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Adiposity and Breast Cancer: The Role of Genetic and Modifiable Risk Factors

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Adiposity and Breast Cancer: The Role of Genetic and Modifiable Risk Factors

By

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M.P.H., Emory University, 2000

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An abstract of
A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
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Abstract

Adiposity and Breast Cancer: The Role of Genetic and Modifiable Risk Factors
By Lauren R. Teras

Obesity has been consistently associated with an increased risk of postmenopausal breast cancer. However, the implications of this association for cancer prevention and the genetic contributions to mechanisms by which obesity may affect breast cancer risk are not fully understood. The goal of this dissertation was to address three unanswered questions: 1) Does weight loss reduce breast cancer risk in overweight women? 2) Do polymorphisms of genes that encode adipokines contribute to risk of postmenopausal breast cancer? 3) Do genetic and non-genetic factors that impact estrogen level interact to affect risk for breast cancer?

Study 1 found that weight loss was not associated with postmenopausal breast cancer risk; however understanding the potential benefit of weight loss is challenged by the rarity, timing, and sustainability of weight loss. Study 2 was a comprehensive examination of the known variability in five genes that code for the two most abundantly produced adipokines (leptin and adiponectin). This second study did not identify any associations with postmenopausal breast cancer. Finally, Study 3 found no evidence of gene-gene or gene-environment joint effects between estrogen-related factors using two analytic methods: multifactor dimensionality reduction and logic regression. Other methods that incorporate all known biological information may be needed to model more complex relationships between the genes and the environment.

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CHAPTER 1: INTRODUCTION AND BACKGROUND

1.1 INTRODUCTION

Obesity has been consistently associated with an increased risk of postmenopausal breast cancer. However, the implications of this association for cancer prevention and the genetic contributions to hormonal mechanisms by which obesity may affect breast cancer risk are not fully understood. The determinants of both obesity and breast cancer are thought to be a combination of genetic and environmental factors. Known breast cancer risk factors include high penetrance genes such as *BRCA1* and *BRCA2* and non-genetic factors thought to operate mostly through hormonal mechanisms, including obesity. Sudden large shifts in the prevalence of obesity in populations are caused by intake of calorie dense foods and inactive lifestyles. However, family, twin, and adoption studies suggest that genetic factors contribute to the obesity phenotype (1). There are three proposed molecular mechanisms linking obesity and breast cancer: insulin and insulin-like growth factors, estrogen, and adipokines (proteins secreted from adipose tissue). The strongest evidence is for the estrogen pathway and the least-studied pathway is adipokines. Adipose tissue is the largest source of estrogen for postmenopausal women and both body mass index and breast cancer risk increase linearly with increasing levels of estrogen. The risk of postmenopausal breast cancer is increased about two-fold for women with the highest compared to the lowest levels of estradiol in many studies (2). Individual polymorphisms in the genes that are involved in estrogen biosynthesis, however, have not been consistently associated with postmenopausal breast cancer (3-5). One plausible explanation for this lack of association is that combinations of factors make a greater contribution to breast cancer risk than an individual factor alone. *In vitro*

studies have provided evidence that leptin promotes cell proliferation, cell survival, cell migration, and angiogenesis (6-13). Very few studies, however, have examined polymorphisms in the genes that produce these proteins.

The goal of this dissertation was to address three of the previously unanswered questions about the relationship between obesity and breast cancer. First, can the increased risk of postmenopausal breast cancer from obesity be reduced by later weight loss? Second, what impact do polymorphisms in adipokine genes have on postmenopausal breast cancer? And third, do combinations of genetic and non-genetic factors related to estrogen increase the risk of postmenopausal breast cancer? Data from the American Cancer Society Cancer Prevention Study-II was used to address these issues.

1.2 BACKGROUND

1.2.1 Breast Cancer

Incidence

Breast cancer is the most commonly diagnosed cancer among women. The lifetime risk of this malignancy is approximately 1 in 8 for U.S. women (14). Approximately 192,370 U.S. women were expected to develop the disease in 2009 (14). U.S. breast cancer incidence rates increased in the 1980s and most of the 90s, but have begun to decrease in more recent years (15-16). Data from the Surveillance, Epidemiology, and End Results (SEER) program show a decrease in age-specific rates between 1999 and 2003 for all U.S. women aged 45 years or older; however, the magnitude and precise timing of the decrease is age-dependent (16). The increase and subsequent decrease of breast cancer rates in the 80s and 90s has been partially attributed to screening mammography (17-20). Mammography rates increased from 29% in 1987 to 70% in 2000 (21). Since then, however, mammography rates have remained stable (16). Birth cohort changes in reproductive patterns and exposure to exogenous hormones are also thought to play a role in the 18 year increase, particularly among postmenopausal women (22). A more recently reported trend is the rapid decrease in breast cancer risk in women aged 50 to 69 years immediately following the Women Health Initiative (WHI) findings of an increase risk of breast cancer for users of postmenopausal hormone replacement therapy (PMH) (16). Breast cancer rates for women in this age group decreased by 12% in the one year between 2002 and 2003 (16). Despite the recent decreases in breast cancer risk, the absolute rates of disease are quite high and they continue to increase with increasing age

(the rates in postmenopausal women are in the range of 240-430 cases per 100,000/year). Incidence rates among black women older than 40 years have been consistently lower than for white women, but patterns of change over time are similar for blacks and whites (23).

Mortality

Breast cancer is the second leading cause of cancer deaths among women (15). The American Cancer Society estimates that 40,170 women will die from breast cancer in 2009 (14). In the early 1980s, breast cancer mortality rates for black women surpassed those of white women and continued to increase until the early 1990s. The rates for black women appear to have reached a plateau during the 1990s and have just begun to decrease since the turn of the century. Mortality rates for white women, however, remained fairly constant until the early 1990s when they began to decrease rapidly. Mortality rates for white women remain much lower than those for black women despite the recent decreases for both groups ((25 vs. 33 per 100,000) (23).

Survival

For women of all races combined, five-year survival rates for breast cancer are 98% for localized tumors, 84% for regional tumors and 23% for distant tumors. Survival rates for black women are considerably lower than for white women: 93% (localized), 72% (regional), and 16% (distant). Although some of the racial disparity in survival may be attributable to factors related to socioeconomic status, there is also some evidence that black women may be more likely to have aggressive tumors that are associated with poorer prognosis (24).

Histopathologic Characteristics of Invasive Breast Cancer

Most breast cancers are still localized to the breast when they are diagnosed (61%). In 31 percent of newly diagnosed cases the disease is found to have spread to regional lymph nodes, six percent of cases present with metastatic disease, and two percent of breast cancers are of unknown stage (23). Most breast cancers (75-80%) develop in the breast ducts, 5-10% percent develop in the in the milk lobules, and the other 10-20% originate from a variety of different cell types. Lobular breast cancers became more common in the 1990s while the frequency of ductal cancers remain the same. This is thought to be due to the increasing use of hormone replacement therapy during that decade (25). A large portion of breast cancers express both estrogen and progesterone receptors (ER and PR respectively). These ER/PR positive tumors have a more favorable prognosis partly because the tumors are more differentiated and partly because they typically respond well to hormonal and anti-estrogen treatment. Approximately 15-30% of breast tumors overproduce Human Epidermal growth factor Receptor 2 (HER2/neu). These tumors tend to grow faster and are more likely to recur than HER2 negative breast cancers (26). Tumors that are ER, PR, *and* HER2 negative are sometimes referred to as "triple negative" breast cancers. Triple negative tumors tend to be diagnosed at a later stage and higher grade than other breast cancers and have a worse prognosis. It is thought that triple negative breast cancers may have an entirely different set of risk factors than the hormone-related breast cancers (27).

International Variation and Migration Studies

According to recent estimates, over a million new breast cancer cases develop and over 400,000 deaths are attributable to breast cancer worldwide each year (28). However, the rates of the disease vary by geographic region. Female breast cancer incidence rates for

2002 ranged from 3.9 cases per 100,000 in Mozambique to 101.1 in the United States. Breast cancer incidence is highest in North America, Australia, and Northern and Western Europe; rates are lowest in central and other parts of Africa and in many parts of Asia. Variability in rates is likely due to differences in risk factor profiles and screening practices. Women who migrate from countries with typically low rates of breast cancer to countries with high rates are much more likely to develop breast cancer than those who do not relocate. A 2002 study from Hawaii and Los Angeles showed that Japanese-American women had almost the same risk of breast cancer as white women (29).

Risk Factors

The strongest risk factors for breast cancer are gender, age, and ionizing radiation. Women have one hundred times the risk of developing breast cancer compared to men. Breast cancer rates increase with increasing age until age 80 (23). The median age at diagnosis is 61 (14). Ionizing radiation is another strong and well-established risk factor for breast cancer. Studies of women exposed to high levels of radiation show consistently significantly increased risk of breast cancer. This risk appears to be the highest among women who were exposed before puberty (30-31).

Reproductive and hormonal factors have been consistently associated with breast cancer. Years of reproductive life: both early age at menarche and late age at menopause have been found to be associated with breast cancer in several studies. Later onset of menarche has been associated with a 5-20% decrease in breast cancer risk for each additional year of age at the first period (32-33). Likewise for each year later a woman reaches menopause her breast cancer risk increases by 3% (32). Having children, particularly at a younger age, also decreases breast cancer risk. Women who have their

first child before age 20 have a thirty percent lower risk than women who give birth after age thirty five (32). Although not all studies have found a relationship between breastfeeding and breast cancer, studies conducted in countries with longer duration of breastfeeding have found it to be a protective factor (34). A reanalysis of 47 studies conducted by the Oxford Collaborative Group in 2002 found a 4% decrease in breast cancer risk for each year of breastfeeding (35). Although this finding was consistent among women in both developing and industrialized countries, the number of women who breastfeed longer than twelve months in industrialized countries is small (36).

Endogenous hormones are associated with an increased risk of breast cancer. Women who have high levels of estrogens and androgens have a 2-3 times higher risk of developing breast cancer than women with low levels of these hormones (2). This increased risk is seen even in women who are at known genetic high risk of breast cancer and those who are taking postmenopausal hormones (although risk was lower than in women who had never used exogenous hormones) (2). Research on oral contraceptives and breast cancer has produced inconsistent results. Variation in results from early studies are hypothesized to be caused by variability in OC formulations or age or timing of use (37). A 1996 meta-analysis found an increased risk of 24% (38). Recently, a large case-control study conducted in the U.S. (39) found no association while a Norwegian cohort study (40) found a statistically significant 60% increased risk. Use of hormone replacement therapy has also been shown to increase risk of breast cancer (41-43). Estrogen alone is thought to increase risk by 0-3% per year and when estrogen is combined with progesterone it is thought to increase risk by approximately 4-8% per year (44).

Other risk factors may also be related to breast cancer, at least in part, through their impact on hormone levels. Increased levels of estrogen and possibly other hormones relate adiposity and breast cancer. This relationship is discussed in more detail in subsequent sections of this dissertation. A pooled analysis of six cohort studies found a 9% increase in breast cancer risk for each 10-gram per day increase in alcohol intake (45). Intervention studies have shown that consumption of alcohol can increase both total and bioavailable estrogen levels in both pre- and postmenopausal women, which may explain the increased risk with breast cancer (29). Although not all studies have found an association, a meta-analysis conducted by the World Cancer Research Fund (WCRF) and the American Institute for Cancer Research (AICR) found a 3% decreased risk of breast cancer for every seven metabolic equivalent (MET) hours of recreational activity per week. Physical activity is thought to impact breast cancer risk both through its impact on weight change and independently by reducing estrogen levels.

There is also evidence that genetic factors play a role in breast carcinogenesis. Having a family history of breast cancer is associated with an increased risk of breast cancer. Women whose mother or sister has a history of breast cancer have a 1.5- to 3-fold increase in breast cancer risk compared to women with no family history. Approximately 5-10% of all breast cancers, and 30% of breast cancers diagnosed before the age of thirty are thought to be caused by high penetrance genetic mutations such as *BRCA1* and *BRCA2* (46-47). Low penetrance genetic changes, most likely in combination with the environmental and other genetic factors, are suspected of playing a role in a larger percentage of breast cancers. Single nucleotide polymorphisms (SNPs) have been examined in many low penetrance genes, but replicable findings have thus

been few. The lack of replication of many studies may be due to the complexity of the relationship between these genes and breast cancer, as well as the fact that many genetic association studies have been underpowered to detect small associations. There have been some SNP associations that have been successfully replicated in genome-wide association studies or a breast cancer consortium. These include SNPs in *FGFR2* (46, 48), *LSP1* (48), *MAP3K1*(48), *TGFB1*(49), *TOX3*(48, 50), *CASP8* (49), 2q35 (50), and 8q24 (48).

Most studies that have identified risk factors have examined them in all breast cancers combined. As with many cancers, however, evidence is mounting that the etiology of breast cancer may vary by tumor characteristics (51). Therefore, the risk factors listed above may be unassociated or weaker with some histopathologic types of breast cancer and stronger with others.

1.2.2 Obesity

In the year 2000, for the first time in human history, the number of adults worldwide who were overweight was greater than the number of adults who were underweight (52). As of 2005, the World Health Organization (WHO) estimated that 1.6 billion adults (aged 15 and older) were overweight (BMI 25+), including 400 million obese (BMI 30+) individuals (53).

In the U.S., the rates of obesity have greatly increased over the past 50 years. The 1960-1962 National Health Examination Survey (NHANES) conducted by the Centers for Disease Control and Prevention (CDC) estimated that 45% of men and women were overweight or obese (54). The latest figures from the CDC estimate that as of 2007-2008, 68% of the country was overweight about half of whom were obese. Women were

slightly more likely to be obese than men (35.5% vs. 32%), although more men than women had a BMI of at least 25: (72.3% vs. 64.1%). There is even more variability in obesity prevalence by race. Non-Hispanic white women have the lowest prevalence (33%) and non-Hispanic black women (49.6%) have the highest prevalence of obesity (55).

The problem of obesity is not limited to the United States, it is now common in all parts of the world except sub-Saharan Africa (56). The prevalence of obesity in the U.S. is the ninth highest worldwide following several countries in the South Pacific and Saudi Arabia. Countries with similar or only slightly lower prevalence of obesity compared to the U.S. include Panama (34.74%), Egypt (30.3%), Kuwait (28.75%), the former Yugoslav Republic of Macedonia (25.1%), Seychelles (25.1%), and Mexico (23.6%) (57). Notably, several of those are low-income countries. Because overweight and obesity are a more recent phenomenon in the developing countries, the rate of change for many of these countries is two to five times greater than in the United States (58). In addition, there appears to be a changing trend in terms of socioeconomic (SES) status and obesity. Although the obesity epidemic began as a phenomenon of higher income people, the burden appears to be shifting towards lower income groups. For example, over a ten-year period in Brazil, the highest prevalence of obesity shifted from the highest to the lowest SES group. This has created a seemingly paradoxical situation where overweight adults and malnourished children are found within the same population strata (52).

Excess body weight contributes to a number of diseases including type 2 diabetes, cardiovascular diseases (coronary heart disease, hypertension, stroke), respiratory diseases, reproductive abnormalities, osteoarthritis, gallbladder disease, sleep apnea,

depression, and many cancers (59). The list of cancers known or strongly suspected of being associated with obesity continues to grow. The AICR/WCRF conducted a comprehensive review of the literature and concluded that the evidence is convincing that body fatness is a cause of the cancers of the esophagus (adenocarcinoma), pancreas, colon and rectum, breast (postmenopausal), endometrium, and kidney (60). In addition, the evidence is convincing that gallbladder cancer may be caused by body fatness and there is limited evidence that it may be a cause of liver cancer.

In addition to its impact on morbidity, obesity also contributes to premature mortality. Currently obesity accounts for 5-15% of U.S. deaths and smoking accounts for 18% (61). However, if obesity continues to increase, it will pass smoking as the leading risk factor for mortality (51). Obesity is estimated to decrease life expectancy by seven years at the age of 40 (56) and this impact may increase as greater number (and proportion) of children are affected (62). In fact, it has been hypothesized that the current trend of increasing life expectancy may level off or even decline if obesity trends continue (63).

1.2.3 Adiposity and Breast Cancer: Association and Potential Mechanisms

There is substantial evidence to show that increases in adiposity increase breast cancer risk among postmenopausal women. A WCRF/AICR meta-analysis found that breast cancer risk increased by about 3% for each 5 kilogram increase in weight. However, this association becomes much stronger when results are stratified on PMH use. Studies have found an approximately two-fold increased risk with 50 or more pounds of weight gain among never PMH users (64-66). Among pre-menopausal women the relationship between adiposity and breast cancer appears to be protective (60). This may

be the reason weight gain appears more harmful in terms of breast cancer risk than body mass index (BMI, kg/m^2). Women who were lean as young adults and heavier postmenopausally would have a higher risk of breast cancer in both time periods. In addition to its impact on weight change, physical activity has been shown to be an independent predictor of breast cancer risk.

Currently there are three main hypothesized molecular pathways between adiposity and breast cancer: increased insulin and insulin-like growth factor (IGF), increased estrogen, and autocrine, paracrine, and endocrine signaling from proteins secreted by adipocytes. While this dissertation mainly focuses on the second and third of these pathways, they are all inextricably linked.

Insulin/IGFs

In obese individuals increased levels of free fatty acids (FFA) are released from adipose tissue. These increases in FFA force the liver and other tissues to focus on storage and oxidation of fats, and as a result, they have a reduced capacity to absorb, store, and metabolize glucose. The result is insulin resistance and hyperinsulinemia. These increased levels of insulin in turn lead to reduced liver synthesis and blood levels of insulin-like growth factor binding proteins (IGFBP) 1 and 2. In the absence of the binding proteins there are excesses of free insulin-like growth factor-1. These increased levels of insulin and IGFs are hypothesized to lead to increased cell proliferation and, therefore carcinogenesis (67).

Estrogen

Biosynthesis of estrone and estradiol from adipose tissue is the major source of estrogen in postmenopausal women. Women who have high levels of circulating estrogen have a

2-3 times increase in breast cancer risk compared to those with low estrogen levels (2). Estradiol (the bioactive form of estrogen) is synthesized from the conversion of androgens and estrone by the enzymes aromatase and 17-β hydroxysteroid dehydrogenase (17-βHSD). In addition, adipokines such as leptin, TNF, and IL6 have been shown to upregulate aromatase and 17-βHSD or transcriptionally activate the estrogen receptor in the absence of estrogen (REF). SNPs in the genes that produce these estrogen biosynthesis factors have been studied in relation to breast cancer risk, but results have identified very few associations (68-72).

Adipokines

Adipose tissue is made up of more than just fat cells. In addition to adipocytes, adipose tissue contains a connective tissue matrix, nerve tissue, stromovascular cells, and immune cells (73). In addition, there are two types of adipocytes: white and brown. Brown adipocytes are mostly responsible for heat production (74). White adipocytes are the primary energy reservoir in humans and provide long term fuel for the body (75). White adipocytes absorb fatty acids from food, as well as those derived from glucose in the liver and contribute to the expanding lipid droplet (76-77). It was previously believed that this was their only function, however, we know now that white adipocytes secrete proteins such as leptin and adiponectin. Other adipose tissue cells also secrete bioactive molecules. Macrophages, for example, secrete cytokines such as TNF-α and IL-6 (78). Because so many adipokines have now been discovered, adipose tissue has been called the body's most prolific endocrine organ (79).

It has been hypothesized that these adipokines may be involved directly and/or indirectly in the development of postmenopausal breast cancer. Animal studies,

microarray analysis, and *in vitro* tumor studies provide evidence that adipose tissue and these adipokines can directly influence tumor growth (79-81). Some of the most compelling evidence comes from a study by Iyengar, et al. Tumors formed from human breast cancer cells injected into adipocytes of mice grew three times larger than tumors from human breast cancer cells injected into the fibroblasts of mice (81).

1.2.4 Summary

In summary, what we now know is that 1) obesity (particularly adult weight gain) is associated with postmenopausal breast cancer, 2) adipose tissue secretes proteins that may promote carcinogenesis, and 3) breast cancer risk is strongly associated with estrogen. What we do not know is 1) whether this increased risk for postmenopausal breast cancer with obesity can be reduced with later weight loss, 2) whether polymorphisms in adipokine genes are associated with breast cancer risk, and 3) whether genetic and non-genetic factors related to estrogen levels combine to impact breast cancer risk. These issues will be addresses by three separate but related studies.

1.3 Dissertation Aims

Aim 1. Describe the association between weight loss and postmenopausal breast cancer in overweight women.

1a. Is adult weight loss among overweight women associated with a reduced risk of incident postmenopausal breast cancer? If so, what is the shape of the dose-response curve?

1b. Does menopausal status, postmenopausal hormone use, or starting body mass index modify the association between weight loss and incident postmenopausal breast cancer in overweight women?

Aim 2. Evaluate association between genes that encode adipokines (proteins secreted from adipose tissue) and postmenopausal breast cancer.

2a. Are single nucleotide polymorphisms (SNPs) in the following genes associated with postmenopausal breast cancer: *leptin*, *leptin receptor*, *adiponectin*, *adiponectin receptor 1 and adiponectin receptor 2*?

2b. Is this relationship different when stratified by BMI, location of weight gain, or physical activity?

Aim 3. Identify joint effects between genetic and non-genetic variables that impact estrogen levels that impact risk of breast cancer.

3a. Are there combinations of SNPs in estrogen biosynthesis genes and estrogen-related non-genetic factors (obesity, physical activity, and postmenopausal hormone use) that affect risk for postmenopausal breast cancer?

3b. If so, are the findings replicable using different analytic methods?

CHAPTER 2: WEIGHT LOSS AND POSTMENOPAUSAL BREAST CANCER IN A PROSPECTIVE COHORT OF U.S. WOMEN

2.1 Abstract

Overweight and obesity are associated with an increase in postmenopausal breast cancer risk; however, it is unclear whether losing excess weight will lower a woman's risk. We analyzed data on overweight and obese women from the Cancer Prevention Study II (CPS-II) Nutrition Cohort to examine the relationship between weight loss and postmenopausal breast cancer risk. Among the 13,055 cancer-free women included in the analysis, 815 postmenopausal breast cancer cases were diagnosed between enrollment in 1992 and June 30, 2007. Self-reported weight was collected prospectively at two different time periods during adulthood ten years apart. Among weight losers the median weight loss was 11 pounds, but only 52% of the women maintained this weight loss through the next five years (1992-1997). We used restricted cubic splines (to explore possible non-linear associations) and multivariate Cox proportional hazards modeling (for categorical analyses) and observed no association between weight loss and postmenopausal breast cancer using either method. The hazard rate ratio (RR) for 30+ pounds weight loss compared to stable weight was 0.95 (0.67-1.35). Restricting analyses to women who had maintained or lost more weight did not change results. There was no evidence of effect modification by postmenopausal hormone (PMH) use, initial BMI, age, menopausal status at the time of weight loss, or previous weight change. In summary, weight loss was not associated with postmenopausal breast cancer risk in this study. This finding warrants cautious interpretation because sustained weight loss is rare and because timing of weight loss may be important.

2.2 Introduction

A 2007 consensus report by the World Cancer Research Fund and the American Institute for Cancer Research concluded that there is convincing and consistent evidence that a clear dose-response relationship between greater body fatness and postmenopausal breast cancer exists in humans (60). We previously found that 60 or more pounds of weight gain during adulthood was associated with a two-fold increased risk of breast cancer (64). It is unknown, however, whether *losing* excess weight will lower a woman's risk of the disease. Modest weight loss (5-10% of starting weight) has been shown to reduce risk of cardiovascular disease and diabetes (82) but most observational studies examining weight loss and postmenopausal breast cancer have reported null results or weak and not statistically significant inverse associations (65-66, 83-96) (Figure 2.1). The magnitude of effect ranged from 0.55 to 0.84 but a wide range is expected since the referent groups and starting and ending weights varied from study to study. An additional study by Trentham-Dietz, et al found that women who reached their highest lifetime weight between the age of 11 and 45 and subsequently lost weight, had a 10% reduction in breast cancer risk per 5kg lost (87). Several studies have attempted to ascertain the importance of timing of weight loss. Researchers from the Iowa Women's Health Study Cohort compared weight change patterns in three time periods: age 18 to age 30, age 30 to menopause, and after menopause. Regardless of the timing of the loss, they found that weight loss decreased the risk of breast cancer compared to those who gained weight (85). This was even true for women who gained weight in one time period and lost weight in the next (compared to women who gained in both time periods), suggesting that the detrimental effects of

weight gain can be reversed by weight loss. Although the results were not statistically significant, Ziegler, et al found that for women in their 50s, recent weight loss was a stronger predictor of breast cancer risk than weight loss throughout adulthood (94). Finally, Eliassen, et al found that weight loss after menopause was associated with lower breast cancer risk. This finding was only significant among women who did not use postmenopausal hormone therapy (10kg lost compared to ±2kg, HR=0.43 (0.21-0.86)) (65). However, another recent cohort study examined weight loss since menopause and was not able to replicate this finding (66).

Additional evidence in support of an inverse association between weight loss and cancer comes from bariatric surgery studies (97-99). A recent Canadian study found that bariatric surgery patients were significantly less likely to be diagnosed with breast cancer ($p=0.001$) over five years of follow-up compared to obese controls (99).

There are several possible explanations for the null (or non-significant) results in many previous studies including power limitations, potential for misclassification due to the lack of sustained weight loss, timing of weight loss, and potential effect modification by PMH or other factors. Since no study other than those focusing on bariatric surgery patients has restricted their analysis to overweight or obese women, another possibility is that the women who lost weight were already at a relatively low risk of breast cancer and, thus, the weight loss did not reduce their risk relative to the comparison group. To help clarify the relationship between weight loss and postmenopausal breast cancer, particularly among women who are overweight or obese, we conducted an analysis of the data from the American Cancer Society Cancer Prevention Study II (ACS CPS-II) Nutrition Cohort.

2.3 Methods

Study Population

Participants in this study were drawn from the CPS-II Nutrition Cohort, a prospective cohort study established by the American Cancer Society in 1992 (100). The Nutrition Cohort is a subset of the 1.2 million men and women enrolled in the CPS-II Mortality Cohort in 1982 (101) who were aged 50–74 years in 1992 and lived in one of 21 U.S. states with a population-based state cancer registry. The 1982 baseline cohort was recruited by 77,000 ACS volunteers in all 50 U.S. states, the District of Columbia, and Puerto Rico. The participants were friends and family of the volunteers and are, therefore, not a random sample of the U.S. population. When participants enrolled in the baseline cohort, they completed a four-page, self-administered questionnaire that included demographic characteristics, personal and family history of cancer and other diseases, reproductive history, and various behavioral, environmental, occupational, and dietary exposures. In 1992, participants completed a more detailed self-administered questionnaire that included further questions on demographic, medical, reproductive, dietary, and behavioral factors. Beginning in 1997, follow-up surveys were sent to cohort members every 2 years to update exposure information and to ascertain newly diagnosed cancers. Follow-up survey response rates among living cohort members have been at least 89%. This analysis is based on fifteen years of follow-up (1992—2007).

Women were excluded from the analysis if they were lost to follow-up (n=3,116), reported having cancer before baseline (n=12,057), had missing or implausible (BMI<15) reported weights (n=1,811), were not postmenopausal at baseline (n=4,594), or had missing breast cancer diagnosis date information (n=82) (Table 2.1). Since the focus of this paper is weight loss among overweight women, participants who gained five or more

pounds during our exposure period (n=37,764) (1982-1992) and those who were normal or underweight in 1982 (BMI<25) (n=25,307) were also excluded from the analysis. The final analytic cohort consisted of 13,055 women who each contributed an average of 11.8 person years.

Breast Cancer Case Ascertainment

Among the women included in this analysis, we identified a total of 815 incident cases of breast cancer diagnosed between enrollment and June 30, 2007. The majority of the breast cancer cases were initially self-reported on one of the follow-up surveys (1997, 1999, 2001, 2003, or 2005) (n=778). Ninety four percent of the cases were subsequently verified by medical record review (n= 564) or cancer registry linkage (n=167). Because self reports of breast cancer have been shown to be very accurate in this cohort (102), we also included a small number of reported breast cancers (n=46) that have not yet been verified. Sixteen additional cases were initially identified from death certificates during routine linkage of the entire cohort to the National Death Index and 12 of these were later verified through registry linkage. 21 additional cases were identified through verification of another cancer reported by the participant.

Weight Loss Measure

Participants were asked to report their current weight on both the 1982 CPS-II Mortality Cohort survey and the 1992 Nutrition Cohort survey. Weight change was calculated as weight in 1992 minus weight in 1982. The categorical analysis subdivided all participants into five groups. The reference group included women who lost no more than 4lbs or gained no more than 5lbs (weight maintenance); the remaining four groups were defined as weight loss of 5-9lbs, 10-19lbs, 20-29 lbs and 30+ lbs. Percent weight lost was

calculated as weight lost between 1982 and 1992 divided by weight in 1982 times one hundred. For the categorical percent weight lost analysis, the reference group was no weight change and the exposure groups were 1-<5%, 5-<10%, 10-<15%, 15-<20%, or 20+% of body weight lost.

Statistical Analyses

All statistical analyses were conducted using SAS v.9.2. We used restricted cubic splines to examine the possible non-linear relation between adult weight loss and postmenopausal breast cancer. The LGTPHCURV8 SAS macro published by Li, et al was used to conduct the spline analyses (103). Tests for non-linearity used the likelihood ratio test comparing the model with only the linear term to the model with the linear and the cubic spline terms. Multivariate Cox proportional hazards models were also used to create hazard rate ratios (RRs) and corresponding 95% confidence intervals (CIs) for the categorical analyses (104).

Spline and Cox models were adjusted for the following potential confounders: baseline body mass index (BMI) in 1982 (25-27, >27-29, >29-31,>31-33, >33-35, >35), BMI at age 18 (18.5-<22.5,22.5-<25, 25+), postmenopausal hormone replacement therapy (PMH) use in 1992 (never, current, former, unknown), parity (none, one, two, three, four or more, unknown), age at first live birth (<21, 21-22, 23-24, 25-29, 30+, unknown), alcohol use (nondrinker, former drinker, 1 drink or less per day, 2-3 drinks per day, 4+ drinks per day, unknown), physical activity (none/slight, moderate, heavy, unknown), race (white, black, other race), education (less than high school, high school graduate, some college, college graduate, unknown), mammography at baseline in 1992 (within the last year, not within the last year, unknown), family history of breast cancer

(yes, no), and oral contraceptive use (never, ever, unknown). In addition, all models were stratified on single year of age at enrollment. Effect modification by age, menopausal status at the time of the weight loss, PMH use, and previous weight change was evaluated using a likelihood ratio test. Models with multiplicative interaction terms for each of the potential effect modifiers were compared to models with no interaction terms and a *p*-value <0.05 was considered statistically significant. Previous weight change was defined as the change in weight between age 18 (reported on the 1992 survey) and current weight in 1982.

To compare the effect of any weight loss to that of sustained weight loss, we conducted a sensitivity analysis that focused on women who maintained their weight or lost more weight through the first follow-up interval (1992-1997). We also conducted a sensitivity analysis restricting cases to invasive cancer only in order to rule out differential effects of weight loss for *in situ* and invasive cases.

All aspects of the CPS-II study protocol have been reviewed and approved by the Emory University Institutional Review Board.

2.4 Results

Selected participant characteristics are described in Table 2.2. The mean age of women in this study in 1992 (at baseline) was 64 years. Weight loss from 1982 to 1992 generally captured the period of 5 to 15 years after menopause. Among the 6,180 women who reported losing five or more pounds between 1982 and 1992, the median weight loss was 11 pounds (inter-quartile range: 7-20 lbs). Ten percent of these women lost 30 or more pounds. Women who lost more weight began follow-up heavier than those who lost less weight. For example, women who lost 30+ pounds during this time period had a median

BMI of 32.8 in 1982 whereas women who maintained their weight (4lbs lost to 5lbs gained) had a median BMI of 27.4 in 1982. Women who lost thirty or more pounds were more likely to have never used PMH, less likely to have a college education, be nondrinkers, have never had a mammogram, and have self-reported diabetes, heart disease or high blood pressure in 1992 than women whose weight remained essentially unchanged between 1982 and 1992.

We observed no association between weight loss and postmenopausal breast cancer whether using continuous or categorical variables. The p-value for non-linearity was p= 0.92 and the p-value for a linear association was p=0.71 from the spline model (Figure 2.3). The multivariate hazard rate ratio (RR) for 30+ pounds of weight loss compared to unchanged weight was 0.95 (0.67-1.35) (Table 2.3). There was still no association between weight loss and postmenopausal breast cancer after further restricting the analyses to women who had maintained their weight or lost more weight during the next interval (1992-1997). Stratifying on PMH use or initial BMI did not change the results (Tables 2.4). There was also no evidence of effect modification by age, menopausal status at the time of the weight loss, or previous weight change. The sensitivity analysis restricting cases to invasive breast cancer cases did not change the results. The analyses focusing on percent weight loss also produced null results (Table 2.5).

2.5 Discussion

In this prospective cohort study, adult weight loss was not related to postmenopausal breast cancer among overweight women. While our results are compatible with most of the observational studies published to date (66, 84, 86-87, 89-90,

93-96), they are not consistent with mechanistic studies that show that weight loss is associated with lower levels of circulating estrogens (62, 105). Circulating estrogen levels have been consistently shown to be linearly associated with breast cancer risk (2).

Studies of women who have undergone bariatric surgery have reported an overall lower cancer incidence and mortality compared to obese controls (97-99). One of these studies found a pronounced reduction in risk of breast cancer (99) but this finding was not confirmed in another study (97). It is important to note that the bariatric surgery studies included relatively young participants (average age: 39-47 years) that may not be directly comparable to our population of older postmenopausal women. Adams, et al (97) tried to examine pre- and postmenopausal breast cancers separately, however, they categorized women as postmenopausal if they were aged 50 and older, which may have resulted in misclassification of menopausal status. Another distinguishing characteristic of the bariatric surgery studies is that they examined the effects of extreme and rapid amounts of weight loss resulting from the procedure. These studies do not inform us about smaller amounts of weight loss, or gradual weight loss that results from exercise and diet modification. Only one of these studies (98) was able to examine the effect of the amount of weight lost after the surgery (rather than just surgery compared to no surgery). Perhaps the most important limitation of this group of studies is that bariatric surgery patients, unlike controls, are screened for cancer and other conditions before the surgery; therefore, they are less likely to have an undetected disease at study entry. Finally, it is also possible that the reduced risk of cancer after bariatric surgery may be linked to physiologic and biochemical changes from the surgery rather than the reduction in adipose tissue. This idea is supported by a prospective study that found a strong effect

for bariatric surgery compared to no surgery but did not find a dose-response relationship with weight loss within either the surgery or the control groups (98).

Although this is a relatively large, prospective study with repeat weight measures, there are limitations to our study that are important to note. In this cohort of overweight women, only 12% of the participants lost 20 or more pounds from 1982 to 1992 and less than 5% lost more than 30 pounds (figure 2.2). Thus, we may not have had enough women losing enough weight to detect an association. Research has shown that women who lose weight often do not maintain their weight loss, regardless of the method used (106). One recent cohort study found a strong statistically significant association between weight loss since menopause and breast cancer only when the reduced weight was maintained for at least two survey cycles (four years). Although we examined weight maintenance over more than one cycle, our first interval was ten years and our second interval was five years, and we were unable to assess weight fluctuations during those intervals. In addition, the relevant timing of the weight loss, latency, and induction periods are unknown. Eliassen, et al found an association with weight loss since menopause but not weight loss since age 18 (65).

It is clear that more research must be done on weight loss and breast cancer in very large studies that include a wide range of weight loss, weight measures at several different life points, and with longer follow-up. A consortium, much like the ones currently studying genetic risk factors, would be most helpful given that weight loss is a relatively rare exposure. Given that body weight is one of the few modifiable risk factors for postmenopausal breast cancer, understanding the potential benefits of weight loss remains a crucial area of research.

2.6 Figures and Tables

Table 2.1. Exclusion cascade to create analytic cohort

	Women n=97,786	Incident breast cancer n=5,299
Lost to follow-up	3,116	0
Prevalent breast cancer	6,230	0
Prevalent other cancer	5,827	386
Missing/Invalid weight	1,811	118
Not postmenopausal by 1992	4,594	336
Unknown diagnosis date	82	82
Weight gain (6+lbs) 1982-1992	37,764	2,638
Body mass index <25	25,307	1,626
Final Analytic Cohort	**13,055**	**815**

Table 2.2. Characteristics of overweight and obese women who lost or maintained weight between 1982-1992

	-4 to 5lbs	-5 to -9lbs	-10 to -19lbs	-20 to -29lbs	<-30lbs
Characteristic*	N=6,868	N=2,174	N=2,384	N=965	N=664
Median weight change 1982-1992 (pounds)	1	-5	-13	-22	-37
Median age in 1992 (yrs)	64	65	65	64	64
Median age at menopause (yrs)	50	50	49.5	50	49
Median body mass index in 1982 ((kg/m^2)	27.4	28	28.5	30	32.8
Median body mass index in 1992 ((kg/m^2)	27.5	26.7	26.5	25.8	25.8
Median weight gain since age 18 (pounds)	37	39	44	49	60
Menopausal Status in 1982					
Pre/Peri Menopausal	27.5%	25.7%	24.5%	29.7%	27.0%
Postmenopausal	72.5%	74.3%	75.5%	70.3%	73.0%
Postmenopausal Hormone Use in 1992					
Never	48.4%	48.7%	50.5%	55.2%	56.5%
Current	26.0%	25.2%	23.0%	20.8%	19.9%
Former	23.1%	23.7%	24.0%	21.6%	20.7%
Education as of 1982					
< High School	7.1%	9.6%	9.2%	7.9%	8.3%
High School Graduate	38.3%	35.7%	36.5%	33.8%	38.4%
Some College	30.5%	30.2%	29.8%	34.4%	31.6%
College Graduate	24.3%	24.5%	24.6%	23.9%	21.7%
Race					
White	96.7%	97%	96.7%	97.3%	96.2%
Black	2.2%	1.8%	2.3%	2.3%	2.9%
Other	1.1%	1.1%	1%	0.4%	0.9%
Physical Activity in 1982					
None/Slight	30.9%	30.5%	33.7%	32.4%	38.0%
Moderate	64.5%	64.8%	62.1%	63.1%	56.2%
Heavy	4.6%	4.7%	4.2%	4.5%	5.8%

Table 2.2. Continued…

Characteristic*	-4 to 5lbs N=6,870	-5 to -9lbs N=2,175	-10 to -19lbs N=2,386	-20 to -29lbs N=965	<-30lbs N=664
Alcohol					
Nondrinker	42.6%	45.4%	47.1%	47.9%	54.7%
<=1/day	39.7%	36.9%	36.1%	36%	31.7%
2-3/day	10.7%	10.8%	8.5%	8.9%	6.7%
4+/day	3.9%	3.8%	4.5%	3.8%	3.3%
Former	3.1%	3.0%	3.8%	3.4%	3.7%
Mammography (as of 1992)					
Never	8.6%	9.4%	10.3%	11.5%	13.5%
Yes, Recent	61.8%	63.1%	60.8%	61.5%	55.9%
Yes, Not Recent	29.6%	27.5%	28.9%	27.0%	30.6%
Diabetes 1992					
No diabetes	89.9%	84.4%	80.4%	75.9%	69.7%
Diabetes 1992	9.7%	15.1%	19.2%	23.7%	30.1%
High Blood Pressure in 1992					
No	53.5%	51.3%	49.8%	49.2%	41.0%
Yes	46.5%	48.7%	50.2%	50.8%	59.0%
High Cholesterol in 1992					
No	56.7%	55.0%	57.2%	59.4%	62.2%
Yes	43.3%	45.0%	42.8%	40.6%	37.8%

*Columns that do not add to 100% reflect missing data

Table 2.3. Hazard ratios of breast cancer incidence according to weight loss between 1982 and 1992 among overweight and obese women

	Weight Loss			Maintained Weight Loss		
Weight Loss (Pounds)	Cases	Person-years	Multivariate Model	Cases	Person-years	Multivariate Model
+5 to -4	428	83,436	1.00 (ref)	132	22,118	1.00 (ref)
-5 to -9	134	25,834	0.99 (0.82-1.20)	62	9,873	1.04 (0.77 -1.42)
-10 to -19	156	27,417	1.08 (0.90-1.30)	46	9,624	0.79 (0.56-1.11)
-20 to -29	61	10,783	1.03 (0.79-1.36)	14	3,385	0.63 (0.36-1.13)
<=-30	36	6,878	0.95 (0.67-1.35)	9	1,476	0.94 (0.46- 1.93)

*Models adjusted for body mass index at baseline, body mass index at age 18, alcohol use, physical activity, menopausal status, oral contraceptive use, parity, age at first birth, race, education, family history of breast cancer, mammography, and postmenopausal hormone use

†Women in the "maintained weight loss" analysis did not gain more than five pounds in the subsequent interval (1992 to 1997).

Table 2.4. Hazard ratios of breast cancer incidence according to weight loss between 1982 and 1992 stratified by postmenopausal hormone (PMH) use and baseline body mass index (BMI) among overweight and obese women

		Weight Loss (Pounds)	Cases	Person-years	Multivariate* Model
PMH Use	**Never**	+5 to -4	193	38,541	1.00 (ref)
		-5 to -9	57	12,149	0.90 (0.67-1.21)
		-10 to -19	73	13,188	1.05 (0.80-1.38)
		-20 to -29	38	5,615	1.22 (0.85-1.74)
		<=-30	18	3,682	0.86 (0.52-1.41)
	Current	+5 to -4	119	21,690	1.00 (ref)
		-5 to -9	36	6,569	0.98 (0.67-1.43)
		-10 to -19	43	6,414	1.18 (0.83-1.68)
		-20 to -29	7	2,331	0.52 (0.24-1.12)
		<=-30	6	1,477	0.73 (0.32-1.68)
	Former	+5 to -4	82	18,221	1.00 (ref)
		-5 to -9	32	5,690	1.26 (0.84-1.90)
		-10 to -19	35	6,182	1.28 (0.86-1.91)
		-20 to -29	10	2,223	0.94 (0.48-1.82)
		<=-30	11	1,321	1.71 (0.90-3.25)
BMI in 1982	**Overweight BMI 26-30**	+5 to -4	324	482.76	1.00 (ref)
		-5 to -9	100	493.15	1.67 (0.38-7.27)
		-10 to -19	87	493.75	0.46 (0.12-1.73)
		-20 to -29	27	420.72	0.56 (0.08-4.00)
		<=-30	10	524.07	0.91 (0.05-15.95)
	Obese BMI 31+	+5 to -4	104	19,723	1.00 (ref)
		-5 to -9	34	7,144	1.42 (0.51-3.98)
		-10 to -19	69	10,198	0.59 (0.23-1.51)
		-20 to -29	34	5,371	0.67 (0.16-2.76)
		<=-30	26	4,899	0.93 (0.11-7.56)

*Models adjusted for body mass index at baseline, body mass index at age 18, alcohol use, physical activity, menopausal status, oral contraceptive use, parity, age at first birth, race, education, family history of breast cancer, mammography, and postmenopausal hormone use

Table 2.5. Hazard ratios of breast cancer incidence according to percent weight loss between 1982 and 1992 among overweight and obese women

Percent Weight Loss	Cases	Person-years	Multivariate* Models
No weight change	347	66,412	1.00 (ref)
1-5%	212	40,471	0.99 (0.83-1.18)
5-<10%	155	27,180	1.11 (0.92-1.34)
10-<15%	53	12,453	0.80 (0.60-1.07)
15-<20%	28	4,720	1.17 (0.79-1.72)
20+%	20	3,111	1.27 (0.81-2.00)

*Models adjusted for body mass index, alcohol use, physical activity, menopausal status, oral contraceptive use, parity, age at first birth, race, education, family history of breast cancer, mammography, and postmenopausal hormone use

Figure 2.1. Previous population-based studies of weight loss and breast cancer

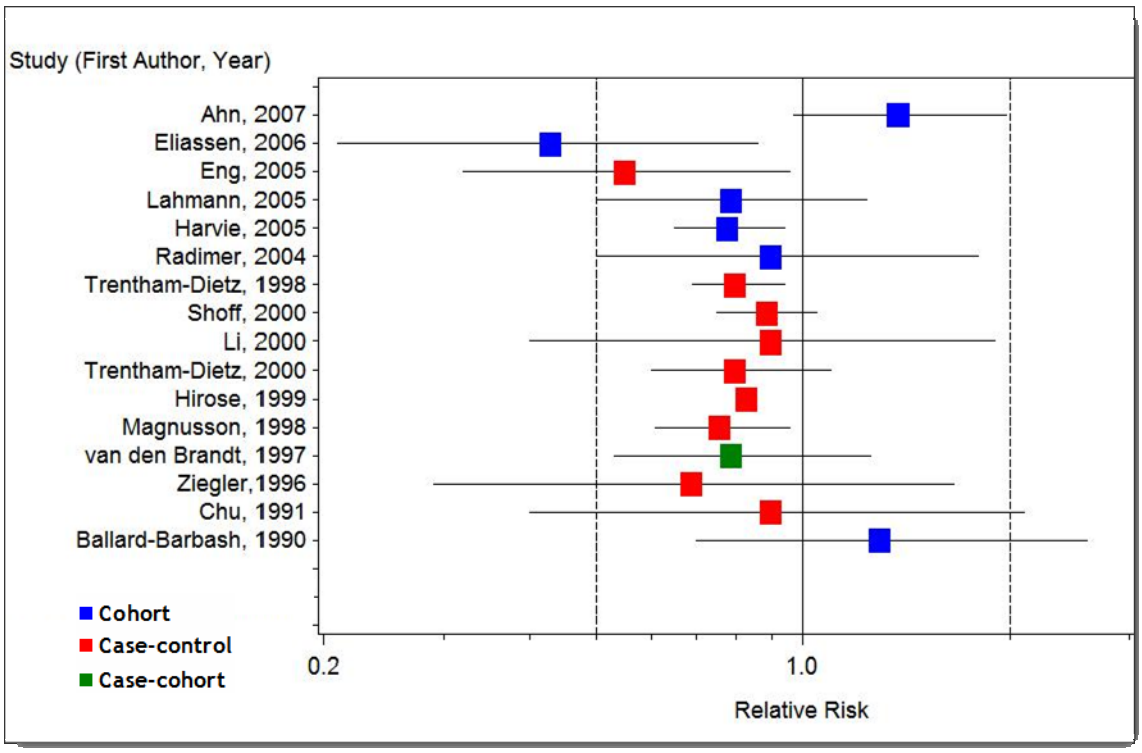


Figure 2.2. Histogram of reported weight loss over ten years during adulthood (1982-1992) in the American Cancer Society Cancer Prevention Study-II Nutrition Cohort

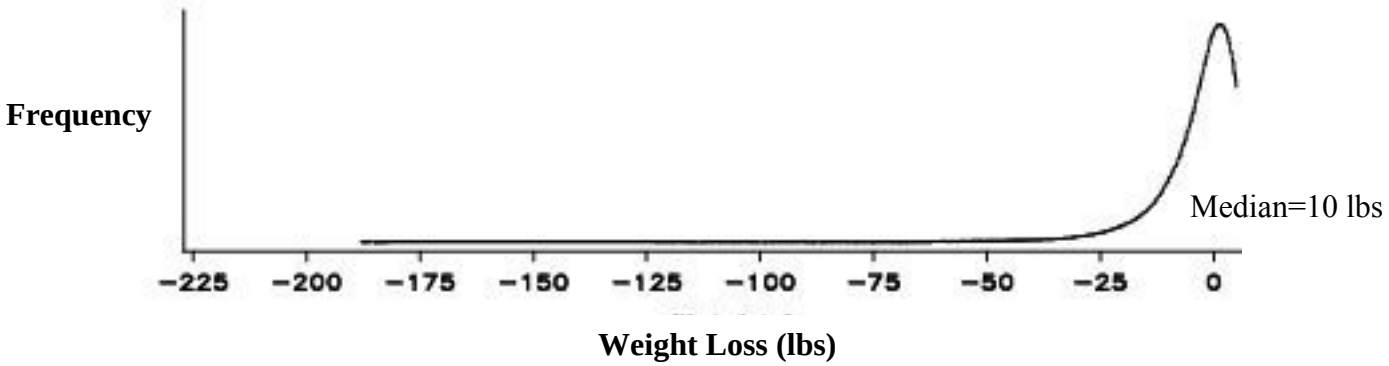


Figure 2.3. Multivariate spline of weight loss between 1982 and 1992 among overweight and obese women

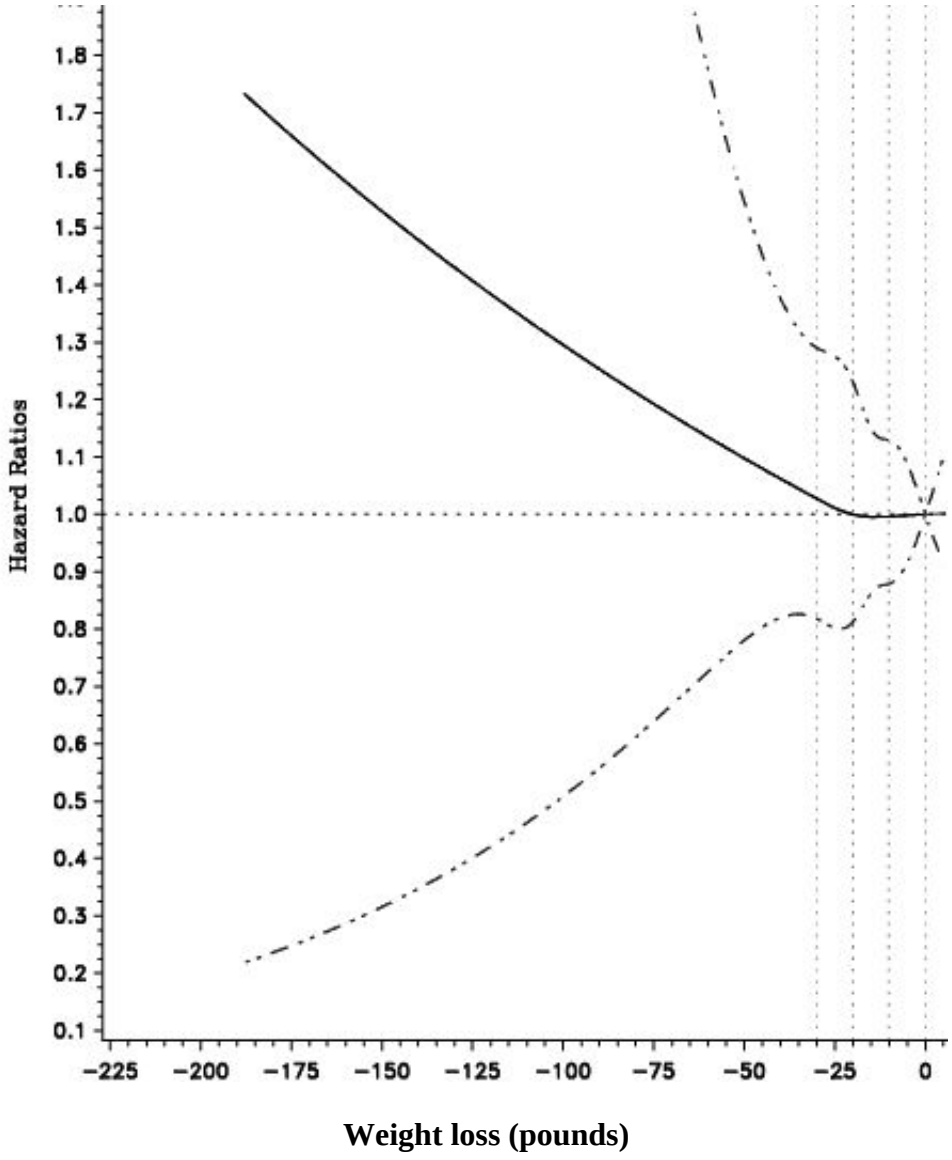
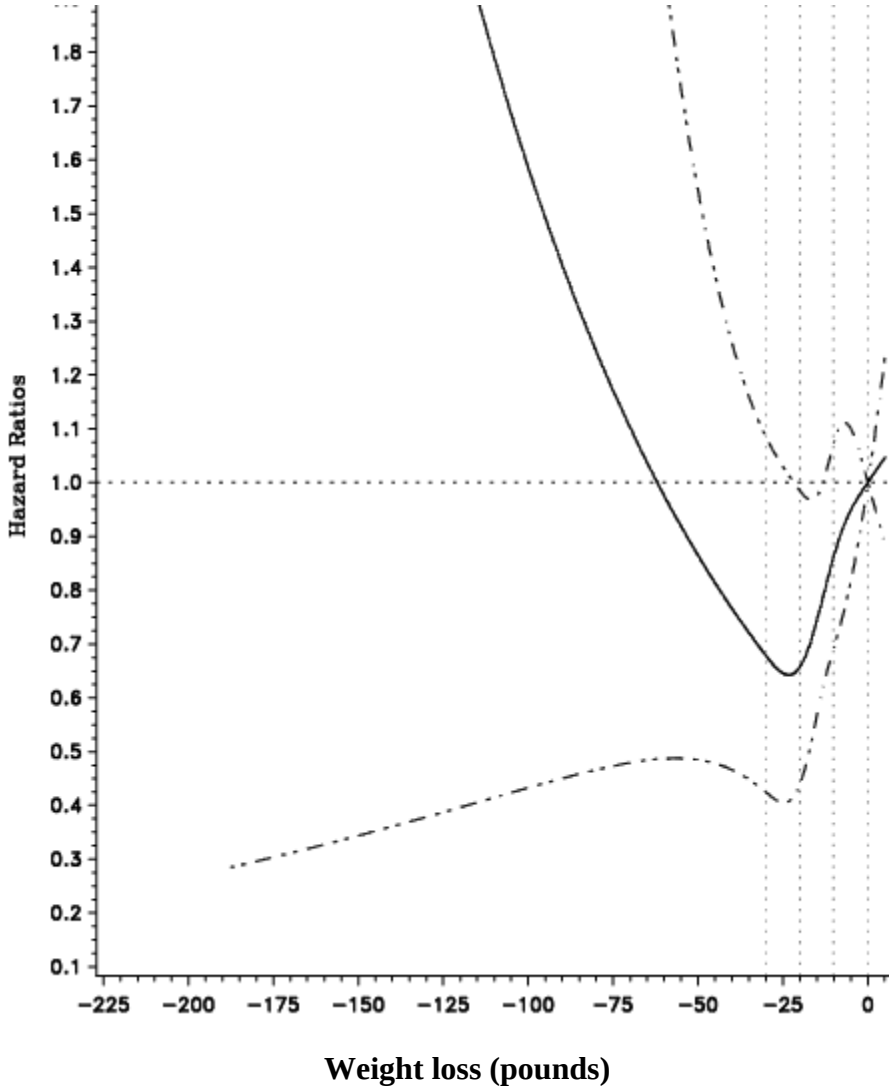


Figure 2.4. Multivariate spline of weight loss between 1982 and 1992 among overweight and obese women who maintained the weight loss



CHAPTER 3: NO ASSOCIATION BETWEEN POLYMORPHISMS IN *LEP, LEPR, ADIPOQ, ADIPOR1,* OR *ADIPOR2* AND POSTMENOPAUSAL BREAST CANCER RISK

3.1 Abstract

There is evidence that adipokines such as leptin and adiponectin may influence breast tumor development. We conducted a nested case control study using women in the American Cancer Society Cancer Prevention Study II to examine the association between postmenopausal breast cancer and variability in the genes encoding leptin, the leptin receptor, adiponectin, adiponectin receptor 1, and adiponectin receptor 2. Using 648 cases and 659 controls, we found no statistically significant ($p<0.05$) associations between breast cancer risk and any of the single nucleotide polymorphisms. Individual odds ratios ranged from 0.92 to 1.05. We found no evidence of effect modification by body mass index, adult weight gain, location of weight gain or physical activity. Although we can not rule out that these genes are involved in gene-gene or gene-environment interactions, our results suggest that individual single nucleotide polymorphisms in these genes do not substantially impact postmenopausal breast cancer risk.

3.2 Introduction

Adipokines (proteins secreted from adipose tissue) such as leptin and adiponectin have been hypothesized to influence breast tumor development (107-109). Leptin is a 167-amino acid, cytokine-like protein secreted mostly from adipocytes. Leptin is produced by the gene also named *leptin* located on chromosome 7 (7q31.3) (110). The gene is made up of three exons and two introns (111). The leptin receptor is a single-transmembrane-domain receptor of the cytokine receptor family (112). The gene that encodes this receptor is on chromosome 1 at 1p31 (113). The receptor has at least six splice variants containing up to 18 coding exons (114). All isoforms have the same extracellular domain that binds leptin but have intracellular domains of different lengths. The long isoform is 1,165 amino acids long and contains two JAK2 binding sites, a binding site for SHP2, and a binding site for STAT3 (115). Its intracellular portion is responsible for recruiting and activating signaling substrates. Other isoforms have some, but not all, of these intracellular features (113, 116), and therefore have reduced or completely disabled signaling capabilities (117).

The name leptin aptly originates from the Greek word *leptos*, meaning thin, as it was the first human gene to be known to be involved in obesity. In 1994, this gene was discovered to be the human homolog of the rat obesity gene, *Ob* through positional cloning (118-119). Rare mutations in both the *leptin* and the *leptin receptor* genes are known to cause severe obesity in humans (120-121). It was originally thought that rising leptin levels in humans would prevent obesity by decreasing appetite. However, it appears that leptin levels actually continue to rise as obese humans become more obese

(121-124). In fact, circulating leptin levels are highly positively correlated with measures of adiposity (122). This seeming inconsistency is thought to be explained by the development of leptin resistance. In a lean body, levels of leptin appear to signal to the hypothalamus to decrease appetite and increase energy burn. In fact, reduction of body weight by 10% results in a greater than 50% decrease in leptin level (56). However, as adiposity increases, the rise in leptin seems to have less of an impact on reducing food intake and avoiding obesity. This phenomenon of leptin resistance has been hypothesized to be caused by impairment of the leptin transport or the presence of negative regulators of leptin (6).

There is mounting evidence that leptin may play a role in carcinogenesis. Leptin receptors have been identified in malignant cells of diverse origin including lung, adrenal, colon, gastric, and white blood cells (125-129). Induction of the leptin receptor activates or upregulates several genes involved in cell proliferation, survival, migration, or angiogenesis including*: c-fos*, *c-jun, jun-B egr-1*, *socs-3,* and *VEGF* (8, 130-135). Leptin has been shown to be a serum growth factor (128, 136), suppress apoptosis through Bcl-2 (137-139), increase the levels and activity of metalloproteinases (angiogensis enzymes) (140-141), and regulate neoangiogensis both on its own and in combination with vascular endothelial growth factor and fibroblast-growth factor two (142-144).

Eleven studies using cell lines have examined the effects of leptin treatment on breast cancer cells (145). These studies report increased cell proliferation (133) and detection of leptin receptors (135, 146-148). Although leptin treatment increased cell proliferation of both normal breast epithelial cells and breast cancer cell lines, the response was more pronounced in the cancer cells (149). Likewise, both leptin and leptin receptors have

been detected in normal mammary tissue (150), but they appear to be overexpressed in tumor tissue (147). Garafolo, et al found this overexpression to be particularly large in high grade tumors (146-147). In addition, Miyoshi, et al found that women whose breast cancer tissue sample expressed both high levels of long and short leptin receptor isoforms were more likely to relapse (151). Higher levels of leptin have been found in cancer tissue compared to normal tissue (152-156) and in high grade and metastatic tumors (157). However, all but one study (158) that measured serum or plasma leptin levels found no association with breast cancer (153, 159-160). One study examined nipple aspirate fluid and did not find an association with breast cancer (156).

Recent evidence suggests that leptin may also impact cancer risk indirectly through modifications of the estrogen pathway. Evidence of an interaction between estrogen and leptin has been found in rats (161-162). In addition, human cell line studies provide evidence that leptin increases aromatase activity in several cell types (8, 130-131) including epithelial breast cancer cells (8). Catalano, et al has also shown that leptin can transcriptionally activate estrogen receptor-alpha in the absence of estradiol (10). Garafolo, et al provided evidence that leptin can interfere with the effects of breast cancer treatment antiestrogen ICI 182,780 which works to degrade estrogen receptor alpha (13).

Adiponectin is exclusively secreted by adipocytes (163) and has been shown to suppress cell growth, induce apoptosis, and inhibit angiogenesis (164-168). The gene that encodes adiponectin (*ADIPOQ*) is located at locus 3q27, spans 16kb, and contains three exons (169-171). Adiponectin is involved in both energy homeostasis and glucose and lipid metabolism. It enhances insulin sensitivity by increasing fatty acid oxidation, glucose uptake, and decreasing rate of gluconeogenesis (172).

Seemingly paradoxically, secretion of adiponectin decreases as adipocyte mass expands. Therefore, low serum levels are associated with obesity both in adults and in children (173-174). In a 2001 study of 22 obese patients who underwent gastric bypass surgery, average adiponectin levels increased by 46% following a 21% change in BMI (175). In this study, changes in serum adiponectin were significantly correlated with BMI, waist and hip circumferences, and glucose levels. This change in adiponectin level in response to changes in adipose tissue suggests a negative feedback loop (174). There is also evidence that adiponectin may also be involved in inflammation. Several studies have found that adiponectin plays a role in regulating the cytokines TNF-α (176) and IL-10 (177). The relationship between TNF and adiponectin is likely a feedback loop as TNF-α has been shown to reduce *ADIPOQ* gene expression (178-182). IL-6 also appears to regulate adiponectin expression, further supporting the hypothesized relationship between adiponectin and the immune system (183).

The mechanism between adiponectin and breast cancer is not well-understood but one hypothesis is that adiponectin up-regulates peroxisome proliferator-activated receptor (PPAR) signaling and, therefore, improves DNA repair capabilities (184). Despite the lack of a clear mechanism, evidence from cell line and serum studies is strongly suggestive of a relationship. Studies have consistently found a reduced risk of postmenopausal breast cancer for increasing levels of serum adiponectin (151, 166, 185-188). Cell line studies have shown that treatment of cells with adiponectin significantly decreases cell proliferation (164-168). Several studies found evidence of inhibition of cell cycle progression (164-165, 167). Some studies have found evidence of increased apoptosis (164-165, 167) although others have not (166, 168). Both adiponectin receptors

have been detected in breast tissue (165-166, 168, 189). Korner, et al examined receptor status in normal breast tissue, breast tumor tissue, and tissue adjacent to tumors. They found the highest expression in breast tumors and the lowest in normal breast tissue (166). Further, serum adiponectin levels have been consistently inversely associated with postmenopausal breast cancer risk (151, 166, 185-188).

Few previous publications have examined genetic variation in leptin and the leptin receptor in relation to breast cancer risk, and the results have been inconsistent (158-159, 190-192). Only one study has examined several single nucleotide polymorphisms (SNPs) in *ADIPOQ* and *ADIPOR1* and found statistically significant associations for two SNPs (193). Previous research on the relation of these two genes to breast cancer risk examined only some of the candidate SNPs. The purpose of this study was to provide a more comprehensive analysis of the association between postmenopausal breast cancer and variability in the genes encoding leptin *(LEP*), the leptin receptor *(LEPR),* adiponectin *(ADIPOQ),* adiponectin receptor 1 *(ADIPOR1),* and adiponectin receptor 2 *(ADIPOR2).*

3.3 Methods

Study Population

We conducted a nested case control study using women in the American Cancer Society Cancer Prevention Study II (CPS-II) who provided a blood sample (n=21, 965 women) after giving informed consent (194). Cases included predominantly white, postmenopausal women diagnosed with breast cancer between 1992 and 2001. Cases were verified through medical records or linkage to state cancer registries. Controls were selected from cohort members who remained cancer-free through 2001 and were matched

to cases on age (±6 months), race (white, black, other), and blood draw date (±6 months). Questionnaire information was collected before the cases were diagnosed.

SNP selection and genotyping

The SNPs of interest for this study were selected using HapMap[1] (Release 21, July 2006). All SNPs in HapMap that had a minor allele frequency (MAF) of at least five percent and were within ten kilobases (kb) of *LEP, LEPR, ADIPOQ, ADIPOR1,* or *ADIPOR2* were identified (n=382 SNPs). Because genotyping this extensive list of SNPs was cost-prohibitive, we used the Tagger program within Haploview (v.3.32) to create linkage disequilibrium (LD) bins and chose tagging SNPs, which reduced the number of SNPs analyzed while maximizing capture of the genetic variability in the genes (195). SNPs in a large intronic region in *LEPR (*n=134 SNPs), as well as singleton, intronic SNPs >1kb from an exon of any of these genes (n=21 SNPs), were excluded. Genotyping was performed on the remaining 53 SNPs using the Beckman SNPstream genotyping system. Forty-eight of the 53 SNPs were successfully genotyped after two attempts. Positive and negative DNA controls and blind duplicates were randomly interspersed among the samples. Concordance among duplicate samples was >99%. Genotyping call rates ranged from 91.3% to 99.3%. One SNP (rs6660481) deviated from Hardy-Weinberg equilibrium at the p=0.01 level and was excluded, leaving 47 SNPs in the final analysis.

Statistical Analysis

We used conditional logistic regression to estimate odds ratios (ORs) for postmenopausal breast cancer. Models included body mass index (BMI), weight change, location of weight gain, and physical activity as potential confounders. Effect modification of the

[1] International HapMap Project: http://www.hapmap.org/

relationship between each SNP and postmenopausal breast cancer by these variables was evaluated.

3.4 Results

This study included 648 cases and 659 controls. Cases and controls were similar in terms of age at blood draw (mean, 69 years) and race (99% White); additional characteristics of the cases and controls have been reported elsewhere (194). We found no statistically significant ($p<0.05$) associations between breast cancer risk and any of the SNPs in *LEP, LEPR, ADIPOQ, ADIPOR1,* or *ADIPOR2* using a dominant, genotypic, or additive genetic model (dominant models are shown in Table 3.2). Individual odds ratios (ORs) ranged from 0.92 to 1.05. We also found no evidence of effect modification by BMI (Figure 3.1), physical activity (Figure 3.2), adult weight gain (Figure 3.3), or location of weight gain (Figure 3.4).

3.5 Discussion

The results from this study do not support an association between postmenopausal breast cancer and individual SNPs in *LEP, LEPR, ADIPOQ, ADIPOR1, or ADIPOR2* in a population of predominately white U.S. women. Our results are consistent with a recent genome-wide association study[2] of breast cancer that did not identify any SNPs in these gene regions as possible risk loci (46). The present study makes an important contribution to our understanding of these genes in relation to breast cancer because it is the first to comprehensively evaluate most of the known variation in these genes.

[2] CGEMS: Cancer Genetic Markers of Susceptibility. http://cgems.cancer.gov/data.

Eight small breast cancer studies previously conducted in different countries (Korea, China, Taiwan, Tunisia, U.S.) evaluated seven candidate *LEP* or *LEPR* SNPs with inconsistent results (Table 2.1) (158-159, 190-192). Only one SNP was examined by more than one study (rs1137101). Two studies found a roughly two fold statistically significant risk of breast cancer for rs1137101 (158, 192). However, two others found no association between this SNP and breast cancer (159, 190). Woo, et al also found no association between breast cancer and three other *LEPR* SNPs (rs8179183, rs805096, and rs1137100) (159). Liu, et al reported a suggestion of an association between rs1137100 and breast tumor size but among premenopausal women only (191).

Only one previous study has looked at adiponectin SNPs and breast cancer. The authors found statistically significant associations between breast cancer and two *ADIPOQ* SNPs (rs2241766 and rs1501299) and two *ADIPOR1* SNPs (rs2232853 and rs7539542) among U.S. women (193). The authors found no association with six other *ADIPOQ* or *ADIPOR1* SNPs. Six of the ten SNPs studied (rs226679, rs822396, rs1501299, rs2232853, rs1342387, rs7539542) were either in our study or in strong LD with SNPs in our study ($r2 \geq 0.9$).

The statistically significant ORs in the studies mentioned above ranged from 1.58 to 2.04. We had 80% power to detect an OR as low as 1.55 for SNPs with a MAF of 6.5% and an OR as low as 1.46 for SNPs with a MAF=49.5%. Thus, we had sufficient power to detect the ORs reported in these studies. Given the very different relationship between obesity and pre- and postmenopausal breast cancer, it may be relevant to note that some of these studies did not conduct separate analyses by menopausal status (158-159, 193) and those that did (190-192) were quite small (≤118 postmenopausal women).

Our study was an adequately powered, population-based, comprehensive assessment of variation across these genes and postmenopausal breast cancer. Although we can not rule out that these genes are involved in gene-gene or gene-environment interactions, our results suggest that individual SNPs in these genes do not substantially impact postmenopausal breast cancer risk.

3.6. Figures and Tables

Table 3.1. Previous studies of LEP, LEPR single nucleotide polymorphisms (SNPs) and breast cancer

		Single Nucleotide Polymorphisms						
		rs1137101 G vs. A	**rs1137100 G vs. A**	**rs8179183 G vs. C**	**rs1805096 G vs. A**	**rs1045895 G vs. A**	**rs7602 G vs. A**	**rs7799039 G vs. A**
Previous Studies	**Woo, 2006 n=90 (45) Korea**	0.59 (0.19-1.81)	1.08 (0.40-2.93)	0.63 (0.14-2.81)	0.65 (0.21-2.01)			
	Snoussi, 2006 N=530 (308) Tunisia	1.87 (1.36-2.56)						
	Gallicchio, 2007 n=994 (61) U.S.	1.30 (0.70-2.44)				0.56 (0.33-0.95)	1.19 (0.69-2.07)	
	Han, 2007 n=222 (94) China	4.87 (1.30-18.22)						
	Liu, 2007 n=88(47) Taiwan		p=0.972					
	Han, 2008 n=740 (240) China	2.04 1.09-3.82						
	Okobia, 2008 n=183(95) Nigeria	0.9 0.4-1.8						
	Cleveland, 2009 n=2173 (1,065) U.S.	1.04 0.81-1.34						1.30 1.01-1.66

Table 3.2. Association between single nucleotide polymorphisms representing linkage disequilibrium bins in *LEP, LEPR, ADIPOQ, ADIPOR1,* and *ADIPOR2* and risk of postmenopausal breast cancer

Gene	Marker	Location*	Genotypes	Genotype Frequencies		OR† (95% CI)	Other SNPs in LD bin‡
				Cases	Controls		
LEP	rs4731423	Regulatory	AA	187	202	1.00 (ref)	rs10954175 rs12706832 rs2278815 rs4731426 rs11761556 rs2060715 rs4731429 rs1349419
			GA OR GG	445	442	1.01 (0.93-1.10)	
LEP	rs13245201	Intron	AA	198	211	1.00 (ref)	rs3828942 rs7799039 rs10487506
			GA OR GG	438	433	0.99 (0.91-1.08)	
LEP	rs10244329	Intron	AA	159	165	1.00 (ref)	rs11763517
			AT OR TT	460	461	1.00 (0.93-1.06)	
LEP	rs7795794	Intron	GG	551	571	1.00 (ref)	rs791606 rs10276311 rs7788818 rs4731430 rs4731424 rs4731427 rs10264361 rs791604 rs791607 rs2060713 rs4236625 rs2122627 rs791608 rs2167271
			GA OR AA	84	78	1.04 (0.95-1.14)	
LEP	rs10954173	Intron	GG	229	232	1.00 (ref)	rs10249476 rs12537573 rs1376268 rs11760956 rs12535747
			GA OR AA	388	402	1.02 (0.96-1.09)	
LEP	rs2071045	Intron	TT	361	388	1.00 (ref)	none
			TC OR CC	278	262	1.03 (0.93-1.13)	
LEPR	rs3806318	Intron	AA	315	353	1.00 (ref)	none

Gene	Marker	Location*	Genotypes	Genotype Frequencies		OR† (95% CI)	Other SNPs in LD bin‡
				Cases	Controls		
			AG OR GG	299	270	1.01 (0.95-1.07)	
LEPR	rs1327118	Intron	GG	181	209	1.00 (ref)	rs12145690
			GC OR CC	456	441	1.06 (0.96-1.17)	rs10493377
LEPR	rs9436739	Intron	TT	449	465	1.00 (ref)	rs9436297
			TA OR AA	167	167	1.04 (0.98-1.11)	rs9436737 rs9436738
LEPR	rs9436740	Intron	AA	323	334	1.00 (ref)	none
			AT OR TT	306	308	1.02 (0.94-1.11)	
LEPR	rs9436301	Intron	TT	358	341	1.00 (ref)	rs9436303
			TC OR CC	282	305	0.94 (0.85-1.03)	
LEPR	rs1887285	Intron	TT	532	532	1.00 (ref)	rs4468199
			TC OR CC	108	116	0.95 (0.86-1.05)	
LEPR	rs3790431	Intron	TT	400	412	1.00 (ref)	rs3790432
			TC OR CC	237	240	1.04 (0.94-1.15)	rs6588152 rs2376016
LEPR	rs1137100	Exon					rs7519977
			AA	346	363	1.00 (ref)	rs4655518 rs11208674
			AG OR GG	242	243	1.01 (0.97-1.06)	rs6657632 rs12033452 rs10789184
LEPR	rs4655517	Intron	TT	158	168	1.00 (ref)	rs10158279
			TC OR CC	478	478	0.99 (0.91-1.09)	rs6673324
LEPR	rs1137101	Intron					rs10736402 rs10889567 rs11208682
			GG	181	211	1.00 (ref)	rs10732836 rs6669117
			GA OR AA	460	439	1.01 (0.91-1.13)	rs12564626 rs4655539 rs7364510 rs2154380 rs10449758
LEPR	rs4655537	Intron	GG	248	271	1.00 (ref)	none
			GA OR AA	384	375	1.04 (0.95-1.14)	
LEPR	rs3762274	Intron	AA	230	257	1.00 (ref)	rs3828033
			AG OR GG	397	382	1.02 (0.95-1.10)	

Gene	Marker	Location*	Genotypes	Genotype Frequencies Cases	Controls	OR† (95% CI)	Other SNPs in LD bin‡
LEPR	rs11585329	Intron	GG	441	468	1.00 (ref)	none
			GT OR TT	197	181	1.03 (0.94-1.14)	
LEPR	rs8179183	Exon	GG	433	420	1.00 (ref)	rs6661050 rs17415296 rs3828034 rs3790438 rs2376018 rs4606347
			GC or CC	188	216	1.02 (0.95-1.09)	rs3790437 rs12077336 rs17127838 rs6665672 rs17406429 rs7545475 rs11801408
LEPR	rs4655556	Intron	GG	422	438	1.00 (ref)	rs1892535 rs12025906 rs6690625
			GA or AA	209	205	1.02 (0.94-1.10)	rs1938484 rs12040007 rs7518632 rs4655555
LEPR	rs10889569	Intron	AA	243	246	1.00 (ref)	rs6700896 rs6588153 rs7531867
			AT or TT	374	382	1.01 (0.94-1.09)	rs6678033 rs1805096 rs7516341 rs1892534
ADIPOQ	rs1063539	3' UTR	GG	471	471	1.00 (ref)	rs2241767 rs1063537
			GC or CC	171	184	1.03 (0.91-1.16)	rs2082940 rs3774262
ADIPOQ	rs864265	Intron	GG	458	455	1.00 (ref)	rs6444168
			GT or TT	179	189	0.96 (0.88-1.05)	
ADIPOQ	rs17300539	Regulatory	GG	534	533	1.00 (ref)	none
			GA or AA	101	110	0.98 (0.90-1.07)	
ADIPOQ	rs266729	Regulatory	CC	357	359	1.00 (ref)	none
			CG or GG	263	279	1.05 (0.98-1.13)	
ADIPOQ	rs182052	Intron	GG	300	293	1.00 (ref)	rs1648707
			GA or AA	338	353	0.97 (0.89-1.07)	

Gene	Marker	Location*	Genotypes	Genotype Frequencies		OR† (95% CI)	Other SNPs in LD bin‡
				Cases	Controls		
ADIPOQ	rs16861210	Intron	GG	516	519	1.00 (ref)	rs822387
			GA or AA	117	127	1.02 (0.94-1.11)	
ADIPOQ	rs822394	Intron	CC	444	426	1.00 (ref)	rs822396
			CA or AA	189	219	0.99 (0.91-1.07)	
ADIPOQ	rs17366568	Intron	GG	485	489	1.00 (ref)	none
			GA or AA	147	152	0.99 (0.92-1.07)	
ADIPOQ	rs3821799	Intron	CC	179	174	1.00 (ref)	none
			CT or TT	461	476	0.98 (0.88-1.09)	
ADIPOQ	rs3774261	Intron	GG	227	214	1.00 (ref)	rs6773957
			GA or AA	409	430	0.96 (0.88-1.05)	
ADIPOQ	rs17366743	Exon	TT	617	621	1.00 (ref)	none
			TC or CC	27	33	0.97 (0.84-1.12)	
ADIPOQ	rs7639352	Intron	CC	349	341	1.00 (ref)	rs6444175
			CT or TT	286	305	0.99 (0.90-1.08)	
ADIPOR1	rs4336908	Regulatory	GG	397	392	1.00 (ref)	rs2185781
			GA or AA	237	253	1.00 (0.92-1.09)	
ADIPOR1	rs7539542	3’ UTR	CC	281	296	1.00 (ref)	none
			CG or GG	356	356	1.05 (0.94-1.17)	
ADIPOR1	rs1342387	Intron	GG	172	184	1.00 (ref)	rs2275737 rs7514221
			GA or AA	458	457	1.01 (0.93-1.09)	
ADIPOR1	rs16850799	Intron	GG	371	390	1.00 (ref)	rs12045862
			GA or AA	256	245	1.00 (0.93-1.07)	
ADIPOR1	rs1418445	Intron	GG	239	255	1.00 (ref)	rs6666089 rs2232854 rs10920533 rs10494839 rs2232847 rs2232853 rs10800886 rs2232852 rs1539355
			GC or CC	389	387	1.03 (0.96-1.11)	

Gene	Marker	Location*	Genotypes	Genotype Frequencies Cases	Controls	OR† (95% CI)	Other SNPs in LD bin‡
ADIPOR2	rs7132033	Intron					rs1029629 rs2058035 rs7297509 rs2370055 rs4766413 rs12810020 rs12230579 rs11061925 rs11061962 rs11832817 rs11061927
			CC	325	323	1.00 (ref)	
			CG or GG	308	317	0.98 (0.91-1.06)	
ADIPOR2	rs11061952	Intron	GG	545	545	1.00 (ref)	rs11061946
			GA or AA	85	94	0.99 (0.92-1.06)	
ADIPOR2	rs10773986	Intron					rs929434 rs12342 rs7313760 rs10773982 rs2286385 rs7978818 rs12831353 rs12813694 rs11061974 rs12828908 rs11061937
			AA	301	309	1.00 (ref)	
			AG or GG	333	335	1.00 (0.93-1.09)	
ADIPOR2	rs2058112	Intron					rs7316374 rs16928751 rs1468491 rs7132184 rs7975375 rs2286380 rs767870 rs7967137 rs10848554 rs10848568
			CC	491	484	1.00 (ref)	
			CT or TT	150	166	0.95 (0.86-1.06)	
ADIPOR2	rs11061973	Intron					rs2058033 rs11061967 rs12230440 rs12821401 rs11061935
			GG	471	466	1.00 (ref)	
			GA or AA	173	185	0.93 (0.82-1.05)	

Gene	Marker	Location*	Genotypes	Genotype Frequencies Cases	Controls	OR† (95% CI)	Other SNPs in LD bin‡
ADIPOR2	rs2108642	Intron					rs10773988 rs7294668 rs6489326 rs12316367 rs9739162 rs4766415
			GG	167	184	1.00 (ref)	rs10773991
			GT or GG	453	445	1.01 (0.94-1.08)	rs9300298 rs10735003 rs11614639 rs10848557 rs9805049 rs2286384 rs2286383 rs2068485 rs10773989
ADIPOR2	rs1044471	3' UTR	CC	163	166	1.00 (ref)	none
			CT or TT	478	482	0.96 (0.86-1.07)	
ADIPOR2	rs13219	Regulatory	AA	208	203	1.00 (ref)	rs2286379 rs1044825
			AG or GG	432	446	0.97 (0.87-1.08)	rs7294540 rs10744552

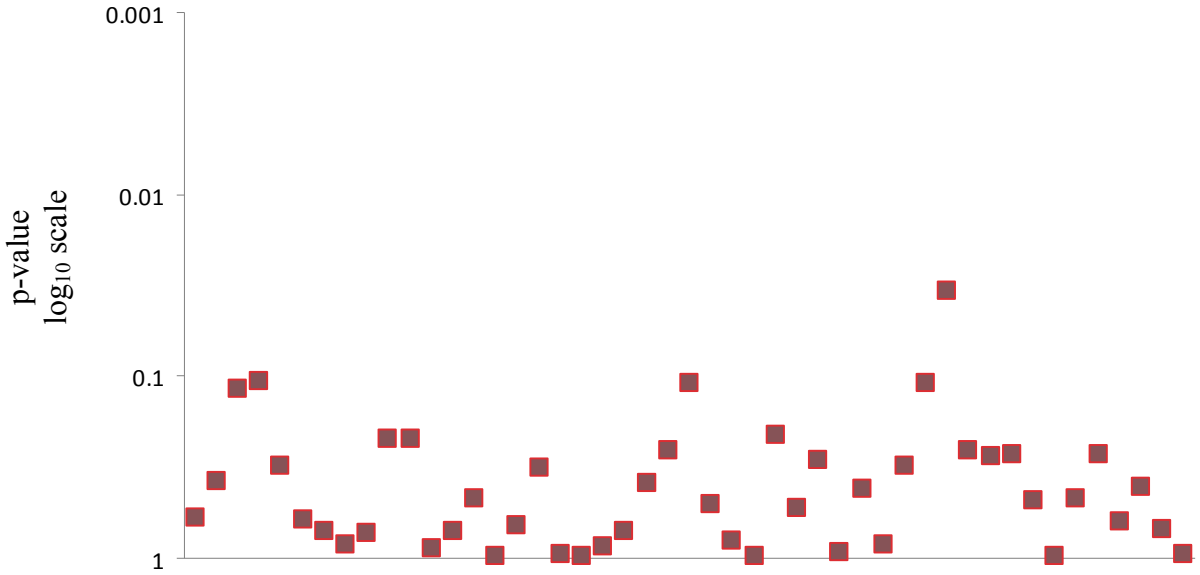
Abbreviations: SNP, single nucleotide polymorphism; UTR, untranslated region; LD, linkage disequilibrium; OR, odds ratio; CI, confidence interval;

*Location of polymorphism within the gene. Regulatory region spans 10 kilobases up and downstream from the first and last exon.

†Odds ratio adjusted for age, race, and date of blood draw.

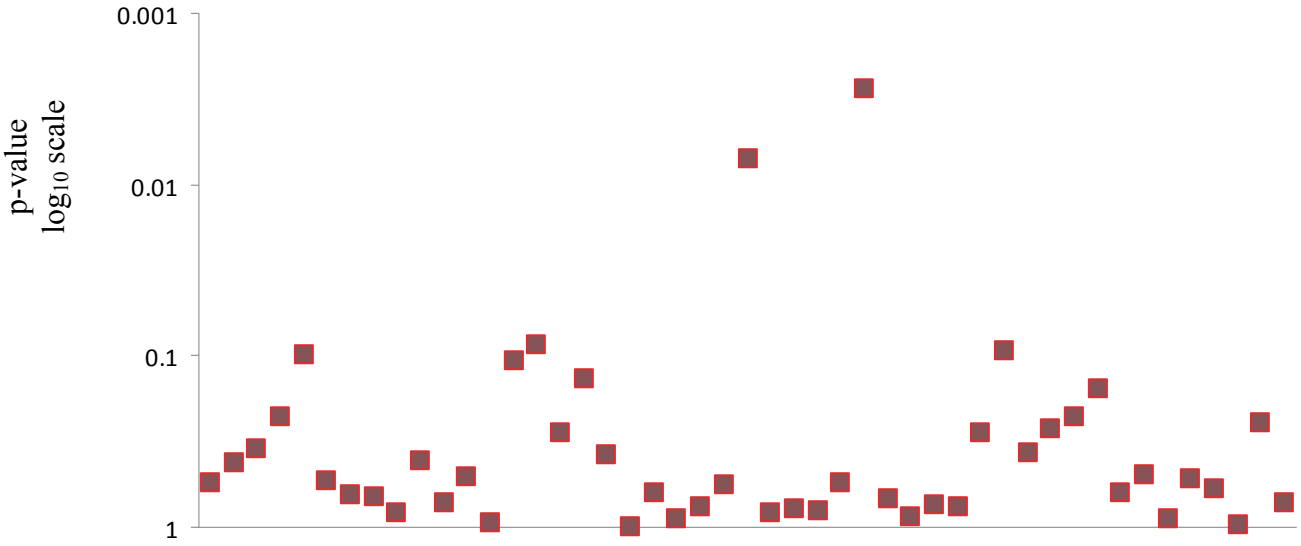
‡Coefficient of determination (r^2) between the marker SNP and the other SNPs in the LD bin was at least 0.8 (mean r^2=0.953).

Figure 3.1. Dominant model p-values for the statistical interaction between single nucleotide polymorphisms in *LEP*, *LEPR*, *ADIPOQ*, *ADIPOR1*, and *ADIPOR2* and body mass index (BMI)



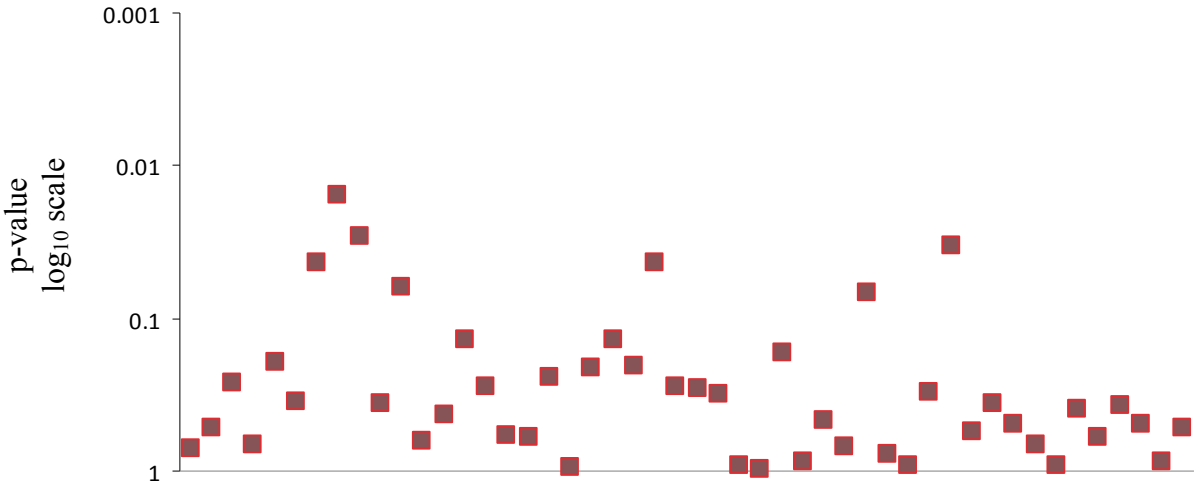
47 Single Nucleotide Polymorphisms in *LEP* (6), *LEPR* (18), *ADIPOQ* (11), *ADIPOR1* (5), and *ADIPOR2* (8)

Figure 3.2 Dominant model p-values for the statistical interaction between single nucleotide polymorphisms in *LEP*, *LEPR*, *ADIPOQ*, *ADIPOR1*, and *ADIPOR2* and physical activity



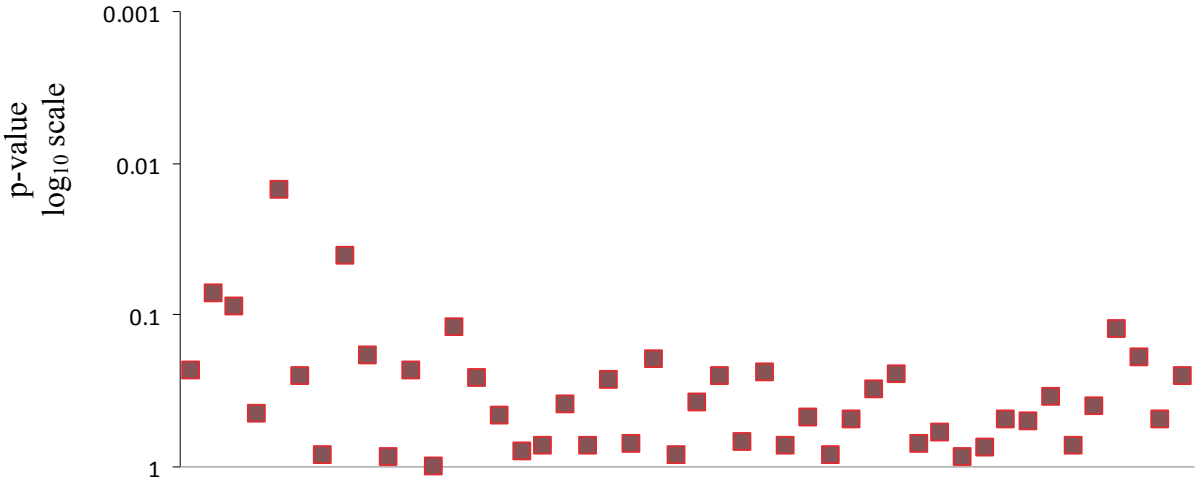
47 Single Nucleotide Polymorphisms in *LEP* (6), *LEPR* (18), *ADIPOQ* (11), *ADIPOR1* (5), and *ADIPOR2* (8)

Figure 3.3. Dominant model p-values for the statistical interaction between single nucleotide polymorphisms in *LEP*, *LEPR*, *ADIPOQ*, *ADIPOR1*, and *ADIPOR2* and weight change



47 Single Nucleotide Polymorphisms in *LEP* (6), *LEPR* (18), *ADIPOQ* (11), *ADIPOR1* (5), and *ADIPOR2* (8)

Figure 3.4. Dominant model p-values for the statistical interaction between single nucleotide polymorphisms in *LEP*, *LEPR*, *ADIPOQ*, *ADIPOR1*, and *ADIPOR2* and location of weight gain



47 Single Nucleotide Polymorphisms in *LEP* (6), *LEPR* (18), *ADIPOQ* (11), *ADIPOR1* (5), and *ADIPOR2* (8)

CHAPTER 4: A SEARCH FOR JOINT EFFECTS OF GENETIC AND ENVIRONMENTAL FACTORS ON BREAST CANCER RISK USING TWO NOVEL PUBLISHED APPROACHES

4.1 Abstract

High circulating estrogen levels have been consistently linked to increased breast cancer risk; however, studies of genes related to estrogen biosynthesis have identified very few associations. Given the complexity of steroid hormone metabolism, joint effects of candidate estrogen pathway loci on breast cancer risk seem probable. Traditional analytic methods are limited in their ability to handle sparse data and, therefore, evaluate higher order interactions. Multifactor Dimensionality Reduction and logic regression are two alternative methods that have been proposed specifically to investigate joint effects. We used these methods to examine gene-gene and gene-environment interactions in 56 single nucleotide polymorphisms in six estrogen biosynthesis genes and three non-genetic exposures known to impact estrogen levels. Data for these analyses were obtained from a case-control study of 465 postmenopausal breast cancer cases and 464 controls nested within the American Cancer Society's Cancer Prevention Study-II Nutrition Cohort. Neither MDR nor logic regression identified any statistically significant multifactor effects among various combinations of genetic and non-genetic factors of interest. It is possible that SNPs of genes regulating estrogen biosynthesis and metabolism truly do not affect breast cancer risk either alone or in combination with other factors. Future studies incorporating multiple levels of biological and environmental information may be needed to confirm these findings.

4.2 Introduction

It has been hypothesized that a lack of clear and consistent associations between low-penetrance genetic mutations and breast cancer observed in epidemiologic studies may be explained by complex interactions among genetic factors as well as possible higher order (three or more variable) gene-environment interactions that affect disease risk (196). Traditional analytic methods are limited in their ability to evaluate higher order interactions because these methods were not designed to handle sparse data – a common problem even in large studies aiming to examine joint effects of multiple factors. To address this issue, various alternative methods have been recently developed. Multifactor Dimensionality Reduction (MDR) (197) and Logic Regression (198) are two such methods that have been proposed specifically to investigate joint effects involving a large number of risk factors, such as single nucleotide polymorphisms (SNPs) of genes regulating a given biologic pathway.

MDR operates by classifying combinations of variables as either "high" or "low" risk for the outcome of interest based on the ratio of cases to controls for each combination. The ability of high/low risk status to predict true case or control status is used to evaluate the importance of the variable combination. Logic regression uses simulated annealing to evaluate binary variable combinations. This method compares a score function before and after each change in the model. The final model is the one that minimizes the score function after a specified number of iterations. Both methods use cross-validation consistency and permutation p-values to test the statistical significance of findings and to account for multiple comparisons. Although the two techniques are

different, both MDR (199-201) and logic regression (201-203) have been reported to identify joint effects. One study of bladder cancer and DNA repair polymorphisms used both methods (201) and found similar results. In addition, simulation studies have demonstrated that both methods can detect high-order joint effects even in the absence of main effects (204-205).

Given the complexity of steroid hormone metabolism, joint effects of candidate estrogen pathway loci on breast cancer risk seem probable. Over the past decade, the search for candidate susceptibility genes associated with breast cancer has logically focused on the steroid hormone metabolism pathway. High circulating estrogen levels have been consistently linked to increased breast cancer risk (2); however, studies of genes related to estrogen biosynthesis and metabolism have identified very few associations (68-72). Two studies attempted to examine the relation between breast cancer and various combinations of SNPs in the estrogen metabolic pathway (206-207). The results of the first study were null (207); however, the analyses only included 18 polymorphisms in 11 different genes, and thus potentially important genetic factors may have been missed. The second was a small study (n=200 cases) that identified an interaction between two SNPs: one in *HSD17B1* and one in *CYP17* (206).

In an effort to more fully investigate the joint effects of various genetic and non-genetic estrogen-related factors we considered 56 SNPs in six adipose tissue estrogen biosynthesis genes and three non-genetic exposures known to impact estrogen levels. In the present analyses we used data from a case-control study of postmenopausal breast cancer nested within the American Cancer Society's Cancer Prevention Study-II

Nutrition Cohort. The data from this study were analyzed using both MDR and logic regression and the results of the two analyses were assessed for consistency.

4.3 Methods

Study Population

Women from the American Cancer Society Cancer Prevention Study II Nutrition Cohort (CPS-II) who provided a blood sample after giving informed consent (n=21,965 women) were eligible for inclusion in the present analysis. These women originally enrolled in the CPS-II cohort in 1992 and filled out a ten-page self-administered questionnaire at the time of enrollment (100). Follow-up surveys to ascertain cancer incidence and update exposure data were sent starting in 1997 and every two years afterwards. DNA was extracted from buffy coat specimens that had been stored in liquid nitrogen. Cases for this analysis included 465 predominantly white, postmenopausal women diagnosed with breast cancer between 1992 and 2001 and verified through medical records or linkage to state cancer registries. For all cases, questionnaire information on risk factors for breast cancer was collected before the diagnosis (that is, in 1992). Collection of DNA samples occurred from 1998 through 2001, in some cases after or only slightly before cancer diagnosis; however, this should not have affected the results since the presented analyses are focused on germline mutations. 464 controls were selected from cohort members who remained cancer-free through 2001 and were matched to cases on age (±6 months), race (white, black, other), and blood draw date (±6 months).

SNP Selection

SNPs selected for this study are found in six genes: *HSD17B1, HSD17B2, CYP19, TNF, IL6 and LEP*). Aromatase (coded by *CYP19)* is the enzyme that catalyzes the conversion

of androstendione and testosterone, into estrone and estradiol (the more bioactive form of estrogen). These two forms of estrogen also convert back and forth and this reaction is catalyzed by the enzymes HSD17B1 and HSD17B2. The adipokines leptin, interluekin 6, and tumor necrosis factor (coded by *LEP, IL6,* and *TNF* respectively*)* all upregulate aromatase and, therefore, upregulate estrogen. For all genes except *TNF*, the SNPs were selected as linkage disequilibrium (LD) bin tagging polymorphisms (195) that allowed us to capture most of the variability in these genes. The SNPs for *TNF* (and three additional *IL6* SNPs) were genotyped because they had been previously identified as potentially functional.

Genotyping

The SNPs for this study were genotyped previously using two different platforms at different laboratories. For all genotyping, DNA controls and blind duplicates were randomly interspersed among the samples. SNPs from *HSD17B1, HSD17B2, CYP19, TNF,* and *IL6* were genotyped using TaqMan (Applied Biosystems, Foster City, CA) in XXyear. SNPs from *LEP* were genotyped using the Beckman SNPstream genotyping system. Genotyping call rates ranged from 92.2% to 99.8%. All SNPs were in Hardy-Weinberg equilibrium among controls.

Non-Genetic factors

Information about height, weight, physical activity, and postmenopausal hormone (PMH) use was collected for the 1992 baseline survey. Women were asked to report their current weight in 1992, and recall their weight at age 18. The weight change variable was divided into five categories: any weight loss, gain of up to 20 pounds, gain of 21 to 60 pounds, gain of more than 60 pounds, and unknown amount of weight change.

Physical activity was measured in metabolic equivalents per week (MET-hours per week) from questions about the average time per week participants spent doing various activities (e.g. walking, running, swimming, aerobics) (208). Categories of physical activity were less than seven METS-hrs/week, seven to 24.5 MET-hrs/week, more than 24.5 MET-hrs/week, or unknown. For reference, 3.5 MET-hrs/week is equivalent to approximately one hour of moderately paced walking. PMH use was categorized as never, current, former, ever (status unknown), or missing.

Statistical Analyses

MDR

As described previously, MDR classifies each combination of variables as either "high" or "low" risk for the outcome of interest based on the ratio of cases to controls. The threshold for defining risk status in this study was defined as the ratio of cases to controls within the study population (1:1.066). Thus, if the ratio of cases to controls with a given genotype, for example, was greater than 0.9939 the genotype was labeled "high risk". The genotypes were then pooled into two groups and the data were reduced to one dimension. This new risk factor (high vs. low risk) was then evaluated by calculating a testing accuracy ([True positives + True negatives]/[True positives + True negatives + False positives+ False negatives]) and a cross-validation consistency. Finally, an empirical p-value was calculated using permutation testing to evaluate how likely the prediction error and cross-validation consistency were for each selected n-variable combination model.

We began the analyses by dividing the data into ten parts. Nine tenths of the data were used to find "high" and "low" risk combinations (called the training dataset). These

high and low risk classifications were then tested in the other one tenth of the data (called the testing dataset). The ability of high/low risk status to distinguish cases from controls was used to evaluate the importance of a given variable combination (using the testing accuracy described above). The cross-validation consistency was calculated by repeating this process ten times with a different tenth of the data as the testing dataset each time and then repeating the entire process ten times (for a total of 100). The variable combination with the lowest average prediction error for each 1-, 2-, 3-, and 4-way combination was selected as the best model for that n-way combination.

Logic Regression

In the logic regression analyses, simulated annealing was used to process and evaluate all potential variable combinations. This method is a stochastic search algorithm that uses a score function (in this case, the deviance) to compare potential models. Using simulated annealing, each time a change to the model was made (e.g., by adding a variable or by exchanging one variable for another), the model was re-evaluated by comparing the scores before and after the change. If the new score was found to be better (i.e., had a lower deviance) then the new model was accepted with a probability that decreased as the process progressed. The final model was compared to a null model by calculating a p-value and by performing cross-validation to evaluate the appropriate model size.

Missing Genotype Data

Although only 2% of genotypes were missing in this dataset overall, 48% of the women were missing at least one genotype. Thus, excluding women with at least one missing genotype would have reduced our dataset by almost a half. To avoid this reduction in sample size we performed imputation of missing genotypes using PHASE (v.2.2)

software (209-210). PHASE uses a Bayesian statistical method for reconstructing haplotypes from population genetic data to impute missing genotypes. In contrast to logic regression, which does not allow for any missing values, MDR allows that missing values be placed in a separate category or be deleted. This feature allowed us to compare the results of the MDR analyses using three alternative approaches – imputation of missing data, inclusion of missing data in a separate category, and deletion of missing data. We found that when the MDR analyses placed missing genotypes in a separate category, the results were similar to those obtained after PHASE imputation. However, the results were different when the missing values were deleted (as half the participants were no longer in the analysis). Thus, all results presented here are based on the PHASE imputation of missing genetic data for both MDR and logic regression. Women with missing physical activity, postmenopausal hormone use, or weight change information were excluded (n=46). The final dataset included 929 women (465 cases and 464 controls).

4.4 Results

Table 4.1 shows selected characteristics of the study population. Cases were on average aged 68 years at diagnosis. Case subjects were more likely to have a family history of breast cancer, have gained 60 or more pounds since age 18, and currently use postmenopausal hormone therapy compared to control subjects. Case subjects were also less physically active than control subjects at baseline. In the univariate analyses eight individual SNPs in *CYP19* were significantly associated with postmenopausal breast

cancer assuming a two-sided alpha error of 0.05 and with no adjustments for multiple testing (Table 4.2). The remaining 48 SNPs showed no association with breast cancer.

We limited analyses for MDR and logic regression to no more than four-variable models to decrease the likelihood of noise predictions. MDR analyses examining genetic factors alone (not including non-genetic variables) identified rs727479 as the strongest single variable predictor of breast cancer (Table 4.3). This SNP, which is found in the *CYP19* gene, was also one of the strongest predictors of breast cancer when the data were analyzed using logistic regression (p=0.0012) (Table 4.2). Results of the MDR analysis for combinations of both genetic and non-genetic variables are shown in Table 4.4. After adding non-genetic variables, PMH use was identified as the strongest single predictor of breast cancer in the MDR model but the testing accuracy of this factor (ability to distinguish cases from controls) was very modest (54%) and not statistically significantly different (p=0.12) from testing accuracy of 50%, which represents absence of any ability to discriminate between cases and control. When examining joint effects, the strongest model was a four variable model that included a combination of PMH and three SNPs located in *HSD17B1, HSD17B2* and *IL6*. Although this combination was the best predictor of breast cancer, the testing accuracy of this four variable combination was still rather low (56%) and not statistically significantly different from 50% (p=0.09).

The results of the logic regression analysis can be found in Table 4.5. Consistent with MDR and logistic regression, logic regression identified both PMH and rs727479 in *CYP19* as potentially important variables. The best model identified by this method was the single SNP, rs727479. However, the global null model permutation test indicated that

no variables statistically significantly impacted risk of breast cancer at the alpha level of 0.05.

4.5 Discussion

Neither MDR nor logic regression identified any statistically significant multifactor combinations among estrogen-related genetic and non-genetic factors that may impact breast cancer risk. Both methods identified PMH use and rs727479 as important variables but all other variables selected by the two methods were different. Our study is not the first attempt to assess joint effects of estrogen-related factors on breast cancer risk. In another recent study of breast cancer, Justenhoven et al evaluated 18 estrogen metabolic pathway SNPs and seven environmental variables using both MDR and logic regression, and also did not identify any important higher order interactions (207). The data used in that study included two of the same SNPs (rs605059 and rs700519) and two of the same non-genetic factors (PMH use and obesity) as were used in our analyses, but the rest of the variables of interest were different. In addition, unlike our study, Justenhoven, et al included both pre and postmenopausal breast cancer cases, and thus the two sets of results are not directly comparable.

The lack of important new findings in our analyses and in the analyses reported by Justenhoven, et al may have several explanations. It is important to point out that genetic pathways are complex and it is likely that neither study was able to include all relevant genes and polymorphisms. Moreover, while estrogen clearly plays an important role in breast carcinogenesis, the germline variants of genes involved in synthesizing or metabolizing estrogen may not have an impact on breast cancer risk. The Breast and

Prostate Cancer Cohort Consortium found that a *CYP19* haplotype involving rs727479 increased estrogen levels by 10-20% but did not affect risk of breast cancer (4). Other biological markers or epigenetic factors (i.e., changes in gene expression caused by something other than DNA sequence) may serve as better predictors of breast cancer risk. The transcription of a gene is not the only factor that influences the resulting level of a protein. Rapid degradation of mRNA (or the protein itself), inefficient translation, and post-translational modifications can all impact protein levels. In addition, the same DNA sequence can lead to multiple protein products through alternative splicing, and many proteins only function after forming polymers (211). As metabolites are the end products of cellular processes, they can also be viewed as the response of biological systems to genetic and/or environmental changes (212). Thus, metabolites may provide an even better way to measure the contributions of inherited and environmental factors that may act and interact as part of the same pathway. Finally, measurement of DNA methylation patterns or other quantitative epigenetic changes may help clarify inherited risk of breast cancer (213).

It is also possible that the methods used in our analyses, although innovative, have limited ability to capture important joint effects and interactions. For example, logic regression requires all variables to be dichotomized. This method is, therefore, better suited for binary factors than for continuous or multilevel variables. SNPs can be dichotomized because two meaningful dichotomous variables can describe dominant and recessive models (homozygous wild type compared to at least one mutant allele and two copies of the mutant allele compared to all other genotypes). Although MDR allows variables to have more than two levels, it does not have a way to identify a referent group

to specify the appropriate comparisons. When we ran a logistic regression model with a single four-level variable (never, current, former, and ever status unknown) for PMH, using never users as the reference category, there was no statistically significant association – a result that is in agreement with our MDR and logic regression analyses. However, a comparison of current PMH users to never users with logistic regression showed an effect (OR=1.60, 95% CI: 1.19-2.15, p=0.0017). Another limitation of MDR is that it does not allow control for confounding. Again, this may be less relevant for studies of germline polymorphisms (particularly in racially homogenous groups), but the need to control for confounding becomes more important when non-genetic variables are included in the models. A limitation that is specific to logic regression is that the results can be sensitive to different model settings. Although the authors of the method suggest a starting point and give some general guidelines, users are required to specify settings for the simulated annealing chain. When we used the default number of iterations, we got different results every time we ran the program. After expanding the number of total iterations, we were able to get relatively stable estimates for up to three variable models, but not for models with larger numbers of variables.

The failure to identify any important joint effects in this study may also have been due to sample size limitations. Although both methods used in our analyses are designed to handle sparse data, we got very different results when a subset of the data was analyzed. A larger dataset would likely have produced more stable results for more than three or four variable models. Given the complexity of the estrogen pathway, the true biological interactions likely involve more than four variables, which may require much larger studies.

This study also has strengths. First (with the exception of *TNF*), this was a comprehensive examination of the genetic variability in the genes of interest. Tagging SNPs were used to characterize all known polymorphisms within the genes and in regulatory regions surrounding them. Although not without limitations the two statistical methods used in our analyses allow investigating many possible combinations of variables relatively quickly and incorporate checks for multiple testing. Also, the coding scheme for two dichotomous variables for each SNP suggested by the authors of logic regression allows the model to select whether the dominant or the recessive model is more predictive of breast cancer for each SNP.

It is likely that complex interactions in these genes and between variants in these genes and non-genetic factors exist. However, the inadequate knowledge of the biological significance of SNPs and the still limited (albeit constantly advancing) statistical tools preclude us from fully understanding these interactions. Future studies using alternative statistical approaches or traditional statistical approaches, but with very large sample sizes, are needed to confirm the conclusion that there is no epistasis among genes that regulate estrogen synthesis and metabolism and between genetic and non-genetic estrogen-related factors.

4.6 Figures and Tables

Table 4.1. Characteristics of cases and controls selected from the American Cancer Society Cancer Prevention Study-II Lifelink Cohort

Characteristic	Cases	Controls
Age at blood draw (yrs)	70 (65-74)	70 (65-74)
Age at diagnosis (yrs)	68 (64-73)	--
Age at menopause (yrs)	50 (46-53)	50 (45-52)
BMI 1992 (kg/m^2)	24.2 (21.8-27.5)	24.7 (22.3-28.1)
Race		
White	476 (99)	483 (98.8)
Black	4 (0.8)	4 (0.8)
Other	1 (0.2)	2 (0.4)
Family History		
No	384 (79.8)	416 (85.1)
Yes	97 (20.2)	73 (14.9)
Adult weight change		
-5 to 20 lbs	219 (45.5)	222 (45.4)
20 to 60 lbs	214 (44.5)	225 (46)
60+ lbs	48 (10)	42 (8.6)
Postmenopausal hormone use		
Never	159 (34.2)	199 (42)
Current	213 (45.8)	169 (35.7)
Former	81 (17.4)	104 (21.9)
Physical activity		
<7 METS	183 (38.1)	192 (39.6)
7-24.4 METS	224 (46.7)	210 (43.3)
24.5+ METS	73 (15.2)	83 (17.1)

Table 4.2. Odds ratios and 95% confidence intervals for estrogen biosynthesis polymorphisms and postmenopausal breast cancer

Gene	SNP	Category	Cases	Controls	Odds Ratio	(95% CI)	p-value
HSD17B1	rs676387	C/C	273	262	1.00	(ref)	
		C/A	181	184	0.98	(0.75-1.29)	0.38
		A/A	32	43	0.74	(0.44-1.24)	
		C/A or A/A	213	227	0.93	(0.72-1.21)	0.60
HSD17B1	rs605059	A/A	158	142	1.00	(ref)	
		A/G	226	244	0.81	(0.60-1.10)	0.29
		G/G	102	103	0.83	(0.58-1.20)	
		A/G or G/G	328	347	0.82	(0.61-1.09)	0.17
HSD17B1	rs598126	G/G	156	140	1.00	(ref)	
		G/A	226	246	0.80	(0.59-1.09)	0.34
		A/A	104	103	0.85	(0.59-1.22)	
		G/A or A/A	330	349	0.82	(0.61-1.09)	0.17
HSD17B1	rs2010750	C/C	185	174	1.00	(ref)	
		C/T	223	237	0.88	(0.66-1.17)	0.44
		T/T	78	78	0.88	(0.60-1.30)	
		C/T or T/T	301	315	0.88	(0.67-1.16)	0.37
HSD17B2	CV411254	C/C	191	190	1.00	(ref)	
		C/T	232	229	1.04	(0.79-1.36)	0.82
		T/T	63	70	0.92	(0.63-1.36)	
		C/T or T/T	295	299	1.01	(0.78-1.30)	0.95
HSD17B2	rs2966245	A/A	166	161	1.00	(ref)	
		A/G	240	240	0.99	(0.75-1.32)	0.71
		G/G	80	88	0.92	(0.63-1.35)	
		A/G or G/G	320	328	0.97	(0.74-1.27)	0.84

Table 4.2. (Continued)

Gene	SNP	Category	Cases	Controls	Odds Ratio	(95% CI)	p-value
HSD17B2	rs2042429	T/T	142	143	1.00	(ref)	
		T/C	248	242	1.05	(0.78-1.41)	0.71
		C/C	96	104	0.92	(0.64-1.32)	
		T/C or C/C	344	346	1.01	(0.76-1.34)	0.94
HSD17B2	rs2955163	G/G	437	440	1.00	(ref)	
		G/C	49	46	1.02	(0.67-1.58)	0.75
		C/C	0	3	-	(*-*)	
		G/C or C/C	49	49	1.00	(0.65-1.53)	1.00
HSD17B2	rs2955162	C/C	269	265	1.00	(ref)	
		C/T	185	175	1.04	(0.80-1.35)	0.25
		T/T	32	49	0.64	(0.40-1.02)	
		C/T or T/T	217	224	0.95	(0.74-1.21)	0.66
HSD17B2	rs996752	A/A	216	207	1.00	(ref)	
		A/G	215	213	0.95	(0.73-1.25)	0.22
		G/G	55	69	0.75	(0.51-1.12)	
		A/G or G/G	270	282	0.90	(0.70-1.16)	0.41
HSD17B2	rs4889459	T/T	145	125	1.00	(ref)	
		T/C	225	238	0.78	(0.58-1.07)	0.13
		C/C	116	126	0.77	(0.55-1.09)	
		T/C or C/C	341	364	0.78	(0.59-1.04)	0.09
CYP19	rs4646	C/C	285	245	1.00	(ref)	
		C/A	173	202	0.74	(0.57-0.97)	0.008
		A/A	28	42	0.58	(0.34-0.99)	
		C/A or A/A	201	244	0.72	(0.55-0.93)	0.012
CYP19	rs10046	A/A	162	127	1.00	(ref)	
		A/G	227	241	0.73	(0.54-0.98)	0.009
		G/G	97	121	0.62	(0.43-0.90)	
		A/G or G/G	324	362	0.69	(0.52-0.92)	0.011

Table 4.2. (Continued)

Gene	SNP	Category	Cases	Controls	Odds Ratio	(95% CI)	p-value
CYP19	rs17601241	G/G	402	408	1.00	(ref)	
		G/A	80	76	1.10	(0.77-1.58)	0.74
		A/A	4	5	0.77	(0.17-3.46)	
		G/A or A/A	84	81	1.08	(0.76-1.55)	0.65
CYP19	rs700519	G/G	455	459	1.00	(ref)	
		G/A	29	29	0.96	(0.55-1.68)	0.89
		A/A	2	1	--	--	
		G/A or A/A	31	30	1.00	(0.57-1.74)	1.00
CYP19	rs28757183	G/G	443	456	1.00	(ref)	
		G/A	42	33	1.31	(0.81-2.12)	0.27
		A/A	1	0	--	--	
		G/A or A/A	43	33	1.31	(0.81-2.12)	0.27
CYP19	rs2414096	A/A	148	115	1.00	(ref)	
		A/G	229	245	0.75	(0.55-1.02)	0.024
		G/G	109	129	0.67	(0.47-0.96)	
		A/G or G/G	338	374	0.72	(0.54-0.96)	0.024
CYP19	rs727479	A/A	238	190	1.00	(ref)	
		A/C	196	231	0.66	(0.50-0.87)	0.003
		C/C	52	68	0.63	(0.42-0.95)	
		A/C or C/C	248	299	0.65	(0.50-0.84)	0.0012
CYP19	rs1008805	A/A	162	164	1.00	(ref)	
		A/G	235	248	0.98	(0.73-1.31)	0.36
		G/G	89	77	1.26	(0.85-1.86)	
		A/G or G/G	324	325	1.04	(0.79-1.38)	0.78
CYP19	rs6493494	G/G	152	163	1.00	(ref)	
		G/A	230	241	1.03	(0.76-1.39)	0.22
		A/A	104	85	1.27	(0.88-1.81)	
		G/A or A/A	334	326	1.11	(0.84-1.46)	0.48

Table 4.2. (Continued)

Gene	SNP	Category	Cases	Controls	Odds Ratio	(95% CI)	p-value
CYP19	rs749292	G/G	146	151	1.00	(ref)	
		G/A	232	251	0.94	(0.70-1.27)	0.29
		A/A	108	87	1.25	(0.87-1.79)	
		G/A or A/A	340	338	1.03	(0.78-1.37)	0.83
CYP19	rs1902586	G/G	438	442	1.00	(ref)	
		G/A	45	43	1.03	(0.65-1.64)	0.92
		A/A	3	4	0.67	(0.11-3.99)	
		G/A or A/A	48	47	1.00	(0.64-1.57)	1.00
CYP19	rs936306	C/C	370	342	1.00	(ref)	
		C/T	107	124	0.78	(0.58-1.07)	0.005
		T/T	9	23	0.33	(0.14-0.77)	
		C/T or T/T	116	147	0.70	(0.53-0.94)	0.017
CYP19	rs2445759	G/G	433	403	1.00	(ref)	
		G/T	52	81	0.58	(0.40-0.85)	0.0015
		T/T	1	5	0.20	(0.02-1.71)	
		G/T or T/T	53	86	0.56	(0.38-0.81)	0.002
CYP19	rs28566535	A/A	440	438	1.00	(ref)	
		A/C	44	46	0.92	(0.58-1.46)	0.35
		C/C	2	5	0.25	(0.03-2.24)	
		A/C or C/C	46	51	0.85	(0.54-1.34)	0.49
CYP19	rs3751591	A/A	352	323	1.00	(ref)	
		A/G	123	152	0.69	(0.51-0.93)	0.018
		G/G	11	14	0.74	(0.33-1.64)	
		A/G or G/G	134	166	0.69	(0.52-0.92)	0.011
CYP19	rs1902584	A/A	399	414	1.00	(ref)	
		A/T	84	71	1.24	(0.86-1.77)	0.35
		T/T	3	4	0.70	(0.12-4.17)	
		A/T or T/T	87	75	1.21	(0.85-1.73)	0.28

Table 4.2. (Continued)

Gene	SNP	Category	Cases	Controls	Odds Ratio	(95% CI)	p-value
CYP19	rs1004984	G/G	173	195	1.00	(ref)	
		G/A	232	215	1.24	(0.93-1.65)	0.31
		A/A	81	79	1.15	(0.79-1.67)	
		G/A or A/A	313	294	1.21	(0.93-1.59)	0.15
CYP19	rs2445762	T/T	236	272	1.00	(ref)	
		T/C	210	180	1.36	(1.03-1.79)	0.053
		C/C	40	37	1.28	(0.79-2.07)	
		T/C or C/C	250	217	1.35	(1.04-1.75)	0.026
CYP19	rs2470144	C/C	133	113	1.00	(ref)	
		C/T	242	252	0.83	(0.61-1.13)	0.18
		T/T	111	124	0.78	(0.54-1.13)	
		C/T or T/T	353	376	0.81	(0.61-1.09)	0.16
CYP19	rs2445765	G/G	327	315	1.00	(ref)	
		G/C	142	160	0.85	(0.65-1.13)	0.50
		C/C	17	14	1.15	(0.55-2.41)	
		G/C or C/C	159	174	0.87	(0.67-1.15)	0.33
CYP19	rs2446405	T/T	326	312	1.00	(ref)	
		T/A	141	162	0.83	(0.63-1.10)	0.50
		A/A	19	15	1.27	(0.61-2.62)	
		T/A or A/A	160	177	0.86	(0.65-1.13)	0.27
IL6	rs4552807	T/T	129	144	1.00	(ref)	
		T/A	253	221	1.29	(0.95-1.74)	0.93
		A/A	104	124	0.96	(0.66-1.38)	
		T/A or A/A	357	345	1.18	(0.89-1.57)	0.25
IL6	rs6969502	G/G	302	309	1.00	(ref)	
		G/A	169	151	1.18	(0.89-1.56)	0.65
		A/A	15	29	0.47	(0.24-0.94)	
		G/A or A/A	184	180	1.07	(0.82-1.39)	0.63

Table 4.2. (Continued)

Gene	SNP	Category	Cases	Controls	Odds Ratio	(95% CI)	p-value
IL6	rs6952003	T/T	260	273	1.00	(ref)	
		T/A	197	178	1.18	(0.90-1.55)	0.83
		A/A	29	38	0.78	(0.45-1.35)	
		T/A or A/A	226	216	1.11	(0.86-1.43)	0.43
IL6	rs10156056	G/G	373	367	1.00	(ref)	
		G/C	106	107	1.01	(0.73-1.40)	0.35
		C/C	7	15	0.47	(0.19-1.15)	
		G/C or C/C	113	122	0.93	(0.68-1.26)	0.64
IL6	rs7776857	T/T	217	228	1.00	(ref)	
		T/G	224	200	1.17	(0.89-1.55)	0.59
		G/G	45	61	0.71	(0.45-1.11)	
		T/G or G/G	269	261	1.05	(0.81-1.36)	0.69
IL6	rs7801617	G/G	389	374	1.00	(ref)	
		G/A	92	109	0.80	(0.58-1.11)	0.19
		A/A	5	6	0.80	(0.24-2.63)	
		G/A or A/A	97	115	0.80	(0.58-1.10)	0.17
IL6	rs7805828	G/G	155	177	1.00	(ref)	
		G/A	253	235	1.21	(0.91-1.60)	0.27
		A/A	78	77	1.17	(0.80-1.72)	
		G/A or A/A	331	312	1.20	(0.92-1.56)	0.18
IL6	rs2056576	C/C	230	227	1.00	(ref)	
		C/T	209	209	0.96	(0.74-1.25)	0.65
		T/T	47	53	0.91	(0.59-1.42)	
		C/T or T/T	256	262	0.95	(0.74-1.23)	0.70
IL6	rs12700386	C/C	324	329	1.00	(ref)	
		C/G	140	151	0.95	(0.72-1.25)	0.34
		G/G	22	9	2.58	(1.14-5.85)	
		C/G or G/G	162	160	1.03	(0.79-1.34)	0.84

Table 4.2. (Continued)

Gene	SNP	Category	Cases	Controls	Odds Ratio	(95% CI)	p-value
IL6	rs1800797	G/G	183	177	1.00	(ref)	
		G/A	211	224	0.86	(0.64-1.15)	0.93
		A/A	92	88	1.04	(0.71-1.52)	
		G/A or A/A	303	312	0.91	(0.69-1.19)	0.49
IL6	rs1800796	G/G	435	440	1.00	(ref)	
		G/C	50	46	1.08	(0.70-1.67)	0.92
		C/C	1	3	-	(*-*)	
		G/C or C/C	51	49	1.02	(0.67-1.58)	0.91
IL6	rs1800795	G/G	179	171	1.00	(ref)	
		G/C	217	228	0.86	(0.64-1.15)	0.71
		C/C	90	90	0.98	(0.67-1.43)	
		G/C or C/C	307	318	0.89	(0.68-1.17)	0.41
IL6	rs2069840	C/C	213	212	1.00	(ref)	
		C/G	214	230	0.91	(0.69-1.19)	0.66
		G/G	59	47	1.27	(0.82-1.96)	
		C/G or G/G	273	277	0.97	(0.75-1.25)	0.79
IL6	rs2069861	C/C	392	394	1.00	(ref)	
		C/T	91	88	1.06	(0.76-1.48)	0.76
		T/T	3	7	0.43	(0.11-1.66)	
		C/T or T/T	94	95	1.00	(0.72-1.38)	1.00
IL6	rs10242595	G/G	246	231	1.00	(ref)	
		G/A	193	202	0.89	(0.69-1.16)	0.23
		A/A	47	56	0.80	(0.52-1.22)	
		G/A or A/A	240	258	0.87	(0.68-1.12)	0.28
IL6	rs11766273	G/G	421	422	1.00	(ref)	
		G/A	60	62	0.90	(0.61-1.34)	0.66
		A/A	5	5	0.98	(0.28-3.39)	
		G/A or A/A	65	67	0.91	(0.62-1.33)	0.63

Table 4.2. (Continued)

Gene	SNP	Category	Cases	Controls	Odds Ratio	(95% CI)	p-value
TNF	rs1799964	T/T	315	301	1.00	(ref)	
		T/C	151	172	0.84	(0.64-1.10)	0.64
		C/C	20	16	1.38	(0.68-2.81)	
		T/C or C/C	171	188	0.88	(0.67-1.15)	0.34
TNF	rs1799724	C/C	390	398	1.00	(ref)	
		C/T	93	85	1.15	(0.82-1.61)	0.75
		T/T	3	6	0.42	(0.08-2.15)	
		C/T or T/T	96	91	1.11	(0.79-1.55)	0.55
TNF	rs1800629	G/G	334	342	1.00	(ref)	
		G/A	143	136	1.07	(0.80-1.42)	0.95
		A/A	9	11	0.74	(0.30-1.85)	
		G/A or A/A	152	147	1.04	(0.79-1.38)	0.78
LEP	rs4731423	A/A	144	151	1.00	(ref)	
		A/G	253	230	1.11	(0.83-1.49)	0.56
		G/G	89	108	0.85	(0.57-1.25)	
		A/G or G/G	342	338	1.04	(0.79-1.38)	0.78
LEP	rs13245201	G/G	145	160	1.00	(ref)	
		G/A	248	231	1.19	(0.87-1.62)	0.64
		A/A	93	98	1.07	(0.74-1.56)	
		G/A or A/A	341	329	1.15	(0.86-1.54)	0.34
LEP	rs7795794	G/G	423	434	1.00	(ref)	
		G/A	61	53	1.19	(0.81-1.74)	0.41
		A/A	2	2	1.00	(0.14-7.10)	
		G/A or A/A	63	55	1.18	(0.81-1.72)	0.39
LEP	rs10954173	G/G	178	175	1.00	(ref)	
		G/A	248	237	1.00	(0.75-1.33)	0.30
		A/A	60	77	0.77	(0.51-1.16)	
		G/A or A/A	308	314	0.94	(0.72-1.24)	0.68

Table 4.2. (Continued)

Gene	SNP	Category	Cases	Controls	Odds Ratio	(95% CI)	p-value
LEP	rs2071045	T/T	274	286	1.00	(ref)	
		T/C	180	171	1.14	(0.85-1.52)	0.59
		C/C	32	32	1.01	(0.59-1.72)	
		T/C or C/C	212	203	1.11	(0.85-1.46)	0.44

Table 4.3. Multifactor dimensionality reduction models for estrogen biosynthesis genes and breast cancer

Variables	**Testing Accuracy**	**100-fold Cross Validation Consistency**	**p-value***
CYP19 SNP7 (rs727479)	0.54	89/100	0.24
IL6 SNP1 (rs4552807), *IL6* SNP13 (rs2069840)	0.53	23/100	0.38
CYP19 SNP7 (rs727479), *IL6* SNP1 (rs4552807), *IL6* SNP9 (rs12700386)	0.50	5/100	0.85
HSD17B2 SNP2 (rs2966245), *CYP19* SNP19 (rs2470144), *IL6* SNP1 (rs4552807), *IL6* SNP13 (rs2069840)	0.51	11/100	0.72

*pvalue calculated from 1000 permutations

Table 4.4. Multifactor dimensionality reduction models for prediction of postmenopausal breast cancer by estrogen biosynthesis genes and non-genetic factors

Variables	**Testing Accuracy**	**100-fold Cross Validation Consistency**	**p-value***
Postmenopausal Hormone (PMH) Use	0.55	84/100	0.12
HSD17B2 SNP5 (rs2955162), PMH use	0.53	21/100	0.29
HSD17B2 SNP5 *(rs2955162)*, *IL6* SNP1 (rs4552807), PMH use	0.51	36/100	0.20
HSD17B1 SNP2 (rs605059), *HSD17B2* SNP1 (rs4445895), *IL6* SNP1 (rs4552807), PMH use	0.56	44/100	0.09

*pvalue calculated from 1000 permutations

Table 4.5. Logic regression models for breast cancer prediction

Model Size	Number of Trees	Model	Null model test p-value
1 variable	1 tree‡	*CYP19* SNP7 (rs727479)	0.088
2 variables	1 tree	*CYP19* SNP7 (rs727479) and never PMH users	
	2 trees	*CYP19* SNP7 (rs727479) and current PMH users	
3 variables	1 tree	*CYP19* SNP5 (rs28757183) or *IL6* SNP3 (rs6952003) or *CYP19* SNP13 (rs2445759)	
	2 trees	*CYP19* SNP13 (rs2445759) or *IL6* SNP3 (rs6952003), not Current PMH users	

‡Cross-validation consistency identified this size model as the best for this data

CHAPTER 5: CONCLUSIONS, STRENGTHS, AND LIMITATIONS, FUTURE DIRECTIONS

The goal of this dissertation was to improve our understanding of the relationship between adiposity and breast cancer risk. Specifically, I set out to examine whether the increased risk of breast cancer from increasing levels of adiposity can be reversed through weight loss and to investigate the molecular pathways between obesity and breast cancer. Although I did not identify any statistically significant associations, this dissertation still makes a valuable contribution to the field of breast cancer epidemiology.

In general, published null results help researchers decide how much credence to give positive findings and help them evaluate associations in a systematic manner. Replicated null results offer an opportunity for researchers to feel confident that future time and resources are best spent in other areas. Conversely, null results that have not been replicated are often viewed as an opportunity to closely re-examine every aspect of the study and perhaps uncover limitations. Selective reporting of only positive results (both publication bias and highlighting of positive results within a study) is a major problem (214) and can harm the credibility of the field of epidemiology as a whole. Unwarranted media attention (215) because of over-interpretation of positive results, particularly without negative results to balance them, can adversely impact public opinion of the value of epidemiologic research. In addition, erroneous scientific evidence can lead to inappropriate governmental and public health decisions including potentially harmful and/or costly measures (215).

Our study of the relationship between weight loss and postmenopausal breast cancer found no association. It is possible that there is truly no association, particularly

for small amount of weight loss. Most population studies on the topic have found no association or a weakly protective effect (65-66, 83-96). However, these findings are not consistent with studies that show that weight loss is associated with lower levels of circulating estrogens (62). In addition, bariatric surgery studies suggest that there may be a protective effect of losing large amounts of weight for overweight women. It may be that the smaller amounts of weight change experienced by most women who lose weight do not reduce estrogen levels enough to impact breast cancer risk. Our study, however, did not find an association between weight loss and postmenopausal for any amount of weight loss. Some possible contributing factors are detailed below.

Lack of power likely plays a role in explaining the null findings in this and other studies. In general, relatively few women lose weight throughout adulthood. In this cohort of heavy women who lost or maintained their weight, only 12% (n=1629) of the women lost 20 or more pounds from 1982 to 1992 and less than 5% (n=664) lost more than 30 pounds. Previous studies reported a statistically significant association with weight *gain* only after women had gained at least 20 pounds (64-66).

Another possible explanation for the observed null results is exposure misclassification. Women who do lose weight often do not maintain their weight loss, regardless of the method used to reduce the weight (106). When examining weight loss and postmenopausal breast cancer in the Nurses' Health Study (NHS), Eliassen, et al found a statistically significant association between adult weight loss and postmenopausal breast cancer only when the weight loss had been maintained for two survey cycles (i.e., 4 years). Harvie, et al reported the lowest rates of breast cancer among women who lost or maintained weight in the initial interval (ages 18 to 30) with additional losses in

subsequent intervals (age 30 to menopause and after menopause) (85). We were unable to replicate the NHS finding when restricting our analysis to women who maintained their weight loss during the first follow-up interval (1992-1997). However, our intervals were much longer (10 and 5 years rather than 2) making it more likely that we missed weight fluctuations during the interval.

There may also be a specific window of time in a woman's life when weight loss is most relevant to breast cancer risk. Eliassen, et al reported a significant association specifically with weight loss since menopause (65) but researchers from the Iowa Women's Health Study (85) and the AARP Cohort Study (66, 85) were unable to confirm this observation. Other specific time periods have been investigated but no clear pattern has emerged. Using participants recruited during two different time periods, Trentham-Dietz, et al reported 1) an odds ratio of 0.9 (95% CI 0.84-0.98) per five kilograms of weight loss before (but not after) age 45 and 2) an odds ratio of 0.8 (95% CI 0.69-0.94) for any weight loss after age 35 compared to weight maintainers (87). Zeigler, et al reported an odds ratio of 0.69 (95% CI 0.29-1.66) for weight loss in the previous decade of life for a cohort of Asian Americans in their 50s (94).

Effect modification simultaneously by postmenopausal hormone (PMH) use and BMI may have obscured an association between weight loss and breast cancer. Many studies, including our previous analysis (64), found a relationship between weight gain and breast cancer only among women not currently using PMH. Likewise the Nurses' Health Study observed an association between menopausal weight loss and breast cancer only among never PMH users (65). Although effect modification by baseline BMI has not been reported previously, half of the women who lost the most weight (30+ pounds)

in our study were still overweight or obese after they lost weight. These women's endogenous estrogen level may still be too high to produce a reduction in risk of postmenopausal breast cancer relative to the women who maintained their weight. Although we examined effect modification by PMH use and BMI separately, we did not have the sufficient statistical power to examine the three-way interactions. Therefore, it is possible the weight maintainers were more likely to be PMH users while the weight losers had a heavier starting BMI.

The second and third parts of this dissertation that studied molecular pathways to breast cancer found no evidence that adipokine or estrogen genes impact risk of breast cancer. We have conducted a comprehensive study of the relation between variability in *LEP, LEPR, ADIPOQ, ADIPOR1*, and *ADIPOR2* genes and postmenopausal breast cancer. Although we may have missed weak associations or complex interactions, it seems unlikely that there are strong, independent effects of SNPs in these genes. This conclusion is supported by results from several genome-wide association studies for breast cancer (46).

Our study of estrogen biosynthesis genes and environmental factors impacting estrogen levels, attempted to address the limitation that most traditional methods are not able to examine higher order interactions due to sparse data. Although we did not find any statistically significant joint effects of various genetic and environmental factors, it may be that the interactions were more complex (we only evaluated up to four-way interactions due to sample size limitations). It is also possible that the relevant SNPs are in the genes that were not considered in this study or in a non-gene region.

Another reason for the null results in both genetic studies may be that other biological products or epigenetics (changes to gene expression caused by something other than DNA sequence) are a better measure of the biological and/or environmental risk factors for breast cancer. The level of transcription of a gene is not the only factor that influences the resulting level of a protein. Rapid degradation of mRNA (or the protein itself), inefficient translation, and post-translational modifications can all impact protein levels. In addition, the same DNA sequence can lead to multiple protein products through alternative splicing, and many proteins only function after forming polymers (Anderson 1998). Taking it a step further, since metabolites are the end products of cellular processes, they can be viewed as the response of biological systems to genetic and/or environmental changes(212). In this way, metabolites may provide an even better way to measure the contributions of inherited factors, environmental factors, and how they interact. Measurement of DNA methylation patterns or other quantitative epigenetic changes may help clarify inherited risk of breast cancer. Statistical methods such as hierarchical models that can simultaneously account for biological processes at every step may be needed to take all of these factors into account.

As a group, these studies shared the strength of prospectively collected environmental data and a population-based source population. The genetic data were assessed with several quality control measures and there was very little missing data overall. Where the data were missing, PHASE (version 2.2) was used to impute genotypes by reconstructing haplotypes from population genotype data. In addition, we had comprehensive coverage of the known genetic variation in nearly all the genes we studied. A common limitation of these three studies was sample size. As we clarify more

specific exposures and outcomes, and try to model higher order interactions, power becomes an increasing problem. A consortial approach may be necessary to answer these questions accurately. Although internally valid, our results may not be generalizable to all populations. Future studies are needed to examine genetic and modifiable associations in non-white, U.S. populations and in other countries.

In summary, the three studies included in my dissertation allow the following three main conclusions. First, based on my findings, overweight women may not be able to reduce their breast cancer risk by losing weight (at least with the weight loss patterns observed in the CPSII cohort). Second, the results of my analyses provided evidence that individual, low-penetrance germline mutations in leptin and adiponectin genes do not substantially impact breast carcinogenesis. Finally, my research illustrated the practical applications of statistical methods aimed at modeling complex biological processes and highlighted the need for further development and refinement of these methods.

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