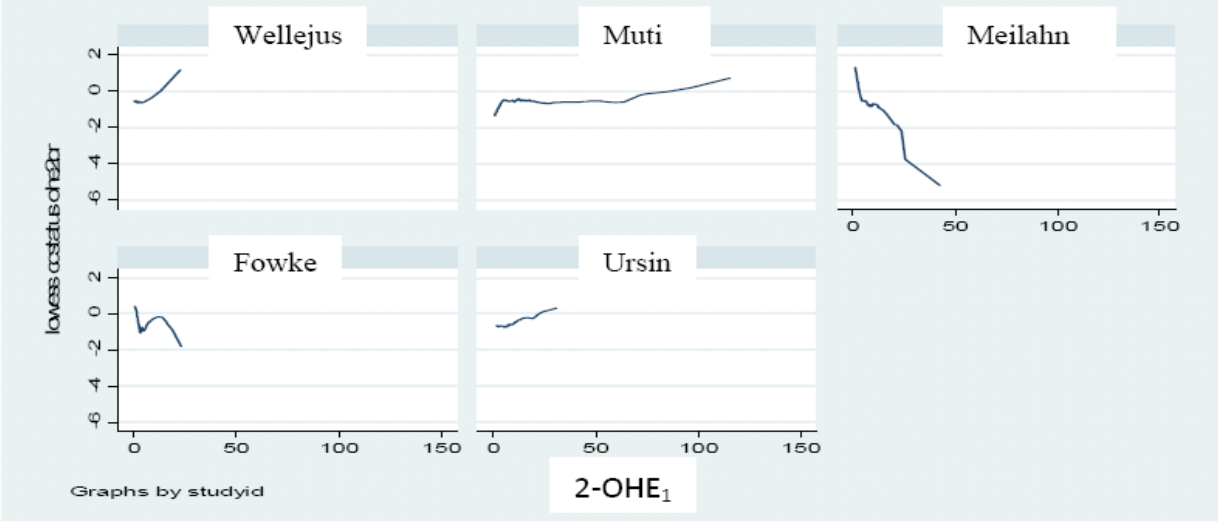


Figure 24 Lowess Smoother Plot for 2-OHE$_1$ by Study, Logit Scale (Postmenopausal Studies)

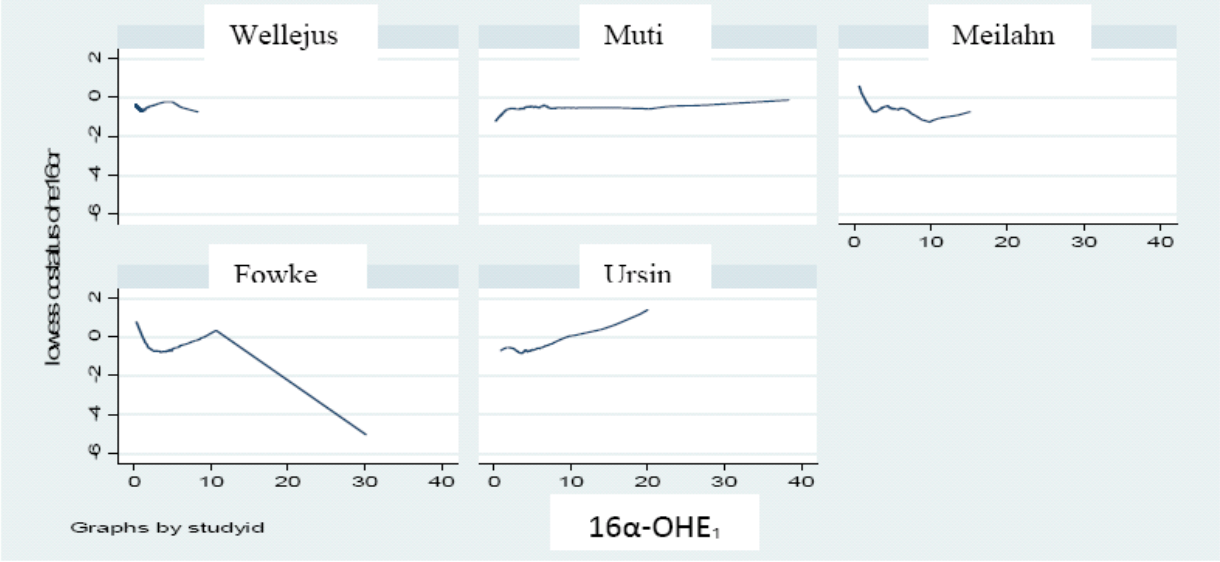


Figure 25 Lowess Smoother Plot for 16α-OHE$_1$ by Study, Logit Scale (Postmenopausal Studies)

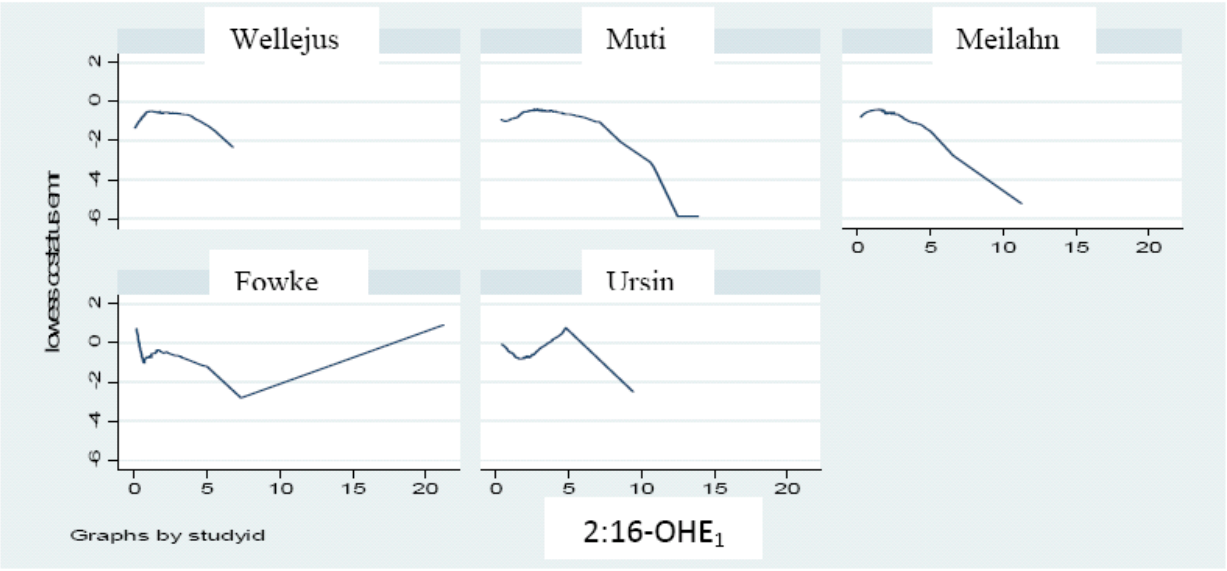
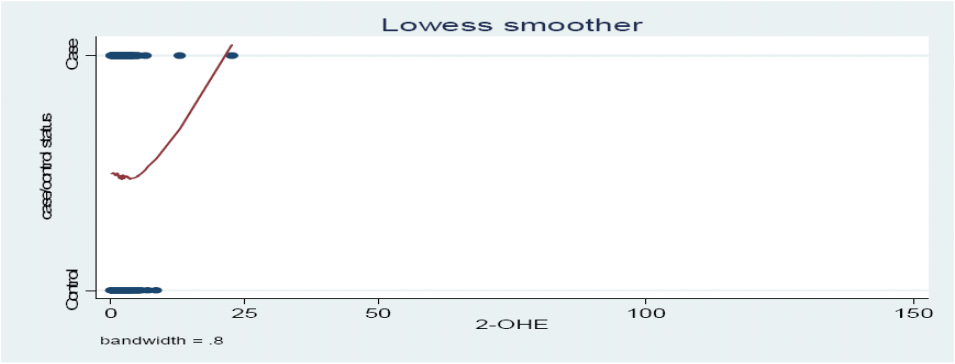


Figure 26 Lowess Smoother Plot for 2:16α-OHE_1 by Study, Logit Scale (Postmenopausal Studies)

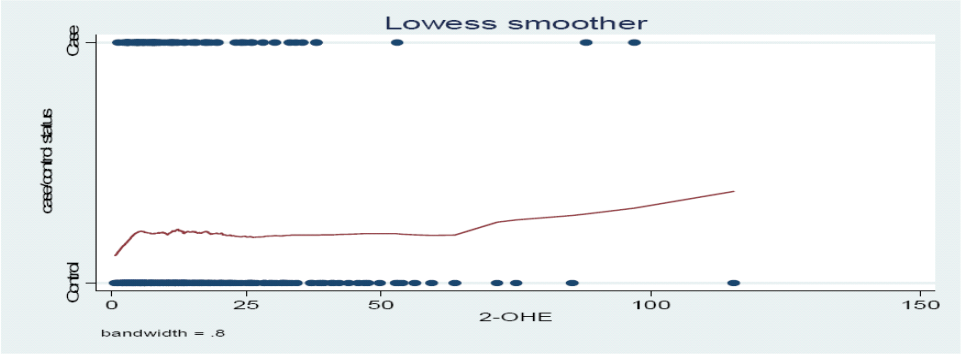
C.5 LOWESS SMOOTHER PLOTS FOR EACH ESTROGEN METABOLITE BY STUDY, P SCALE

2-OHE$_1$ ng/ml per mg/dl of creatinine

Wellejus et al.



Muti et al.



Meilahn et al.

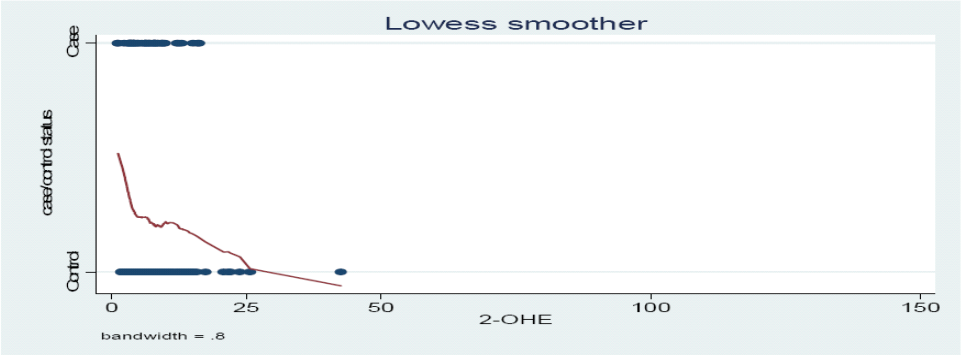
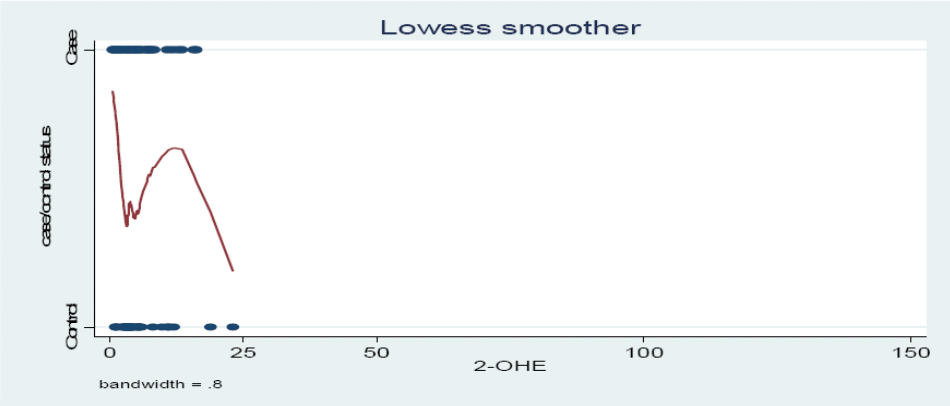


Figure 27 Lowess Smoother Plots for 2-OHE$_1$ by Study, P Scale (Postmenopausal Studies)

Fowke et al.



Ursin et al.

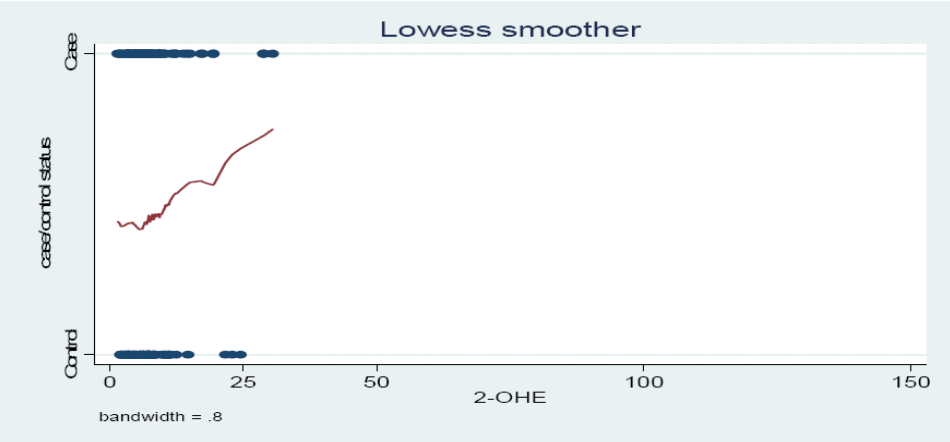
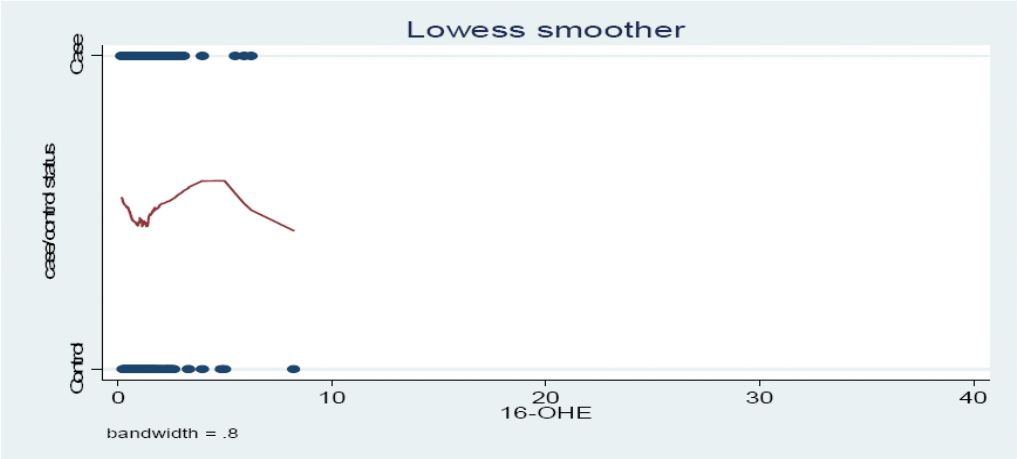


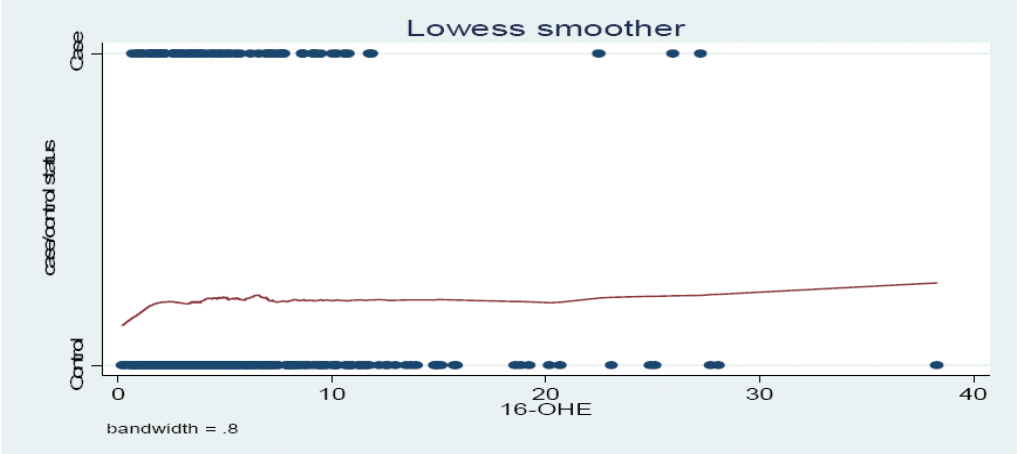
Figure 27 continued.

16α-OHE$_1$ ng/ml per mg/dl of creatinine

Wellejus et al.



Muti et al.



Meilahn et al.

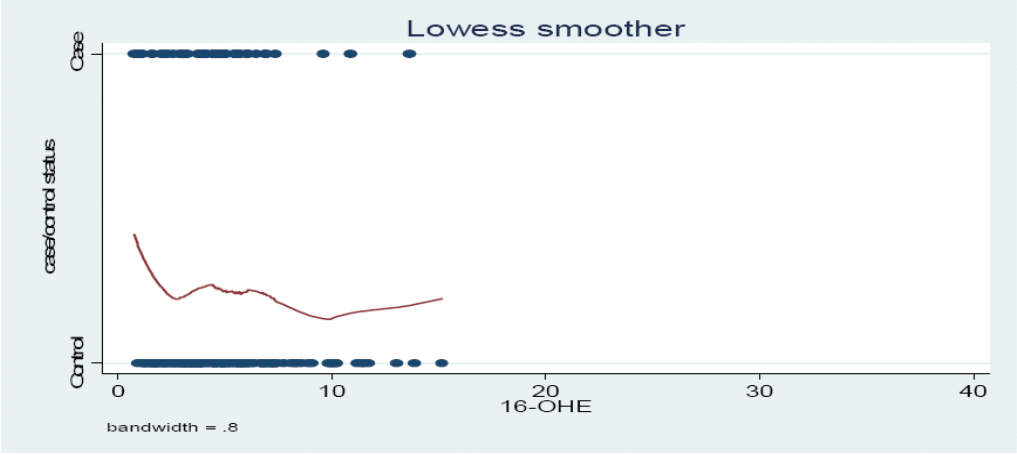
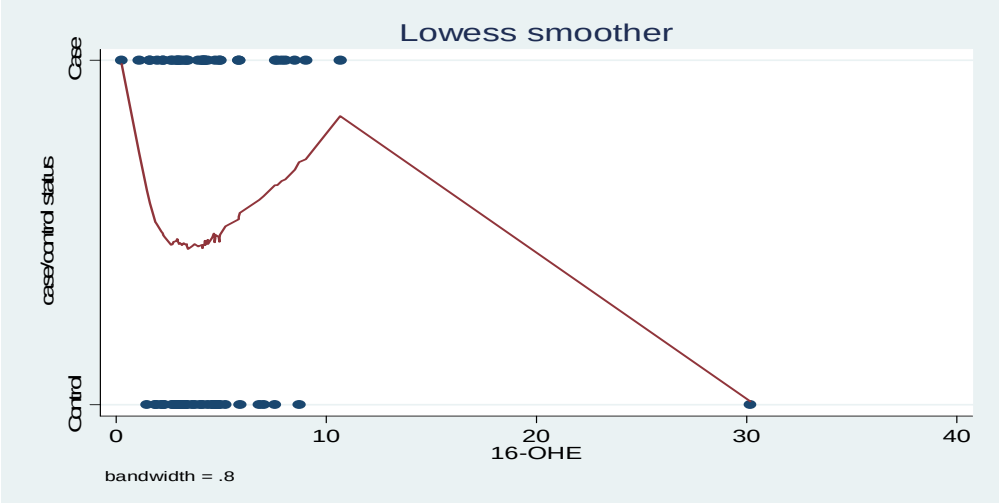


Figure 28 Lowess Smoother Plots for 16α-OHE$_1$ by Study, P Scale (Postmenopausal Studies)

Fowke et al.



Ursin et al.

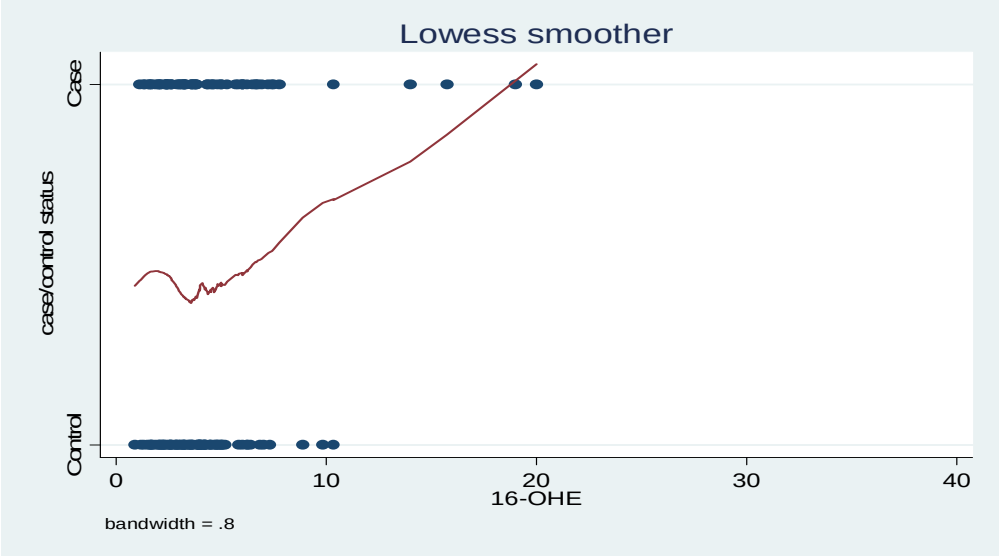
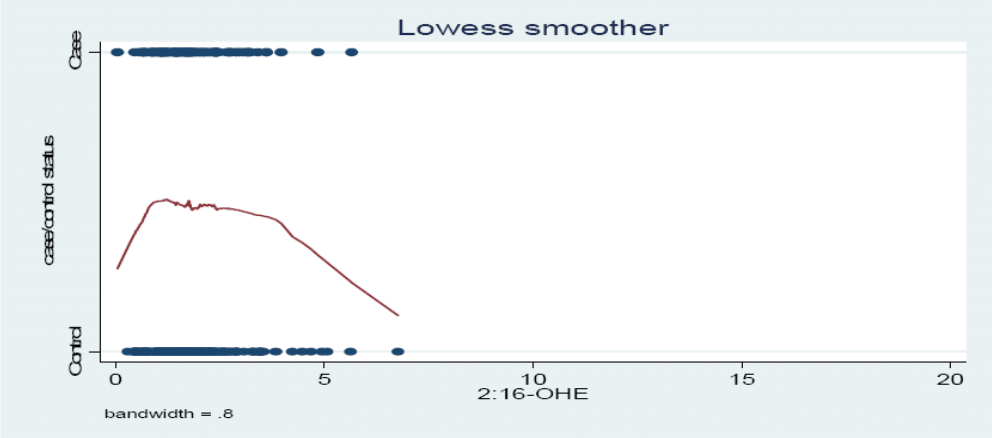


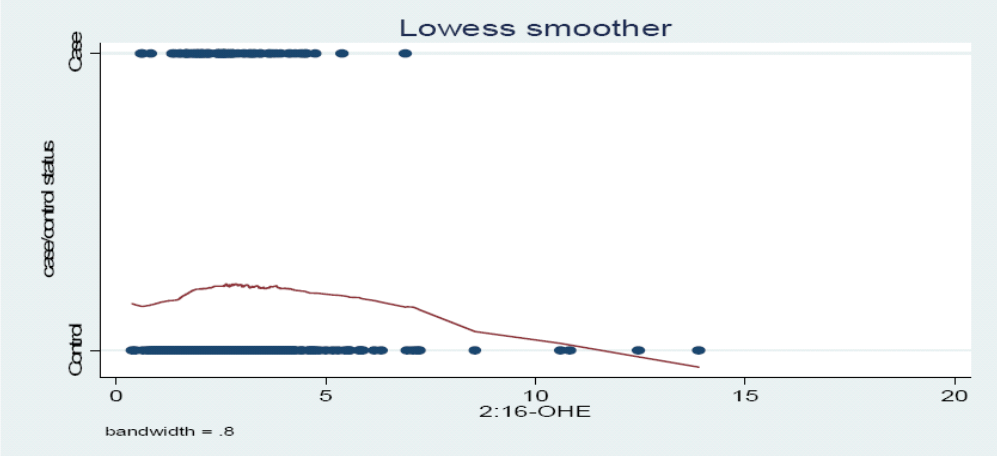
Figure 28 continued.

2:16α-OHE$_1$

Wellejus et al.



Muti et al.



Meilahn et al.

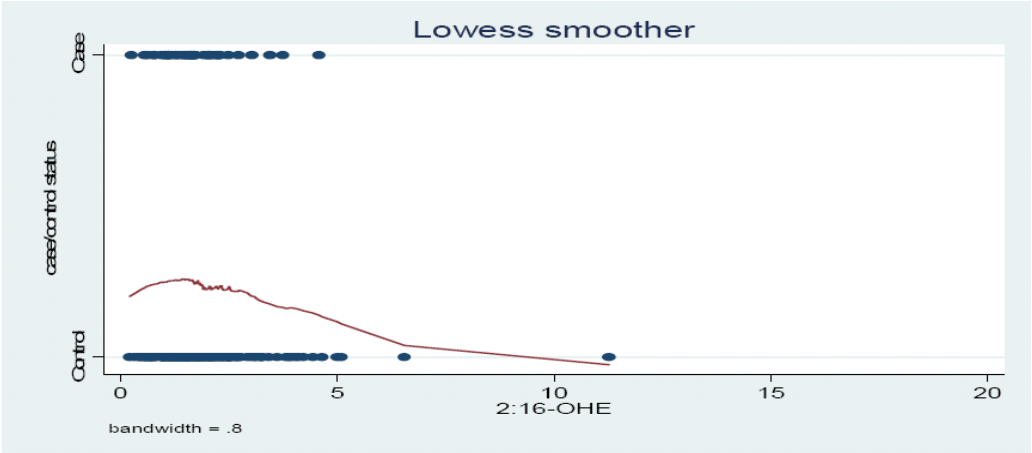
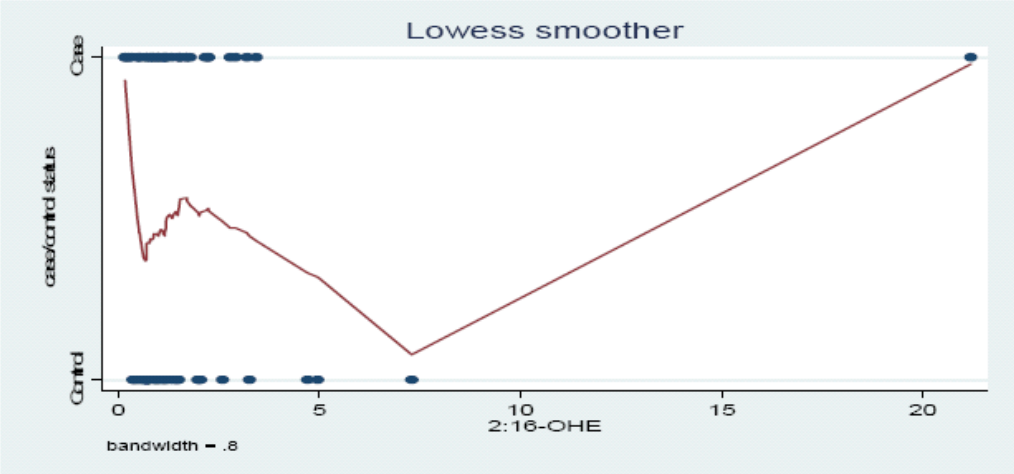


Figure 29 Lowess Smoother Plots for 2:16α-OHE$_1$ by Study, P Scale (Postmenopausal Studies)

2:16α-OHE$_1$

Fowke et al.



Ursin et al.

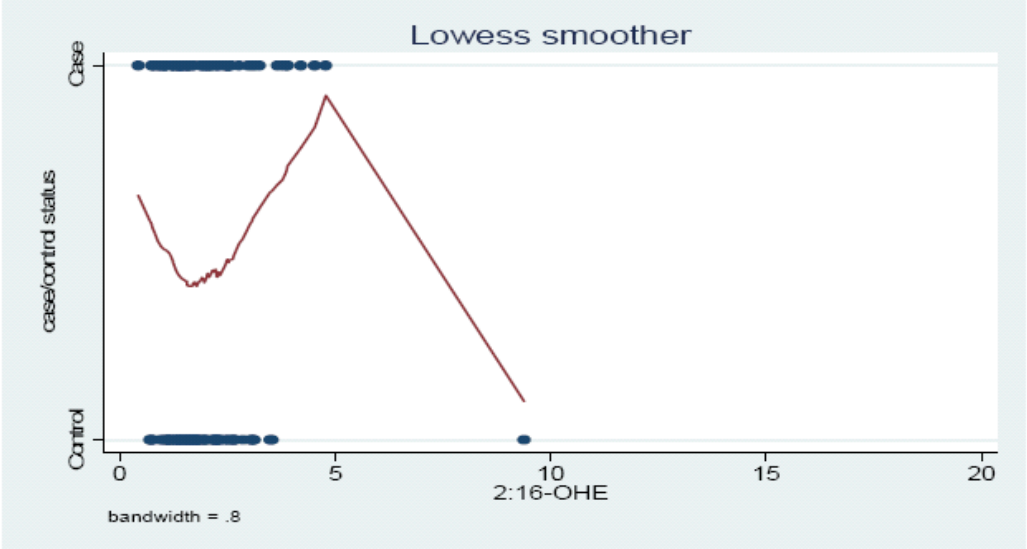


Figure 29 continued

APPENDIX D: URINARY ESTROGEN METABOLITES IN RELATION TO BREAST CANCER FACTORS AMONG HEALTHY WOMEN

D.1 RESULTS FROM ADDITIONAL ANALYSES

Table 46 Results from separate linear regression models estimating the relationship between 2-OHE_1, 16α-OHE_1 and 2:16-OHE_1 and each listed characteristic among premenopausal women.

Variable	2-OHE_1			16α-OHE_1			2:16α-OHE_1	
	B (SE)	p-value		B (SE)	p-value		B (SE)	p-value
Age	-0.0011 (0.0077)	0.89		-0.0097 (0.0073)	0.19		0.0087 (0.0044)	0.05
Age at first menstrual period	-0.0037 (0.0053)	0.49		-0.0075 (0.0084)	0.14		-0.014 (0.0047)	0.003
Age at first pregnancy	0.0033 (0.0087)	0.70		-0.0037 (0.0084)	0.66		0.0070 (0.0051)	0.17
BMI (kg/m^2)	-0.018 (0.0081)	0.03		-0.0042 (0.0077)	0.58		0.0038 (0.0031)	0.21
Waist to hip ratio	-1.85 (0.77)	0.02		-0.92 (0.73)	0.21		-0.92 (0.41)	0.03
Family history of breast cancer								
Yes	0.17 (0.17)	0.30		0.0202 (0.16)	0.90		0.15 (0.96)	0.11
History of benign breast disease								
Yes	0.026 (0.090)	0.77		0.023 (0.85)	0.79		0.0041 (0.051)	0.94
Smoking								
Former	-0.11 (0.104)	0.28		-0.11 (0.089)	0.27		-0.0045 (0.60)	0.94
Current	0.081 (0.094)	0.39		-0.097 (0.089)	0.27		-0.016 (0.054)	0.76
Unknown	-0.038 (0.154)	0.80		-0.0905 (0.15)	0.54		0.052 (0.089)	0.56
Alcohol use								
Yes	-0.056 (0.098)	0.57		-0.018 (0.093)	0.85		-0.038 (0.057)	0.50
Unknown	-0.428 (0.413)	0.30		-0.44 (0.39)	0.26		0.012 (0.238)	0.96

Note: Metabolite outcomes on log scale

1=Among parous women (N=489), 2= waist to hip ratio available in studies by Muti and Fowke et al., not in Meilahn study (N=360)

Each model includes terms for age and separate intercepts for the studies

Table 47 Results from separate linear regression models estimating the relationship between 2-OHE_1, 16α-OHE_1 and 2:16-OHE_1 and each listed characteristic among postmenopausal women.

Variable	2-OHE_1			16α-OHE_1			2:16α-OHE_1	
	B (SE)	p-value		B (SE)	p-value		B (SE)	p-value
Age	-0.073 (0.0055)	0.19		-0.013 (0.0052)	0.0096		0.0062 (0.0040)	0.12
Age at first menstrual period	-0.014 (0.0076)	0.07		-0.0074 (0.0072)	0.31		-0.018 (0.012)	0.15
Age at menopause	-0.0075 (0.0056)	0.18		0.00084 (0.0053)	0.87		-0.0083 (0.0040)	0.04
Age at first pregnancy[1]	-0.013 (0.0065)	0.05		0.00064 (0.00620	0.92		-0.013 (0.0047)	0.005
BMI (kg/m^2)	-0.011 (0.0068)	0.10		-0.0062 (0.0065)	0.34		-0.0052 (0.0050)	0.29
Waist to hip ratio[2]	-0.58 (0.50)	0.25		0.097 (0.46)	0.83		-0.67 (0.33)	0.04
Family history of breast cancer								
Yes	0.076 (0.11)	0.50		-0.039 (0.11)	0.71		0.12 (0.078)	0.14
History of benign breast disease								
Yes	0.050 (0.078)	0.52		0.18 (0.080)	0.023		-0.12 (0.061)	0.05
Smoking								
Former	-0.070 (0.083)	0.40		-0.13 (0.079)	0.091		0.063 (0.061)	0.30
Current	0.14 (0.81)	0.09		0.102 (0.076)	0.18		0.037 (0.059)	0.52
Unknown	0.000082 (0.16)	1.00		0.22 (0.15)	0.14		-0.22 (0.11)	0.06
Alcohol use								
Yes	0.31 (0.086)	0.0004		0.24 (0.083)	0.0045		0.070 (0.063)	0.27
Unknown	-0.402 (0.42)	(0.34)		-0.38 (0.40)	0.35		-0.026 (0.31)	0.93

Note: metabolite outcomes on log scale

1=Among parous women, 2= waist to hip ratio available in studies by Muti et al. and Fowke et al., not in Meilahn study

Each model includes terms for age and separate intercepts for the studies.

D.2 DISTRIBUTION OF ESTROGEN METABOLITES BY STUDY

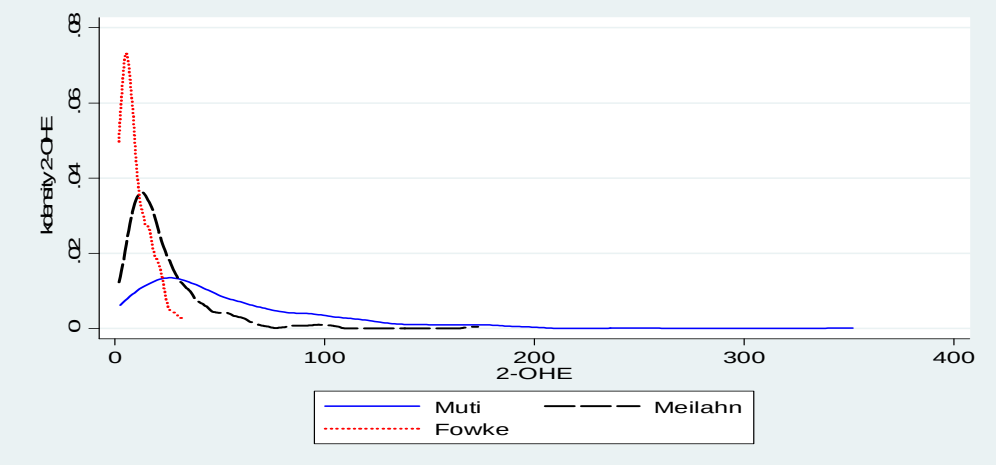


Figure 30 Distribution of 2-OHE$_1$* among Premenopausal Women by Study
*ng/ml adjusted for creatinine (mg/dl)

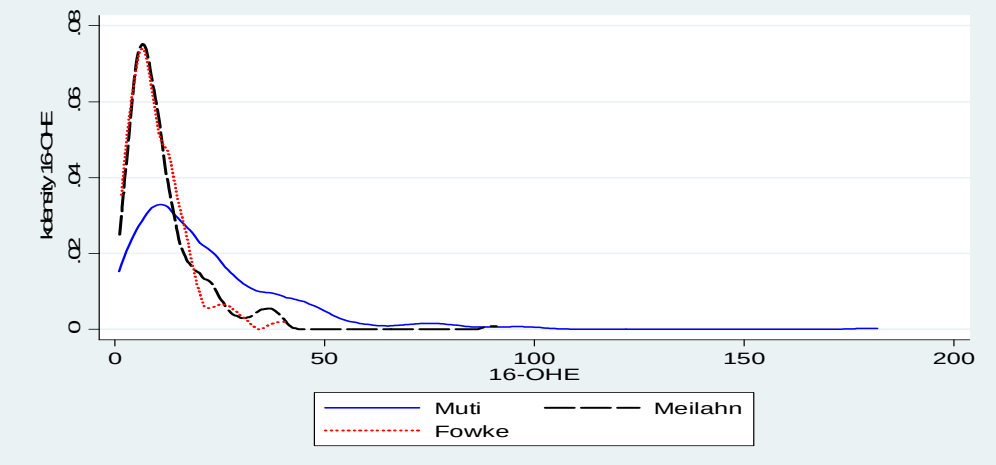


Figure 31 Distribution of 16α-OHE$_1$* among Premenopausal Women by Study
*ng/ml adjusted for creatinine (mg/dl)

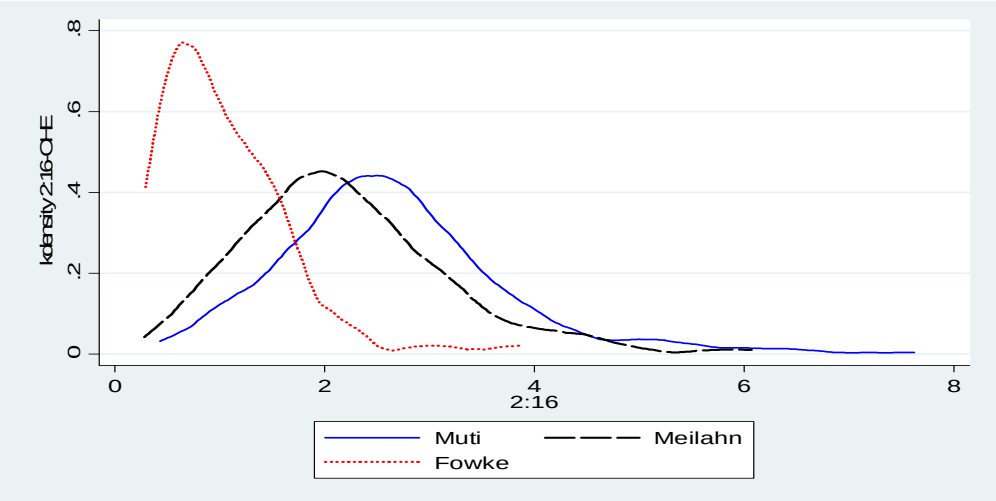


Figure 32 Distribution of 2:16α-OHE$_1$ among Premenopausal Women by Study

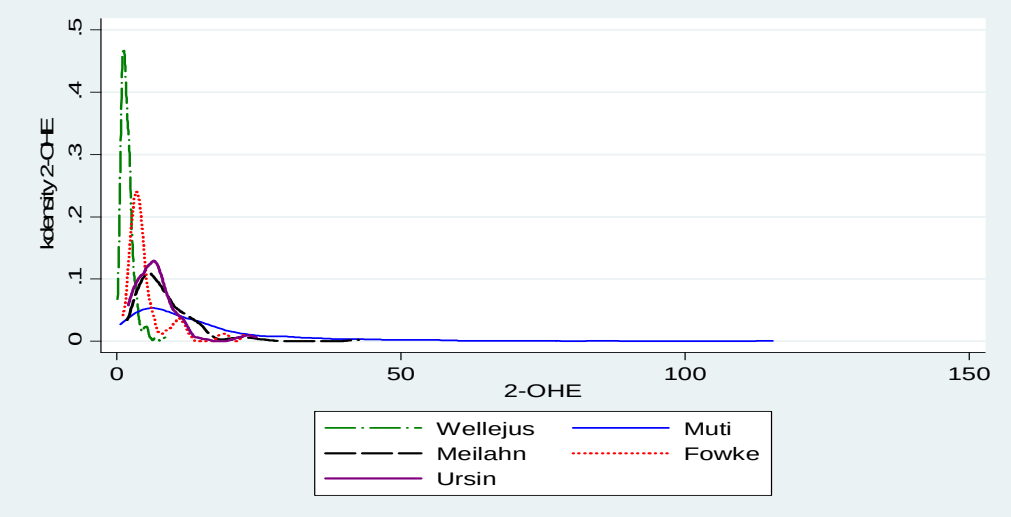


Figure 33 Distribution of 2-OHE$_1$* among Postmenopausal Women by Study
*ng/ml adjusted for creatinine (mg/dl)

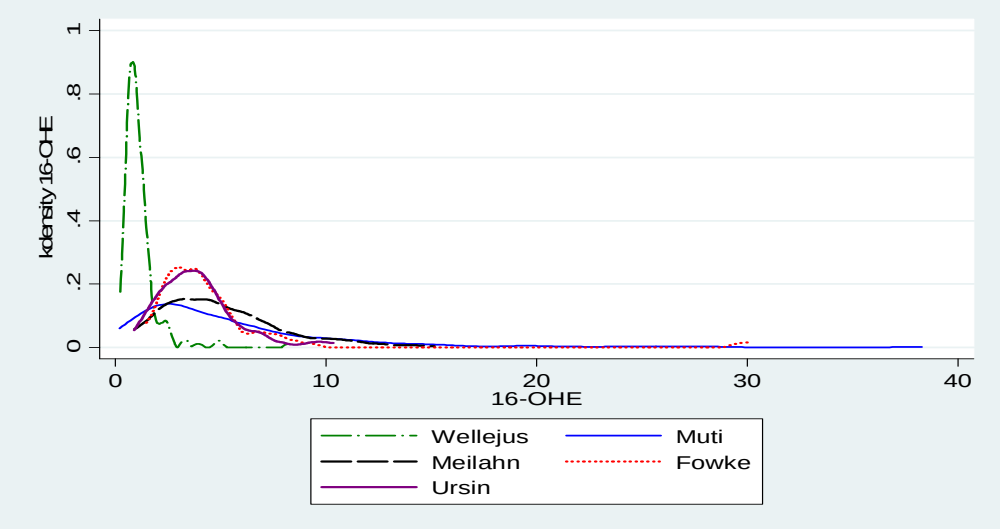


Figure 34 Distribution of 16α-OHE$_1$* among Postmenopausal Women by Study
*ng/ml adjusted for creatinine (mg/dl)

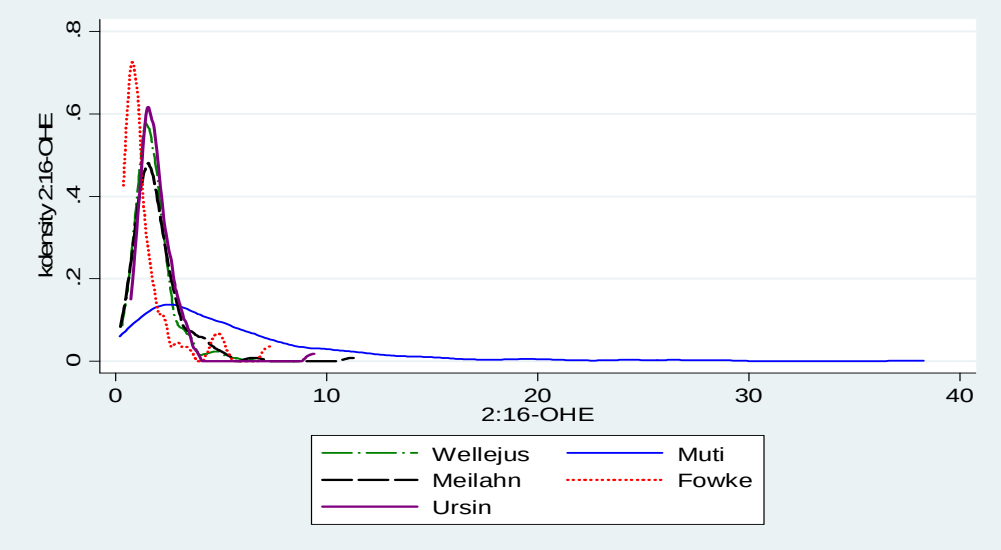


Figure 35 Distribution of 2:16α-OHE$_1$* among Postmenopausal Women by Study

D.3 STUDY SPECIFIC CORRELATIONS

Table 48 Correlation between Estrogen Metabolite Levels and Various Factors Among Premenopausal Women (Fowke et al.) (N=72)

Variable	Estrogen Metabolites Correlation Coefficient* (p-value)		
	2-OHE	**16α-OHE**	**2:16α-OHE**
Age (years)	0.029 (0.81)	-0.050 (0.68)	0.066 (0.58)
Body Mass Index (kg/m_2)	-0.042 (0.73)	-0.032 (0.79)	-0.10 (0.40)
Waist-to-hip ratio	-0.090 (0.46)	-0.0082 (0.95)	-0.19 (0.12)
Age at menarche	-0.043 (0.72)	0.22 (0.067)	-0.25 (0.033)
Age at First Pregnancy†	-0.080 (0.52)	-0.068 (0.59)	-0.013 (0.91)

Note: Urinary $2\text{-}OHE_1$ and $16\alpha\text{-}OHE_1$ metabolites (ng/ml) are standardized to urinary creatinine levels (mg/dl).
*Spearman correlation coefficients and corresponding p-value
†Among women ever pregnant

Table 49 Correlation between Estrogen Metabolite Levels and Various Factors Among Postmenopausal Women (Fowke et al.) (N=37)†

Variable	Estrogen Metabolites Correlation Coefficient* (p-value)		
	2-OHE	**16α-OHE**	**2:16α-OHE**
Age (years)	-0.09 (0.58)	-0.17 (0.30)	0.12 (0.48)
Age at menopause	-0.26 (0.12)	0.03 (0.86)	-0.15 (0.37)
Body Mass Index (kg/m_2)	0.15 (0.36)	-0.11 (0.53)	0.16 (0.34)
Waist-to-hip ratio	-0.086 (0.61)	-0.20 (0.24)	0.051 (0.77)
Age at menarche	-0.033 (0.85)	0.044 (0.79)	-0.049 (0.77)
Age at First Pregnancy†	-0.25 (0.14)	-0.059 (0.74)	-0.22 (0.20)

Note: Urinary $2\text{-}OHE_1$ and $16\alpha\text{-}OHE_1$ metabolites (ng/ml) are standardized to urinary creatinine levels (mg/dl).*Spearman correlation coefficients and corresponding p-value
†Among women ever pregnant

Table 50 Correlation between Estrogen Metabolite Levels and Various Factors Among Premenopausal Women (Meilahn et al.) (N=184)

Variable	Estrogen Metabolites Correlation Coefficient* (p-value)		
	2-OHE	**16α-OHE**	**2:16α-OHE**
Age (years)	0.17 (0.022)	0.18 (0.013)	-0.070 (0.36)
Weight (kg)	-0.23 (0.0017)	-0.15 (0.040)	-0.12 0.09
Body Mass Index (kg/m_2)	-0.15 (0.044)	-0.077 (0.30)	-0.11 (0.14)
Age at menarche	-0.055 (0.46)	-0.082 (0.27)	0.036 (0.62)
Age at First Pregnancy†	0.0066 (0.93)	0.020 (0.79)	-0.040 (0.59)

Note: Urinary 2-OHE and 16α-OHE metabolites (ng/ml) are standardized to urinary creatinine levels (mg/dl).
*Spearman correlation coefficients and corresponding p-value
†Among women ever pregnant

Table 51 Correlation between Estrogen Metabolite Levels and Various Factors Among Postmenopausal Women (Meilahn et al.) (N=139)†

Variable	Estrogen Metabolites Correlation Coefficient* (p-value)		
	2-OHE	**16α-OHE**	**2:16α-OHE**
Age (years)	0.11 (0.20)	-0.0083 (0.92)	0.094 (0.27)
Age at menopause	-0.12 (0.16)	-0.10 (0.25)	-0.020 (0.83)
Weight (kg)	0.054 (0.53)	0.15 0.081	-0.11 0.19
Body Mass Index (kg/m_2)	0.17 0.047	0.20 0.016	-0.093 (0.27)
Age at menarche	-0.20 (0.021)	-0.069 (0.42)	-0.17 (0.043)
Age at First Pregnancy†	-0.12 (0.17)	0.0052 (0.95)	-0.14 (0.10)

Note: Urinary 2-OHE_1 and 16α-OHE_1 metabolites (ng/ml) are standardized to urinary creatinine levels (mg/dl).
*Spearman correlation coefficients

Table 52 Correlation between Estrogen Metabolite Levels and Various Factors Among Premenopausal Women (Muti et al.) (N=292)

Variable	Estrogen Metabolites Correlation Coefficient* (p-value)		
	2-OHE	**16α-OHE**	**2:16α-OHE**
Age (years)	-0.074 (0.21)	-0.18 (0.0023)	0.17 (0.0050)
Weight (kg)	-0.13 (0.023)	-0.087 (0.14)	-0.16 0.0084
Body Mass Index (kg/m_2)	-0.048 (0.42)	-0.022 (0.71)	-0.13 (0.035)
Waist-to-hip ratio	-0.15 (0.011)	-0.12 (0.044)	-0.12 (0.040)
Age at menarche	-0.067 (0.26)	-0.083 (0.16)	0.034 (0.56)
Age at First Pregnancy†	0.0066 (0.93)	-0.064 (0.31)	0.11 (0.086)

Note: Urinary 2-OHE and 16α-OHE metabolites (ng/ml) are standardized to urinary creatinine levels (mg/dl).
*Spearman correlation coefficients and corresponding p-value
†Among women ever pregnant

Table 53 Correlation between Estrogen Metabolite Levels and Various Factors Among Postmenopausal Women (Muti et al.) (N=139)†

Variable	Estrogen Metabolites Correlation Coefficient* (p-value)		
	2-OHE	**16α-OHE**	**2:16α-OHE**
Age (years)	-0.11 (0.076)	-0.16 (0.0069)	0.077 (0.21)
Age at menopause	-0.094 (0.12)	-0.020 (0.75)	-0.15 (0.012)
Weight (kg)	-0.19 (0.0018)	-0.17 (0.0039)	-0.094 0.12
Body Mass Index (kg/m_2)	-0.16 (0.010)	-0.16 (0.0067)	-0.062 (0.30)
Waist-to-hip ratio	-0.085 (0.16)	-0.017 (0.78)	-0.12 (0.056)
Age at menarche	-0.13 (0.035)	-0.078 (0.20)	-0.11 (0.071)
Age at First Pregnancy†	0.0078 (0.90)	0.016 (0.81)	-0.044 (0.50)

Note: Urinary 2-OHE and 16α-OHE metabolites (ng/ml) are standardized to urinary creatinine levels (mg/dl).
*Spearman correlation coefficients and corresponding p-value
†Among women ever pregnant

Table 54 Correlation between Estrogen Metabolite Levels and Various Factors Among Postmenopausal Women (Ursin et al.) (N=76)†

Variable	Estrogen Metabolites Correlation Coefficient* (p-value)		
	2-OHE	**16α-OHE**	**2:16α-OHE**
Age	0.032 (0.78)	0.096 (0.41)	-0.060 (0.60)
Age at menopause			
Weight (kg)	0.042 (0.72)	0.051 (0.66)	-0.060 0.61
Body Mass Index (kg/m_2)	-0.0080 (0.95)	0.11 (0.36)	-0.18 (0.12)
Age at menarche	0.045 (0.70)	-0.035 (0.76)	0.079 (0.49)
Age at First Pregnancy†	-0.063 (0.59)	-0.061 (0.60)	0.0066 (0.95)

Note: Urinary 2-OHE and 16α-OHE metabolites (ng/ml) are standardized to urinary creatinine levels (mg/dl).
*Spearman correlation coefficients and corresponding p-value
†Among women ever pregnant

Table 55 Correlation between Estrogen Metabolite Levels and Various Factors Among Postmenopausal Women (Wellejus et al.) (N=200)†

Variable	Estrogen Metabolites Correlation Coefficient* (p-value)		
	2-OHE	**16α-OHE**	**2:16α-OHE**
Age	-0.032 (0.65)	-0.096 (0.17)	-0.30 (0.67)
Age at menopause	0.016 (0.83)	-0.075 (0.30)	0.084 (0.24)
Weight (kg)	-0.086 0.22	-0.064 0.37	-0.033 0.64
Body Mass Index (kg/m_2)	-0.032 (0.66)	-0.014 (0.84)	-0.024 (0.73)
Waist-to-hip ratio	-0.018 (0.80)	-0.023 (0.75)	0.044 (0.53)
Age at menarche	0.047 (0.51)	0.085 (0.24)	-0.031 (0.67)
Age at First Pregnancy†	-0.12 (0.13)	-0.14 (0.074)	-0.018 (0.82)

Note: Urinary 2-OHE and 16α-OHE metabolites (ng/ml) are standardized to urinary creatinine levels (mg/dl). This table excludes current HRT users.
*Spearman correlation coefficients and corresponding p-value
†Among women ever pregnant

D.4 STUDY SPECIFIC MEDIAN ESTROGEN METABOLITE LEVELS BY CATEGORIES OF BREAST CANCER RISK FACTORS

Table 56 Median Estrogen Metabolite Levels by Selected Factors: Premenopausal (Fowke et al.) (N=72)

Variable	N	2-OHE_1		16α-OHE_1		2:16α-OHE_1
Family History of Breast Cancer						
Yes	0	-		-		-
No	72	6.9		8.7		0.84
p-value		n/a		n/a		n/a
History of Benign Breast Disease						
Yes	6	6.9		5.1		0.98
No	66	7.0		9.1		0.83
p-value		0.57		0.56		0.58
Smoking						
Never	70	6.9		8.6		0.83
Former	2	13.3		11.0		1.2
Current	0	-		-		-
p-value		0.31		0.55		0.47
Alcohol Consumption						
Yes	72	6.9		8.7		0
No	0	n/a		n/a		n/a
p-value						
BMI (kg/m^2)						
< 25	61	6.5		8.5		0.84
≥ 25	11	9.0		9.1		0.68
p-value		0.51		0.68		0.83
Oral Contraceptive Use						
Yes	12	9.0		9.6		1.1
No	60	6.9		8.4		0.81
p-value		0.35		0.77		0.39
Parity						
Yes	68	7.0		9.1		0.88
No	4	4.0		7.1		0.57
p-value		0.19		1.00		0.04

Note: Urinary 2-OHE_1 and 16α-OHE_1 metabolites (ng/ml) are standardized to urinary creatinine levels (mg/dl).

Table 57 Median Estrogen Metabolite Levels by Selected Factors: Postmenopausal (Fowke et al.) (N=37)

Variable	N	2-OHE_1		16α-OHE_1		2:16α-OHE_1
Family History of Breast Cancer						
Yes	1	-		-		-
No	36	3.6		3.5		0.98
p-value		n/a		n/a		n/a
History of Benign Breast Disease						
Yes	1	-		-		-
No	36	3.6		3.5		0.94
p-value		0.11		0.85		0.13
Smoking						
Never	36	3.6		3.7		0.94
Former	1	-		-		-
Current	0	-		-		-
p-value		n/a		n/a		n/a
Alcohol Consumption						
Yes	37	3.7		3.6		0.95
No	0	-		-		-
p-value		n/a		n/a		n/a
BMI (kg/m^2)						
< 25	25	3.6		4.1		0.90
≥ 25	12	3.9		3.1		1.2
p-value		0.80		0.13		0.21
Oral Contraceptive Use						
Yes	16	3.8		3.3		1.1
No	21	3.6		3.7		0.90
p-value		0.78		0.33		0.50
Parity						
Yes	35	3.7		3.4		0.95
No	2	-		-		-
p-value		n/a		n/a		n/a

Note: Urinary 2-OHE_1 and 16α-OHE_1 metabolites (ng/ml) are standardized to urinary creatinine levels (mg/dl).

Table 58 Median Estrogen Metabolite Levels by Selected Factors: Premenopausal (Meilahn et al.) (N=184)

Variable	N	2-OHE_1		16α-OHE_1		2:16α-OHE_1
Family History of Breast Cancer						
Yes	7	24.0		9.8		2.1
No	177	16.7		8.4		2.1
p-value		0.43		0.56		0.93
History of Benign Breast Disease						
Yes	12	27.7		10.9		2.2
No	172	17.1		8.5		2.1
p-value		0.18		0.18		0.99
Smoking						
Never	24	18.7		7.9		2.1
Former	41	18.1		10.0		2.0
Current	86	14.9		7.9		2.2
p-value		0.89		0.22		0.44
BMI (kg/m^2)						
< 25	117	18.4		8.8		2.2
≥ 25	67	15.9		8.3		2.0
p-value		0.37		0.82		0.51
Oral Contraceptive Use						
Yes	118	15.8		8.0		2.2
No	66	19.9		10.3		2.0
p-value		0.12		0.031		0.19
Parity						
Yes	167	16.6		8.4		2.1
No	17	26.6		10.6		2.2
p-value		0.15		0.34		0.28

Note: Urinary 2-OHE_1 and 16α-OHE_1 metabolites (ng/ml) are standardized to urinary creatinine levels (mg/dl).

Table 59 Median Estrogen Metabolite Levels by Selected Factors: Postmenopausal (Meilahn et al.) (N=139)

Variable	N	2-OHE_1		16α-OHE_1		2:16α-OHE_1
Family History of Breast Cancer						
Yes	14	7.2		3.7		2.1
No	124	7.1		4.6		1.6
p-value		0.65		0.45		0.15
History of Benign Breast Disease						
Yes	10	6.8		3.7		1.4
No	129	7.1		4.5		1.7
p-value		0.52		0.61		0.55
Smoking						
Never	64	7.1		4.6		1.8
Former	26	7.6		3.5		2.0
Current	21	6.7		3.8		1.8
p-value		0.48		0.37		0.75
BMI (kg/m^2)						
< 25	70	6.3		3.7		1.8
≥ 25	69	8.0		4.9		1.6
p-value		0.02		0.02		0.44
Oral Contraceptive Use						
Yes	15	6.9		3.4		1.8
No	124	7.1		4.5		1.7
p-value		0.61		0.09		0.44
Parity						
Yes	120	7.1		4.5		1.7
No	19	7.8		4.2		1.7
p-value		0.65		0.48		0.50

Note: Urinary 2-OHE_1 and 16α-OHE_1 metabolites (ng/ml) are standardized to urinary creatinine levels (mg/dl).

Table 60 Median Estrogen Metabolite Levels by Selected Factors: Premenopausal (Muti et al.) (N=288)

Variable	N	2-OHE_1		16α-OHE_1		2:16α-OHE_1
Family History of Breast Cancer						
Yes	18	34.0		12.5		3.0
No	268	41.1		17.3		2.5
p-value		0.89		0.43		0.02
History of Benign Breast Disease						
Yes	103	37.3		17.8		2.5
No	182	41.4		16.9		2.5
p-value		0.74		0.94		0.76
Smoking						
Never	166	41.0		16.3		2.6
Former	53	27.8		12.3		2.4
Current	67	43.8		19.0		2.4
p-value		0.17		0.14		0.18
Alcohol Consumption						
Yes	171	44.1		17.4		2.5
No	113	39.2		17.1		2.5
p-value		0.38		0.98		0.52
BMI (kg/m^2)						
< 25	172	40.6		16.5		2.6
≥ 25	116	41.1		17.2		2.4
p-value		0.82		0.98		0.27
Oral Contraceptive Use						
Yes	107	43.8		17.5		2.6
No	179	39.2		16.3		2.5
p-value		0.24		0.35		0.45
Parity						
Yes	257	41.5		17.1		2.5
No	31	37.9		16.1		2.2
p-value		0.32		0.67		0.04

Note: Urinary 2-OHE_1 and 16α-OHE_1 metabolites (ng/ml) are standardized to urinary creatinine levels (mg/dl).

Table 61 Median Estrogen Metabolite Levels by Selected Factors: Postmenopausal (Muti et al.) (N=272)

Variable	N	2-OHE_1		16α-OHE_1		2:16α-OHE_1
Family History of Breast Cancer						
Yes	27	8.9		3.3		2.8
No	245	10.4		4.3		2.5
p-value		0.81		0.19		0.16
History of Benign Breast Disease						
Yes	59	10.3		4.9		2.3
No	210	10.4		3.9		2.6
p-value		0.34		0.08		0.13
Smoking						
Never	214	10.2		4.1		2.5
Former	29	8.1		2.8		2.6
Current	29	14.8		5.1		2.7
p-value		0.02		0.03		0.82
Alcohol Consumption						
Yes	109	7.3		3.6		2.5
No	160	11.7		4.6		2.6
p-value		0.006		0.03		0.32
BMI (kg/m^2)						
< 25	103	12.5		4.7		2.7
≥ 25	169	8.9		3.6		2.4
p-value		0.008		0.03		0.03
Oral Contraceptive Use						
Yes	31	12.4		5.0		2.6
No	241	10.1		3.9		2.6
p-value		0.05		0.04		0.87
Parity						
Yes	237	10.3		3.9		2.6
No	35	10.3		4.9		2.5
p-value		0.43		0.23		0.63

Note: Urinary 2-OHE_1 and 16α-OHE_1 metabolites (ng/ml) are standardized to urinary creatinine levels (mg/dl).

Table 62 Median Estrogen Metabolite Levels by Selected Factors: Postmenopausal (Ursin et al.) (N=76)

Variable	N	2-OHE_1		16α-OHE_1		2:16α-OHE_1
Family History of Breast Cancer						
Yes	11	7.3		4.0		1.8
No	65	6.4		3.6		1.7
p-value		0.56		0.46		0.70
BMI (kg/m^2)						
< 25	56	6.3		3.5		1.8
≥ 25	20	7.0		4.0		1.6
p-value		0.42		0.18		0.28
Parity						
Yes	68	6.5		3.7		1.8
No	8	6.3		3.7		1.6
p-value		0.38		0.64		0.62

Note: Urinary 2-OHE_1 and 16α-OHE_1 metabolites (ng/ml) are standardized to urinary creatinine levels (mg/dl).

Table 63 Median Estrogen Metabolite Levels by Selected Factors: Postmenopausal (Wellejus et al.) (N=200)

Variable	N	2-OHE_1		16α-OHE_1		2:16α-OHE_1
History of Benign Breast Disease						
Yes	26	1.4		0.91		1.4
No	169	1.6		0.94		1.7
p-value		0.67		0.35		0.05
Smoking						
Never	78	1.4		0.85		1.7
Former	48	1.5		0.92		1.6
Current	73	1.81		1.10		1.6
p-value		0.15		0.15		0.77
Alcohol Consumption						
Yes	189	1.6		0.93		1.7
No	4	0.91		1.20		0.94
p-value		0.027		0.59		0.01
BMI (kg/m^2)						
< 25	78	1.5		0.92		1.6
≥ 25	120	1.5		0.94		1.6
p-value		0.88		0.77		0.86
Oral Contraceptive Use						
Yes	99	1.5		0.94		1.6
No	93	1.5		0.94		1.6
p-value		0.83		0.78		0.70
Parity						
Yes	170	1.5		0.91		1.6
No	29	1.5		1.10		1.6
p-value		0.86		0.34		0.66

Note: Urinary 2-OHE_1 and 16α-OHE_1 metabolites (ng/ml) are standardized to urinary creatinine levels (mg/dl).

D.5 HISTOGRAMS BY VARIOUS TRANSFORMATIONS AMONG ALL PREMENOPAUSAL CONTROLS (N=544)

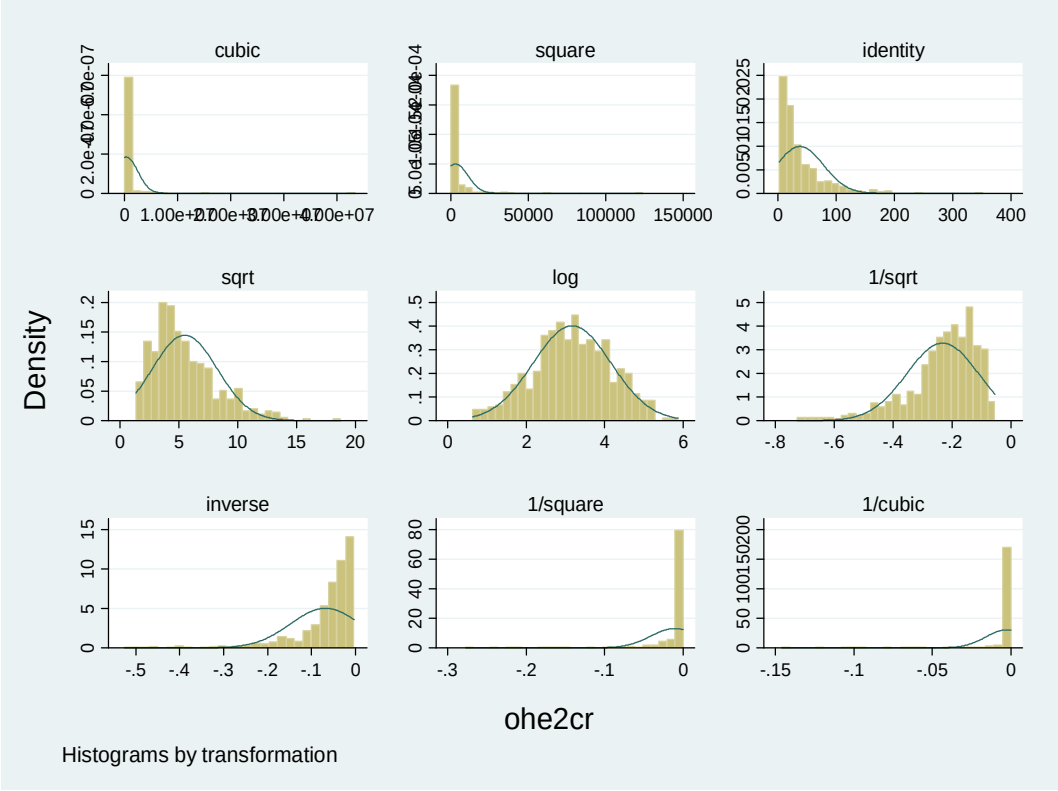


Figure 36 Metabolite: 2-OHE$_1$: Histograms by Various Transformations Among All Premenopausal Controls (N=544)

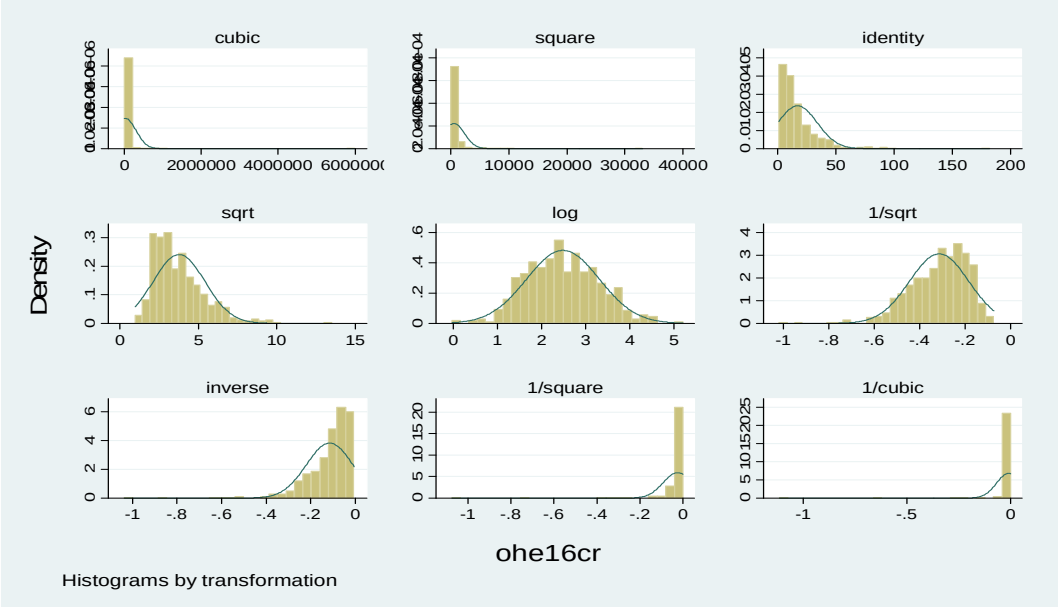


Figure 37 Metabolite: 16α-OHE$_1$: Histograms by Various Transformations Among All Premenopausal Controls (N=544)

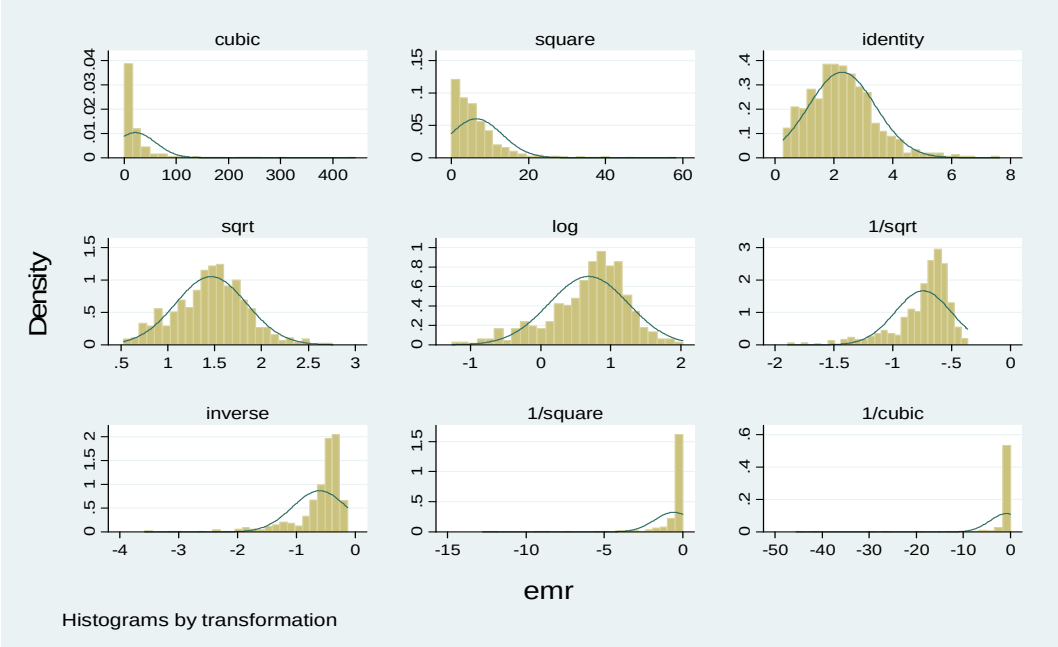


Figure 38 Metabolite: 2:16α-OHE$_1$: Histograms by Various Transformations Among All Premenopausal Controls (N=544)

D.6 HISTOGRAMS BY VARIOUS TRANSFORMATIONS AMONG ALL POSTMENOPAUSAL CONTROLS (N=720)

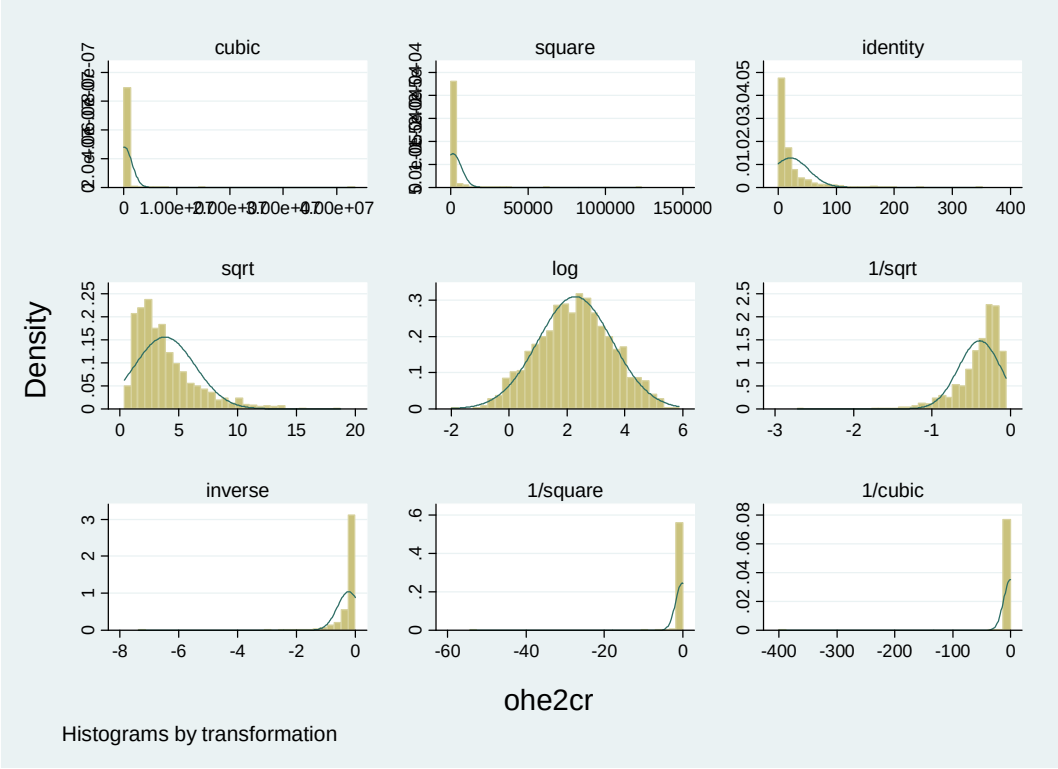


Figure 39 Metabolite: 2-OHE$_1$: Histograms by Various Transformations Among All Postmenopausal Controls (N=720)

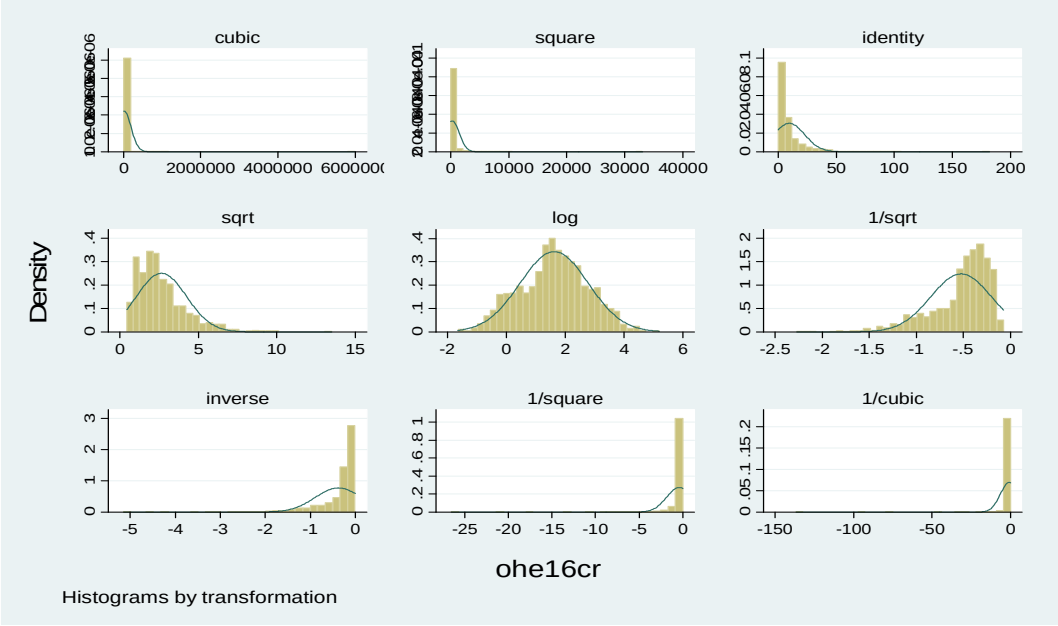


Figure 40 Metabolite: 16α-OHE$_1$: Histograms by Various Transformations Among All Postmenopausal Controls (N=720)

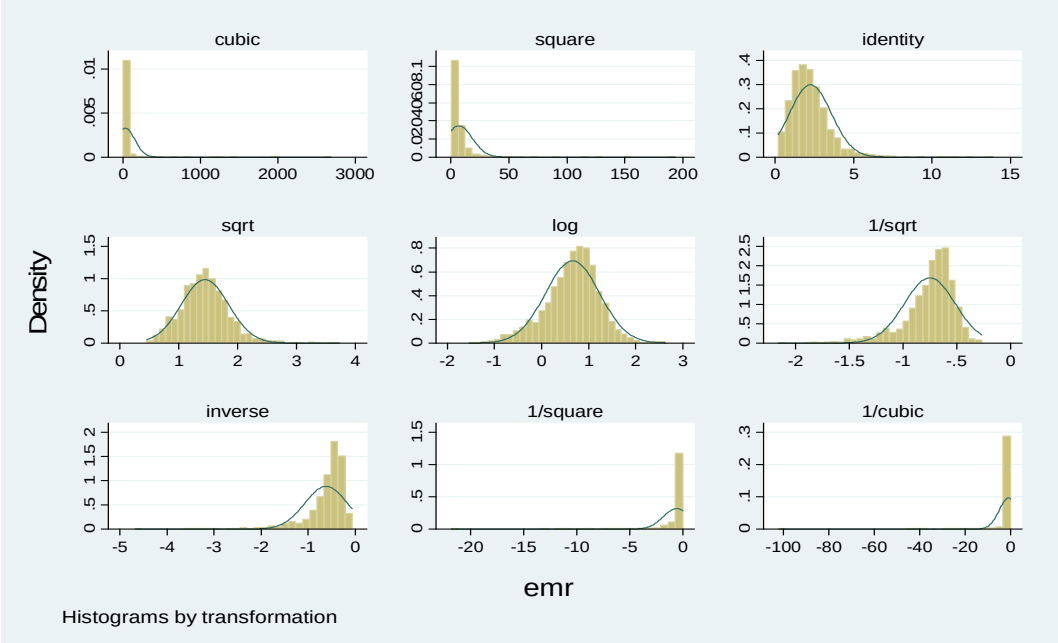


Figure 41 Metabolite: 2:16α-OHE$_1$: Histograms by Various Transformations Among All Postmenopausal Controls (N=720)

D.7 MODEL DIAGNOSTICS

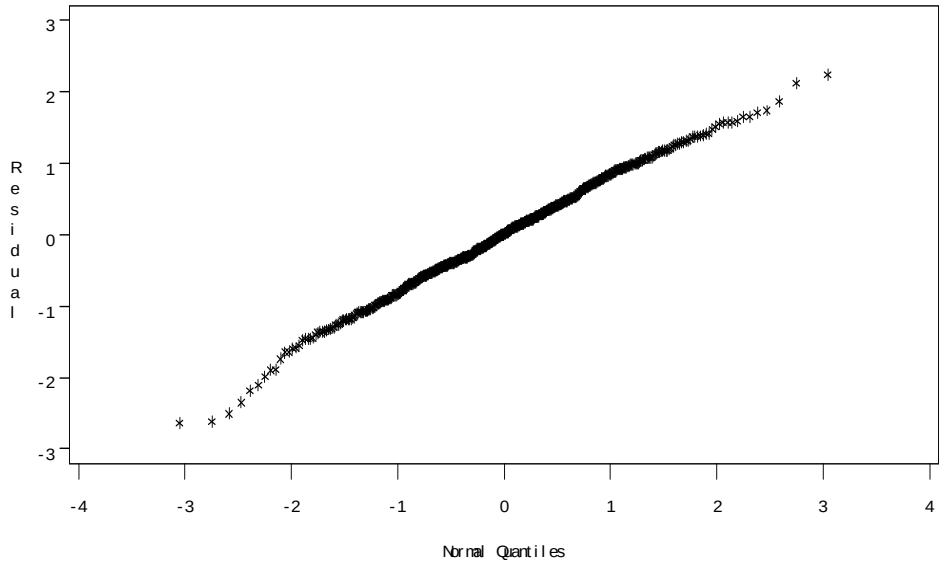


Figure 42 Normal Quantile Plot for the final multivariable model for 2-OHE_1: Premenopausal Women

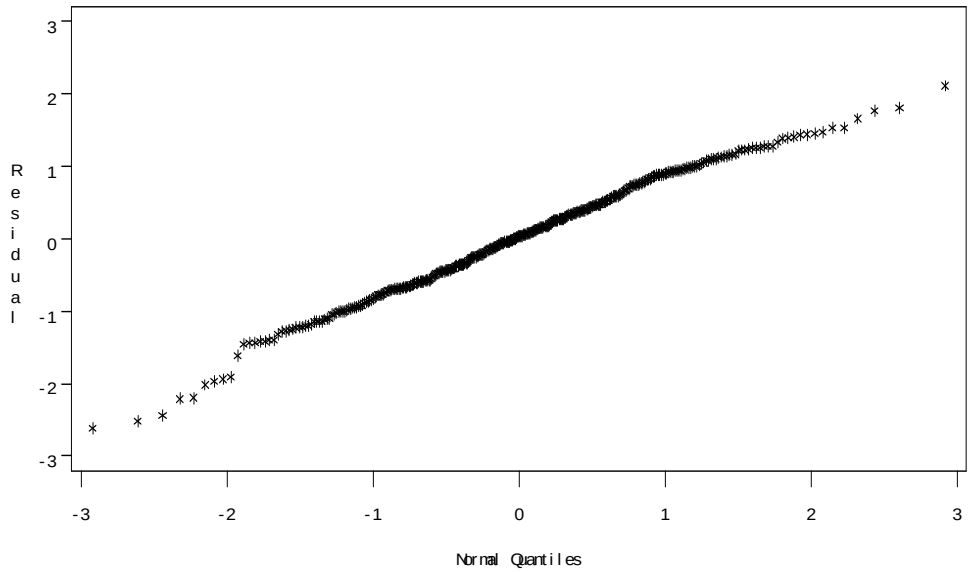


Figure 43 Normal Quantile Plot for the final multivariable model for 2-OHE_1 including both BMI and WHR: Premenopausal Women

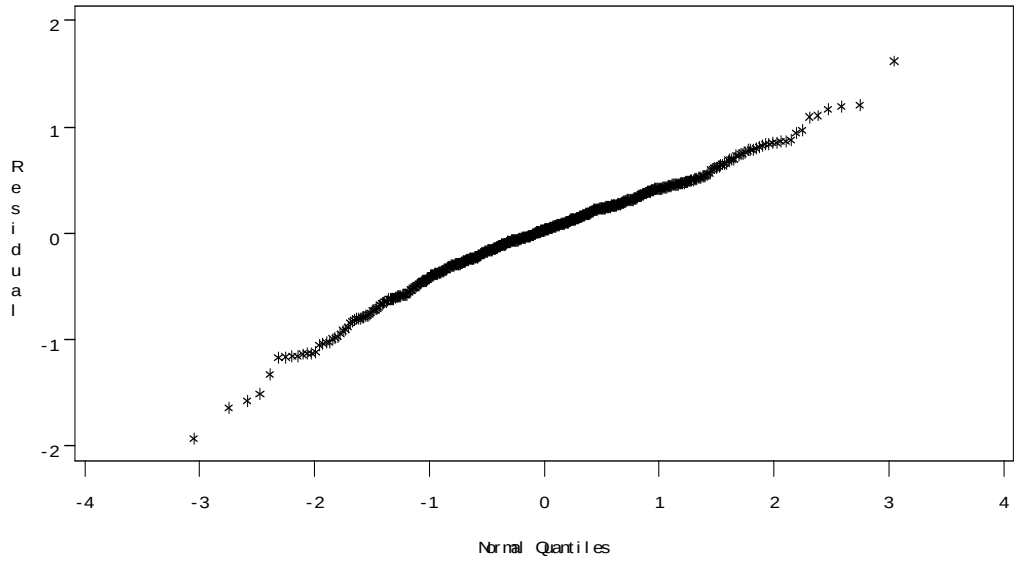


Figure 44 Normal Quantile Plot for the final multivariable model for 2:16α-OHE$_1$: Premenopausal Women

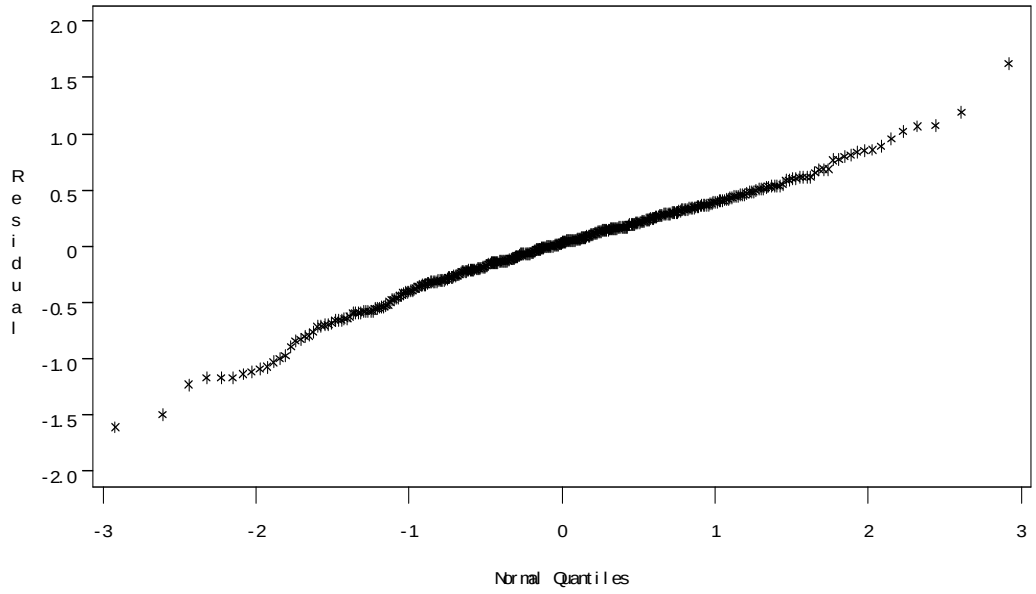


Figure 45 Normal Quantile Plot for the final multivariable model for 2:16α-OHE$_1$ including both BMI and WHR: Premenopausal Women

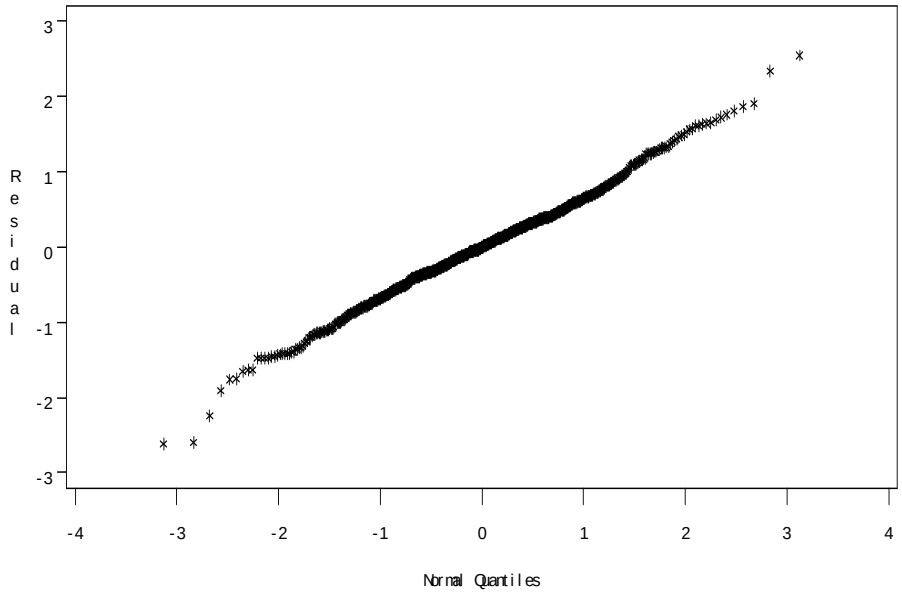


Figure 46 Normal Quantile Plot for the final multivariable model for 2-OHE_1: Postmenopausal Women

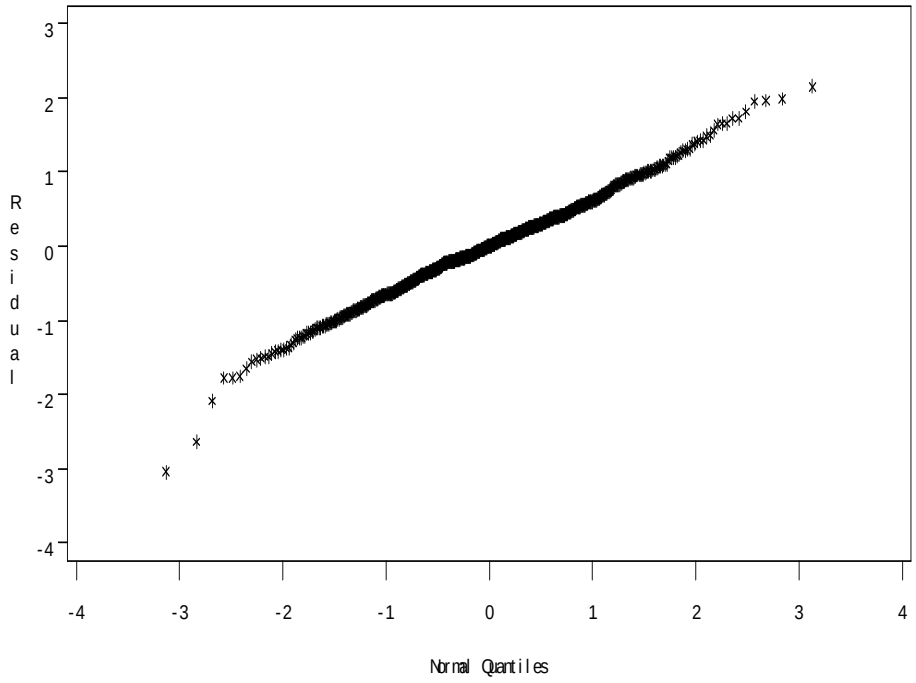


Figure 47 Normal Quantile Plot for the final multivariable model for 16α-OHE_1: Postmenopausal Women

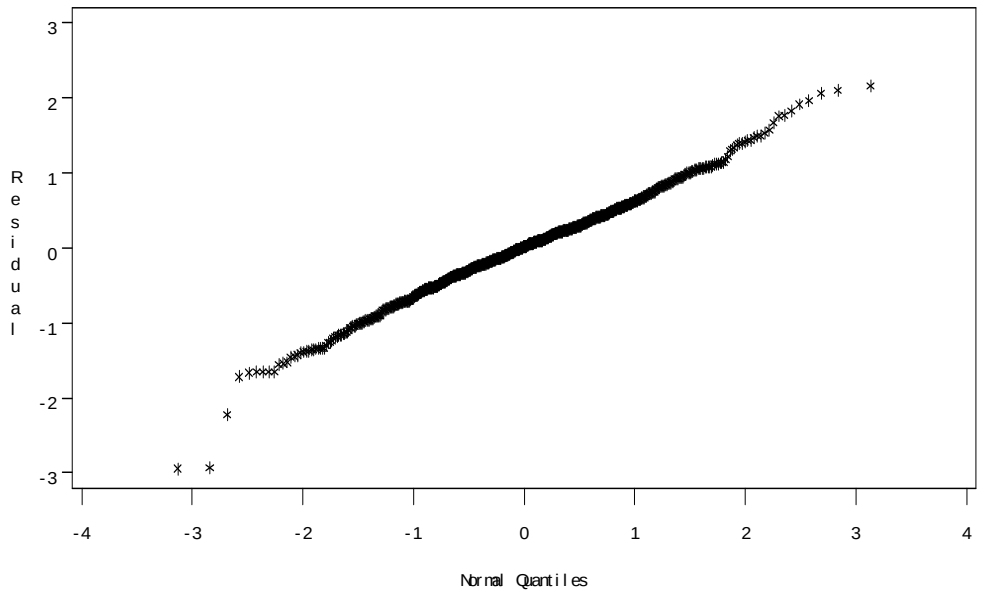


Figure 48 Normal Quantile Plot for the final multivariable model for 2:16α-OHE_1: Postmenopausal Women

BIBLIOGRAPHY

Aldercreutz H, Martin F, Wahlroos O, and Soini E. (1975) Mass spectrometric and mass fragmentographic determination of natural and synthetic steroids in biological fluids. Steroid Biochem Molec Biol. 6: 247-259.

Aldercreutz H, Fotsis T, Bannwart C, et al. (1986) Urinary estrogen profile determination in young Finnish vegetarian and omnivorous women. J Steroid Biochem. 24(1): 289-296.

Aldercreutz H, Gorbach SL, Goldin BR, et al. (1994) Estrogen metabolism and excretion in Oriental and Caucasian women. J Natl. Cancer Inst. 86(14): 1076-1082.

Althuis MD, Fergenbaum JH, Garcia-Closas M, et al. (2004) Etiology of hormone receptor-defined breast cancer: a systematic review of the literature. Cancer Epidemiol Biomarkers Prev. 13(10): 1558-68.

Alvarez-Vasquez RB, Axelrod D, Frenkel K, et al. (2003) Influence of postmenopausal hormone replacement therapy on an estrogen metabolite biomarker of risk for breast cancer. Horm Metab Res. 35: 358-361.

American Cancer Society. *Cancer Facts & Figures 2008.* Atlanta: American Cancer Society: 2008.

American Cancer Society. *Cancer Facts & Figures 2007.* Atlanta: American Cancer Society: 2007.

Anderson TJ, Ferguson DJ and Raab GM. (1982) Cell turnover in the 'resting' human breast: influence of parity, contraceptive pill, age and laterality. Br J Cancer. 46:376-382

Armamento-Villareal RC, Napoli N, Klug T, et al. (2004) The oxidative metabolism of estrogen modulates response to ERT/HRT in postmenopausal women. Bone. 35: 682-688.

Atkinson C, Skor HE, Fitzgibbons ED, et al. (2003) Urinary equol excretion in relation to 2-hydroxyestrone and 16α-hydroxyestrone concentrations: an observational study of young to middle-aged women. J Steroid Biochem Mol Biol. 71-77.

Atkinson C, Lampe JW, Tworoger SS, et al. (2004) Effects of moderate intensity exercise intervention on estrogen metabolism in postmenopausal women. Cancer Epidemiol Biomarkers Prev. 13(5): 868-74.

Barlow WE, White E, Ballard-Barbash R, et al. (2006) Prospective Breast Cancer Risk Prediction Model for Women Undergoing Screening Mammography. J Natl. Cancer Inst. 17(98): 1204 -1214.

Begg CB and Mazumdar M. (1994) Operating characteristics of a rank correlation test for publication bias. Biometrics. 50(4): 1088-101.

Bentz AT, Schneider CM and Westerlind KC. (2005) The relationship between physical activity and 2-hydroxyestrone, 16α-hydroxyestrone, and the 2/16 ratio in premenopausal women (United States). Cancer Causes and Control. 16: 455-461.

Berrino F, Muti P, Micheli A, et al. (1996) Serum sex hormone levels after menopause and subsequent breast cancer. J Natl Cancer Inst. 88: 291-296.

Bernstein L, Yuan JM, Ross RK, et al. (1990) Serum hormone levels in pre-menopausal Chinese women in Shanghai and white women in Los Angeles: results from two breast cancer case-control studies. Cancer Causes Control. 1(1): 51-8.

Bernstein L. (2002) Epidemiology of Endocrine-Related Risk Factors for Breast Cancer. Journal of Mammary Gland Biology and Neoplasia. 7(1): 3-15.

Bernstein L. (2006) The risk of breast, endometrial and ovarian cancer in users of hormonal preparations. Basic Clin Pharmacol Toxicol. 98(3): 288-96.

Blettner M, Sauerbrei W, Schlehofer V, et al. (1999) Traditional reviews, meta-analyses and pooled analyses in epidemiology. Int J of Epidemiol. 28:1-9.

Boyd NF, Greenberg C, Lockwood G, et al. (1997) Effects at two years of a low-fat high-carbohydrate diet on radiologic features of the breast: Results from a randomized trial. J Natl Cancer Inst. 89(7): 488-96.

Boyd NF, Lockwood GA, Byng JW, et al. (1998) Mammographic densities and breast cancer risk. Cancer Epidemiol Biomarkers Prev. 7(12): 1133-44.

Boyd NF, Rommens JM, Vogt K, et al. (2005) Mammographic breast density as an intermediate phenotype for breast cancer. Lancet Oncol. 6(10): 798-808.

Boyd NF, Guo H, Martin LJ, et al. (2007) Mammographic density and the risk and detection of breast cancer. N Engl J Med.. 356(3): 227-36.

Bradlow HL, Sepkovic DW, Klug T, and Osborne MP. (1998) Application of an improved ELISA assay to the analysis of urinary estrogen metabolites. Steroids. 63: 406-413.

Bradlow LH, Jernstrom H, Sepkovic DW, et al. (2006) Comparison of plasma and urinary levels of 2-hydroxyestrogen and 16α-hydroxyestrogen metabolites. Mol Genet Metab. 87:135-146.

Breslow NE, Daly NE. (1980) Statistical methods in Cancer Research, Vol 1: The Analysis of Case-control studies. IARC Scientific Publication. No. 32. Lyon: International Agency for Research on Cancer.

Brisson J, Morrison AS, Kopans DB, et al. (1984) Height and weight, mammographic features of breast tissue, and breast cancer risk. Am J Epidemiol. 119(3): 37-81.

Brooks JD, Ward WE, Lewis JE, et al. Supplementation with flaxseed alters estrogen metabolism in postmenopausal women to a greater extent than does supplementation with an equal amount of soy. Am J Clin Nutr. 79(2): 318-25.

Byrne C, Schairer C, Wolfe J, et al. (1995) Mammographic features and breast cancer risk: effects with time, age, and menopause status. J Natl Cancer Inst. 87: 1622-9.

Byrne C, Schairer C, Brinton LA, et al. (2001) Effects of mammographic density and benign breast disease on breast cancer risk (United States). Cancer Causes Control. 12: 103-10.

Calle EE and Kaaks R. (2004) Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. Nat Rev Cancer. 4(8): 579-91.

Campbell KL, Westerlind KC, Harber VJ, et al. (2005) Associations between Aerobic Fitness and Estrogen Metabolites in Premenopausal Women. Med Sci Sports Exerc. 37(4): 585-592.

Cauley JA, Lucas FL, Kuller LH, et al. (1999) Elevated serum estradiol and testosterone concentrations are associated with a high risk for breast cancer. Ann Intern Med. 130: 270-277.

Cauley JA, Zmuda JM, Danielson ME, et al. (2003) Estrogen metabolites and risk of breast cancer in older women. Epidemiology. 14(6): 740-744.

Cavalieri E, Chakravarti D, Guttenplan J, et al. (2006) Catechol estrogen quinones as initiators of breast and other human cancers: implications for biomarkers of susceptibility and cancer prevention. Biochim Biophys Acta. 1766(1): 63-78.

Chen Z, Zheng W, Dunning LM, et al. (1999) Within-person variability of the ratios of urinary 2-hydroxyestrone to 16α-hydroxyestrone in Caucasian women. Steroids. 64: 856-859.

Chlebowski RT, Aiello E, McTiernan A, et al. (2002) Weight loss in breast cancer patient management. J Clin Oncol. 20: 1128-43.

Chlebowski RT, Chen Z, Anderson GL, et al. (2005) Ethnicity and breast cancer: factors influencing differences in incidence and outcome. J Natl Cancer Inst. 97(6): 439-48.

Collaborative Group on Hormonal Factors in Breast Cancer (CGHFBC). (2001) Familial breast cancer: collaborative reanalysis of individual data from 52 epidemiological studies including 58,209 women with breast cancer and 101, 986 women without the disease. Lancet. 358: 1389-1399.

Colditz GA. (1993) Epidemiology of breast cancer. Findings from the nurses' health study. Cancer. 71(4 suppl): 1480-1490.

Cotterchio M, Kreiger N, Theis B, et al. (2003) Hormonal Factors and the Risk of Breast Cancer According to Estrogen- and Progesterone-Receptor Subgroup. Cancer Epidemiol Biomarkers Prev. 12: 1053-1060.

Cummings SR, Tice JA, Bauer S, et al. (2009) Prevention of Breast Cancer in Postmenopausal Women: Approaches to Estimating and Reducing Risk. J Natl Cancer Inst. 101: 384-398.

Dorgan JF, Longcope C, Stephenson HE Jr, et al. (1996) Relation of prediagnostic serum estrogen and androgen levels to breast cancer risk. Cancer Epidemiol Biomarkers Prev. 5: 533-539.

Egger M, Davey Smith G, Altman DG, et al. (2001) Systematic Reviews in Health Care: Meta-Analyses in Context, 2nd Edition. BMJ, London, UK

Eliassen AH, Colditz GA, Rosner B, et al. (2006) Adult weight change and risk of postmenopausal breast cancer. JAMA. 296(2): 193-201.

Eliassen AH, Missmer SA, Tworoger SS, et al. (2006) Endogenous steroid hormone concentrations and risk of breast cancer among premenopausal women. J Natl Cancer Inst. 98(19): 1406-15.

Eliassen AH and Hankinson SE. (2008) Endogenous hormone levels and risk of breast, endometrial and ovarian cancers. Adv Exp Med Biol. 630: 148-65.

Ellison RC, Zhang Y, McLennan CE, Rothman KJ. (2001) Exploring the relation of alcohol consumption to risk of breast cancer. Am J Epidemiol. 154: 740-7.

Endogenous Hormones and Breast Cancer Collaborative Group (EHBCCG). (2002) Endogenous sex hormones and breast cancer in postmenopausal women: Reanalysis of nine prospective studies. J Natl Cancer Inst. 94: 606-616.

Endogenous Hormones and Breast Cancer Collaborative Group. (2003) Body mass index, serum sex hormones, and breast cancer risk in postmenopausal women. J Natl Cancer Inst. 95 (16): 1218-26.

Falk RT, Rossi SC, Fears TR, et al. (2000) A new ELISA Kit for Measuring Urinary 2-Hydroxyestrone, 16α-Hydroxyestrone, and their ratio: reproducibility, validity, and assay

performance after freeze-thaw cycling and preservation by boric acid. Cancer Epidemiol Biomarkers Prev. 9: 81-87.

Falk RT, Fears TR, Xu X, et al. (2005) Urinary estrogen metabolites and their ratio among Asian american women. Cancer Epidemiol Biomarkers Prev. 14(1): 221-6.

Feigelson HS and Henderson BE. (1996) Estrogens and breast cancer. Carcinogenesis. 17: 2279-84.

Feigelson HS, Jonas CR, Teras LR, et al. (2004) Weight gain, body mass index, hormone replacement therapy, and postmenopausal breast cancer in a large prospective study. Cancer Epidemiol Biomarkers Prev. 13:220-224.

Fishman J, Boyar RM, Hellman L, et al. (1975) Influence of body weight on estradiol metabolism in young women. J Clin Endocrinol Metab. 41: 989-991.

Fishman J, Schneider J, Hershcopf, et al. (1984) Increased estrogen-16α-hydroxylase activity in women with breast and endometrial cancer. J Steroid Biochem. 20(4B):1077-1081.

Fotsis T and Aldercreutz H. (1987) The multicomponent analysis of estrogens in urine by ion exchange chromatography and GC-MS I. Quantitation of estrogens after initial hydrolosis of conjugates. J Steroid Biochem. 28: 203-218.

Fowke JH, Longcope C, and Hebert JR. (2000) *Brassica* vegetable consumption shifts estrogen metabolism in healthy postmenopausal women. Cancer Epidemiol Biomarkers Prev. 9:773-779.

Fowke JH, Longcope C, and Herbert JR. (2001) Macronutrient intake and estrogen metabolism in healthy postmenopausal women. Breast Cancer Research and Treatment. 65: 1-10.

Fowke JH, Qi Dai, Bradlow LH, et al. (2003) Urinary estrogen metabolites and breast cancer: differential pattern of risk found with pre- versus post-treatment collection. Steroids. 1: 65-72.

Frankenfeld CL, McTiernan A, Tworoger SS, et al. (2004) Serum steroid hormones, sex hormone-binding globulin concentrations and urinary hydroxylated estrogen metabolites in post-menopausal women in relation to daidzein-metabolizing phenotypes. J Steroid Biochem Mol Biol. 88:399-408.

Friedenreich CM. (1993) Methods for Pooled Analyses of Epidemiologic Studies. Epidemiology. 4(4): 295-302.

Froguel P and Boutin P. (2001) Genetics of pathways regulating body weight in the development of obesity in humans. Exp Biol Med (Maywood). 226:991-6.

Gaikwad NW, Yang L, Muti P, et al. (2007) The molecular etiology of breast cancer: Evidence from biomarkers of risk. Int J Cancer. 122(9): 1949-1957.

Gail MH, Brinton LA, Byar DP, et al. (1989) Projecting individualized probabilities of developing breast cancer for White females who are being examined annually. J Natl Cancer Inst. 81: 1879-1886.

Gill J. (2000) The effects of moderate alcohol consumption on female hormone levels and reproductive function. Alcohol. 35: 417-23.

Greenlee H, Chen Y, Kabat G, et al. (2006) Variants in estrogen metabolism and biosynthesis genes and urinary estrogen metabolites in women with a family history of breast cancer. Breast Cancer Res Treat. 102(1): 111-7.

Haggans CJ, Travelli EJ, Thomas W, et al. (2000) The effect of flaxseed and wheat bran consumption on urinary estrogen metabolites in premenopausal women. Cancer Epidemiol Biomarkers Prev. 9: 719-725.

Haiman CA, Bernstein L, Van Den Berg D, et al. (2002) Genetic determinants of mammographic density. Breast Cancer Research. 4(3): R5

Hamajima N, Hirose K, Tajima K, et al. (2002) Alcohol, tobacco and breast cancer-collaborative reanalysis of individual data from 53 epidemiological studies, including 58,515 women with breast cancer and 95,067 women without the disease. Br J Cancer. 87:1234- 45.

Hayes CL, Spink DC, Spink BC, et al. (1996) 17β-Estradiol hydroxylation catalyzed by human cytochrome P450 1B1. Prc. Natl. Acad. Sci. 93: 9776-9781.

Helzlsouer KJ, Alberg AJ, Bush TL, et al. (1994) A prospective study of endogenous hormones and breast cancer. Cancer Detect Prev. 18: 79-85.

Henderson BE, Ross R, and Bernstein L. (1998) Estrogens as a cause of human cancer: the Richard and Hinda Rosenthal Foundation award lecture. Cancer Res. 48: 246-53.

Henderson BE and Fiegelson HS. (2000) Hormonal carcinogenesis. Carcinogeneisis. 21: 427-433.

Ho GH, Luo XW, Ji CY, et al. (1998) Urinary 2/16α-hydroxyestrone Ratio: Correlation with Serum Insulin-like Growth Factor Binding Protein-3 and a Potential Biomarker of Breast Cancer Risk. Ann Acad Med Singapore. 27:294-9.

Horn-Ross PL, Hoggatt KJ, West DW, et al. (2002) Recent diet and breast cancer risk: the California Teachers Study (USA). Cancer Causes Control. 13:407-15.

Huang WY, Newman B, Millikan RC. (2000) Hormone-related factors and risk of breast cancer in relation to estrogen receptor and progesterone receptor status. Am. J. Epidemiol. 151:703–714.

Hulka BS and Moorman PG. (2001) Breast cancer: hormones and other risk factors. Maturitas. 38(1): 103-113.

Hunter DJ and Willet WC. (1993) Diet, body size, and breast cancer. Epidemiol Rev. 15:110-32.

Jefcoate CR, Liehr JG, Santen RJ, et al. (2000) Tissue-Specific Synthesis and Oxidative Metabolism of Estrogens. 27:95-112.

Jemal A, Siegel R, Ward E, et al. (2007) Cancer Statistics, 2007. Cancer J Clin. 57:43-66.

Jernstrom H and Barrett-Connor E. (1999) Obesity, weight change, fasting insulin, proinsulin, C-peptide, and IGF-I levels in women with and without breast cancer: the Rancho Bernardo Study. J Women's Health Gender Based Med. 8:1265-72.

Jernstrom H, Klug TL, Sepkovic DW, et al. (2003) Predictors of the plasma ratio of 2-hydroxyestrone to 16α-hydroxyestrne among pre-menopausal, nulliparous women from four ethnic groups. Carcinogenesis. 24 (5): 991-1005.

Kabat GC, Chang CJ, Sparano JA, et al. (1997) Urinary Estrogen Metabolites and Breast Cancer: A Case-Control Study. Cancer Epidemiol Biomarkers Prev. 6:505-509.

Kabat GC, O'Leary ES, Gammon MD, et al. (2006) Estrogen Metabolism and Breast Cancer. Epidem. 17(1): 80-88.

Kerlan V, Dreano Y, Bercovici JP, et al. (1992) Nature of cytochrome P450 involved in the 2-/4-hydroxylations of estradiol human liver microsomes. Biochem Pharmacol. 44: 1745-1756.

Key TJ, Appleby PN, Barnes I, et al. (2002) Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies. J Natl Cancer Inst. 94: 606-616.

Key TJ, Appleby PN, Reeves GK, et al. (2003) Endogenous hormones breast cancer collaborative group. Body mass index, serum sex hormones, and breast cancer risk in postmenopausal women. J Natl Cancer Inst. 95:1218-1226.

Klug T, Bradlow HL, and Sepkovic DW. (1994) Monoclonal antibody-based enzyme immunoassay for simultaneous quantitation of 2- and 16α-hydroxyestrone in urine. Steroids. 59: 648-655.

Kuller LH, Matthews KA and Meilahn EN. (2000) Estrogens and women's health: interrelation of coronary heart disease, breast cancer and osteoporosis. J Steroid Biochem Mol Biol. 74:297-309.

Leelawattana R, Ziambaras K, Roodman-Weiss J, et al. (2000) The oxidative metabolism of estradiol conditions postmenopausal bone density and bone loss. J Bone Miner Res. 15(12):2513-20.

Liehr JG and Roy D. (1990a) Free radical generation by redox cycling of estrogens. Free Radical Biol Med. 8:415-423.

Liehr JG. (1990b) Genotoxic effects of estrogens. Mutation Research. 238: 269-276.

Lim SK, Won YJ, Lee JH, et al. (1997) Altered hydroxylation of estrogen in patients with postmenopausal osteopenia. J Clin Endocrinol Metab. 82: 1001-1006.

Lippert TH, Seeger H, Mueck AO. (1999) Metabolism of endogenous estrogens. In: Oettel M, Schillinger E, editors. Estrogens and antiestrogens – handbook of experimental pharmacology. Berline: springer, 1999: 243-71.

Lippert TH, Seeger H, and Mueck AO. (2000) The impact of endogenous estradiol metabolites on carcinogensis. Steroids. 65: 357-369.

Lippert C, Seeger H, and Mueck. (2003) The effect of endogenous estradiol metabolites on the proliferation of human breast cells. Life Sciences. 72: 877-883.

Longcope C, Gorbach S, Goldin B, et al. (1987) The effect of a low fat diet on estrogen metabolism. J Clin Endocrinol Metab. 64(6): 1246-50.

Lord RS, Bongiovanni B and Bralley JA. (2002) Estrogen metabolism and the diet-cancer connection: Rationale for assessing the ratio of urinary hydroxylated estrogen metabolites. Altern Med Rev. 7(2): 119-129.

Lu LJ, Cree M, Josyula S, et al. (2000) Increased urinary excretion of 2-hydroxyestrone but not 16α-hydroxyestrone in premenopausal women during a soya diet containing isoflavones. Cancer Research. 60:1299-1305.

Ma H, Bernstein L, Ross RK and Ursin G. (2006) Hormone-related risk factors for breast cancer in women under age 50 years by estrogen and progesterone receptor status: results from a case-control and a case-case comparison. Breast Cancer Res. 8(4): R39.

Markushin Y, Zhong W, Cavalieri EL, et al. (2003) Spectral characterization of catechol estrogen quinone (CEQ)-derived DNA adducts and their identification in human breast tissue extract. Chem Res Toxicol. 16: 1107-1117.

Markushin Y, Gaikwad, Zhang H, et al. (2006) Potential biomarker for early risk assessment of prostate cancer. The Prostate. 66:1565-1571.

Martini MC, Dancisak BB, Haggans CJ, et al. (1999) Effects of soy intake on sex hormone metabolism in premenopausal women. Nutr Cancer. 34(2): 133-139.

Masi CM, Hawkley LC, Berry JD, et al. (2006) Estrogen metabolites and systolic blood pressure in a population-based sample of postmenopausal women. J Clin Endocrinol Metab. 91: 1015-1020.

Matthews CE, Fowke JH, Dai Q, et al. (2004) Physical activity, body size, and estrogen metabolism in women. Cancer Causes and Control. 15:473-481.

McCann SE, Wactawski-Wende J, Kufel K, et al. (2007) Changes in 2-hydroxyestrone and 16α-hydroxyestrone metabolism with flaxseed consumption: modification by COMT and CYP1B1 genotype. Cancer Epidemiol Biomarkers Prev. 16)256-62.

McCormack VA and Silva I. (2006) Breast density and parenchymal patterns as markers of breast cancer risk: a meta-analysis. Cancer Epidemiol Biomarkers Prev. 15(6) 1159-1169.

McTiernan A, Rajan KB, Tworoger SS, et al. (2003) Adiposity and sex hormones in postmenopausal breast cancer survivors. J Clin Oncol. 21: 1961-6.

McTiernan A, Kooperberg C, White E, et al. (2003) Recreational physical activity and the risk of breast cancer in postmenopausal women: the Women's Health Initiative Cohort Study. JAMA. 290 (10): 1331-1336.

Meilahn EN, De Stavola B, Allen DS, et al. (1998) Do urinary oestrogen metabolites predict breast cancer? Guernsey III cohort follow-up. Br J Cancer. 78(9): 1250-1255.

Michnovicz JJ, Hershcopf RJ, Naganuma H, et al. (1986) Increased 2-hydroxylation of estradiol as a possible mechanism for the anti-estrogenic effect of cigarette smoking.

Michnovicz JJ, Nagnuma H, Hershcopf RJ, et al. (1988) Increased urinary catechol estrogen excretion in female smokers. Steroids. 52: 69-83.

Michnovicz JJ, Aldercruetz H and Bradlow HL. (1997) Changes in levels of urinary estrogen metabolites after oral indole-3-carbinol treatment in humans. 89(10): 718-723.

Missmer SA, Eliassen AH, Barbieri RL, et al. (2004) Endogenous estrogen, androgen, and testosterone concentrations and breast cancer risk among postmenopausal women. J Natl Cancer Inst. 96: 1856-1865.

Modugno F, Kip K, Cochrane B, et al. (2006) Obesity, hormone therapy, estrogen metabolism and risk of postmenopausal breast cancer. Int J. Cancer. 118:1292-1301.

Moreno V, Martin ML, Bosch FX, et al. (1996) Combined Analysis of Matched and Unmatched Case-Control Studies: Comparison of Risk Estimates from Different Studies. Am J Epidemiol. 143(3): 293-300.

Morimoto L, White E, Chen Z, et al. (2002) Obesity, body size, and risk of postmenopausal breast cancer: the Women's Health Initiative (United States). Cancer Causes and Control. 13: 741-751.

Mueck AO, Seeger H, Graser T, et al. (2001) The Effects of postmenopausal hormone replacement therapy and oral contraceptives on the endogenous estradiol metabolism. Horm Metab Res. 33:744-747.

Mueck AO, Seeger H and Lipper T. (2002) Estradiol metabolism and malignant disease. Maturitas. 43: 1-10.

Mueck AO, Seeger H, and Wallwiener D. (2004) Endogenous estradiol metabolism during treatment with oral contraceptives. Int J Clin Pharmacol Ther. 42(3): 160-4.

Muti P, Bradlow HL, Micheli A, et al. (2000) Estrogen metabolism and risk of breast cancer: a prospective study of the 2:16α-hydroxyestrone ratio in premenopausal and postmenopausal women. Epidem. 11:635-640.

Napoli N, Villareal DT, Mumm S, et al. (2005) Effect of CYP1A1 Gene Polymorphisms on Estrogen Metabolism and Bone Density. J Bone Miner Res. 20:232-239.

Nettleton JA, Greany KA, Thomas W, et al. (2005) The effect of soy consumption on the urinary 2:16-hydroxyestrone ratio in postmenopausal women depends on equol production status but is not influenced by probiotic consumption. J Nutr. 135: 603-608.

Ogden CL, Carroll MD, Curtin LR, et al. (2006) Prevalence of overweight and obesity in the United States, 1999-2004. JAMA. 295(13):1549-55.

Parkin DM, Bray F, Ferlay J, and Pisani P. (2005) Global Cancer Statistics, 2002. CA Cancer J Clin. 55:74-108.

Pasagian-Macaulay A, Meilahn EN, Bradlow HL, et al. (1996) Urinary markers of estrogen metabolism 2- and 16α-hydroxylation in premenopausal women. Steroids. 61:461-467.

Petitti D. Meta-Analysis, Decision Analysis, and Cost Effectiveness Analysis: Methods for Quantitative Synthesis in Medicine. USA: Oxford Press, 2000.

Preston-Martin S, Pike MC, Ross RK, et al. (1990) Increased cell division as a cause of human cancer. Cancer Research. 50:7415-7421.

Ravdin PM, Cronin KA, Howlader N, et al. (2007) The decrease in breast-cancer incidence in 2003 in the United States. N Engl J Med. 356:1670-1674.

Riza E, Silva I, Stavola B, et al. (2001) Urinary estrogen metabolites and mammographic parenchymal patterns in postmenopausal women. Cancer Epidemiol Biomarkers Prev. 10:627-634.

Reeves KW, Gierach G, and Modugno F. (2007) Recreational physical activity and mammographic breast density characteristics. Cancer Epidemiol Biomarkers Prev. 16(5):934-42.

Rock CL and Demark-Wahnefried W. (2002) Nutrition and survival after the diagnosis of breast cancer: a review of the evidence. J Clin Oncol. 20(15):3302-16.

Rogan EG, Badawi AF, Devanesan PD, et al. (2003) Relative imbalances in estrogen metabolism and conjugation in breast tissue of women with carcinoma: potential biomarkers of susceptibility to cancer. Carcinogenesis. 24:697-702.

Rose DP, Komninou D, and Stephenson GD (2004). Obesity, adipocytokines, and insulin resistance in breast cancer. Obesity Reviews. 5(3): 153-165.

Rossouw JE, Anderson GL, Prentice RL, et al. (2002) Risk and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the Women's Health Initiative randomized controlled trial. JAMA. 288: 321-333.

Sala E, Warren R, McCann J, et al. (1999) High-risk mammographic parenchymal patterns and anthropometric measures: a case-control study. Br J Cancer. 81: 1257-61.

Schneider J, Kinne D, Fracchia A, et al. (1982) Abnormal oxidative metabolism of estradiol in women with breast cancer. Proc Natl Acad Sci. 79:3047-3051.

Schneider J, Bradlow HL, Strain G, et al. (1983) Effects of obesity on estradiol metabolism: decreased formation of nonuterotropic metabolites. J Clin Endocrinol Metab. 56: 973-978.

Schneider J, Huh MM, Bradlow HL, et al. (1984) Antiestrogen action of 2-hydroxyestrone on MCF-7 human breast cancer cells. J Biol Chem. 259: 4840-4845.

Seeger H, Wallwiener D, Kraemer E, et al. (2006) Comparison of possible carcinogenic estradiol metabolites: Effects on proliferation, apoptosis and metastasis of human breast cancer cells. Maturitas, 54: 72-77.

Sepkovic D and Bradlow L. (2009) Estrogen Hydroxylation – The Good and the Bad. Steroid Enzymes and Cancer. Ann. N.Y. Acad. Sci. 1155: 57-67.

Sitteri PK. (1987) Adipose tissue as a source of hormones. Am J Clin Nutr. 45: 277-282.

Smith-Warner SA, Spiegelman D, Yaun SS, et al. (1998) Alcohol and breast cancer in women: a pooled analysis of cohort studies. J Am Med Assoc. 279:535 – 40.

Snyder EE, Walts B, Perusse L, et al. (2004) The human obesity gene map: the 2003 update. Obes Res. 12:369-439.

Sowers MR, Wilson AL, Kardia SR, et al. (2006) CYP1A1 and CYP1B1 polymorphisms and their association with estradiol and estrogen metabolites in women who are premenopausal and perimenopausal. Am J Sports Med. 119(9A): S44-S51.

Sowers MR, Crawford S, McConnell DS, et al. (2006) Selected diet and lifestyle factors are associated with estrogen metabolites in a multiracial/ethnic population of women. J. Nutr. 136: 1588-1595.

Sturgeon SR, Potischman N, Malon KE, et al. (2004) Serum levels of sex hormones and breast cancer risk in premenopausal women: a case-control study. Cancer Causes Control. 15: 45-53.

Surveillance, Epidemiology and End Results (SEER) Program. SEER* Stat Database: Incidence-SEER 17 Registries. Available at: http://www.seer.cancer.gov. Accessed September 30, 2008.

Swaneck GE and Fishman J. (1988) Covalent binding of the endogenous estrogen 16α-hydroxyestrone to estradiol receptor in human breast cancer cells: characterization and intranuclear localization. Proc Natl Acad Sci USA. 85: 7831-5.

Taioli E and Garte SJ. (1996) Ethnic differences in estrogen metabolism in healthy women. J Natl Cancer Inst. 88(9): 617.

Taioli 1999. Role of estradiol metabolism and Cyp1a1 polymorphism in breast cancer risk. Cancer Detect Prev. 23(3): 232-7.

Taioli E and Bonassi S. (2002) Methodological issues in pooled analysis of biomarker studies. Mutation Research. 512: 85-92.

Tang YM, Wo Y-YP, Stewart J, et al. (1996) Isolation and characterization of the human cytochrome P450 *CYP1B1* gene. J Biol Chem. 271**:**28324–30.

Telang NT, Suto A, Wong GY, et al. (1992) Estrogen metabolite 16α-hydroxyestrone induces genotoxic damage and aberrant cell proliferation in mouse mammary epithelial cells in culture. J Natl Cancer Inst. 82: 634-638.

Thomas HV, Key TJ, Allen DS, et al. (1997) A prospective study of endogenous serum hormone concentrations and breast cancer risk in postmenopausal women on the island of Guernsey. Br J Cancer. 76: 401-405.

Toniolo PG, Levitz M, Zeleniuch-Jacquotte A, et al. (1995) A prospective study of endogenous estrogens and breast cancer in postmenopausal women. J Natl Cancer Inst. 87: 190-197.

Ursin G, Longnecker MP, Haile RW, et al. (1995) A meta-analysis of body mass index and risk of premenopausal breast cancer. Epidemiology. 6:137-41.

Ursin G, London S, Stanczyk, et al. (1999) Urinary 2-hydroxyestrone/16α-hydroxyestrone ratio and risk of breast cancer in postmenopausal women. J Natl Cancer Inst. 91(12):1067-1072.

Ursin G, Wilson M, Henderson BE, et al. (2001) Do Urinary estrogen metabolites reflect the differences in breast cancer risk between singapore Chinese and United States African-American and White women? Cancer Research. 61: 3326-3329.

Ursin G, London S, Yang D, et al. (2002) Urinary 2-hydroxyestone/16α-hydroxyestrone ratio and family history of breast cancer in premenopausal women. Breast Cancer Res Treat. 72: 139-143.

Ursin G, Ma H, Wu AH, et al. (2003) Mammographic density and breast cancer in three ethnic groups. Cancer Epidemiol Biomarkers Prev. 12: 332-8.

Vainio H, Bianchini F, eds. *IARC Handbooks on Cancer Prevention: Weight Control and Physical Activity.* Lyon (France): IARC Press; 2002.

Van den Brandt PA, Speifelman D, Yaun SS, et al. (2000) Pooled analysis of prospective cohort studies on height, weight, and breast cancer risk. Am J Epidemiol. 152: 514-527.

Verkasalo PK, Thomas HV, Appleby PN, et al. (2001) Circulating levels of sex hormones and their relation to risk factors for breast cancer: a cross-sectional study in 1092 pre- and post-menopausal women (United Kingdom). Cancer Causes Control. 12: 47-59.

Warren R, Skinner J, Sala E, et al. (2006) Associations among mammographic density, circulating sex hormones, and polymorphisms in sex hormone metabolism genes in postmenopausal women. Cancer Epidemiol Biomarkers Prev. 15(8): 1502-8.

Wellejus A, Olsen A, Tjonneland A, et al. (2005) Urinary hydroxyestrogens and breast cancer risk among postmenopausal women: a prospective study. Cancer Epidemiol Biomarkers Prev 14(9):2137-42.

Westerlind KC, Gibson KJ, Wolfe P. (1999) The effect of diurnal and menstrual cyclicity and menopausal status on estrogen metabolites: implications for disease-risk assessment. Steroids. 64: 856-9.

Westerlind KC and Williams NI. (2007) Effect of Energy Deficiency on Estrogen Metabolism in Premenopausal Women. Med Sci Sports Exerc. 39(7): 1090-1097.

Williams AE, Maskarinec G, Franke A, et al. (2002) The temporal reliability of serum estrogens, progesterone, gonadotropins, SHBG and urinary estrogen and progesterone metabolites in premenopausal women. BMC Women's Health. 2(1):13.

Wolfe JN. (1976a) Breast patterns as an index of risk for developing breast cancer. Am J Roentgenol. 126: 1130-39.

Wolfe JN. (1976b) Risk for breast cancer development determined by mammographic parenchymal pattern. Cancer. 37(5): 2486-2492

.

Xu X, Duncan AM, Merz BE and Kurzer MS. (1998) Effects of soy isoflavones on estrogen and phytoestrogen metabolism in premenopausal women. Cancer Epidemiol Biomarkers Prev. 7(12): 1101-8

Xu X, Duncan AM, Merz-Demlow, et al. (1999) Menstrual cycle effects on urinary estrogen metabolites. J Clin Endocrinol Metab. 84: 3914-3918.

Xu X, Duncan AM, Wangen KE, and Kurzer MS. (2000) Soy consumption alters endogenous estrogen metabolism in postmenopausal women. Cancer Epidemiol Biomarkers Prev. 9:781-796.

Yager JD. (2000) Chapter 3: Endogenous estrogens as carcinogens through metabolic activation. Cancer Inst Monogr. 27:67-73.

Yager JD and Davidson NE. (2006) Estrogen carcinogenesis in breast cancer. N Engl J Med. 354:270-82.

Zahid M, Kohli E, Saeed M, et al. (2006) The Greater reactivity of estradiol-3,4-quinone vs estradiol-2,3-quinone with DNA in the formation of depurinating adducts: Implications for tumor-initiating activity. Chem Res Toxicol. 19:164-172.

Zaleniuch-Jacquotte A, Toniolo P, Levitz M, et al. (1995) Endogenous estrogens and risk of breast cancer by estrogen receptor status: a prospective study in postmenopausal women. Cancer Epidemiol Biomarkers Prev. 4: 857-860.

Zeleniuch-Jacquotte A, Shore RE, Koenig KL, et al. (2004) Postmenopausal levels of oestrogen, androgen, and SHBG and breast cancer: Long term results of a prospective study. Br J Cancer 90: 153-159.

Zhang Y, Gaikwad N, Olson K, et al. Cytochrome P450 isofroms catalyze formation of catechol estrogen quinines that react with DNA. Metabolism Clinical and Experimental. 56:887-894 (2007).

Zheng W, Dunning L, Jin F, and Holtzman J. (1998) Correspondence re: G.C. Kabat et al., Urinary estrogen metabolites and breast cancer: a case-control study. Cancer Epidemiol., Biomark. Prev., 6: 505-509, 1997. Cancer Epidemiol Biomarkers Prev. 7: 85-86.

Zhu BT and Conney AH. (1998) Functional role of estrogen metabolism in target cells: review and perspectives. Carcinogenesis. 19:1-27.

Ziegler RG, Russi SC, Fears TR, et al. (1997) Quantifying estrogen metabolism an evaluation of the reproducibility and validity of enzyme immunoassays for 2-hydroxyestrone and 16α-hydroxyestrone in urine. Environ. Health Perspect. 105 (Suppl. 3): 607-614.