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LIST OF ABBREVIATIONS

MTHFR	methylenetetrahydrofolate
MS	methionine synthase
CBS	cystathione- β -synthase
HDL	high density lipoprotein
ER	estrogen receptor
SAM	S-adenosylmethionine
THF	tetrahydrofolate
dUMP	deoxyuridine monophosphate
TMP	thymidine monophosphate
RFLP	restriction fragment length polymorphism
TCC	transitional cell carcinoma
DNMT	DNA methyltransferase
HMEC	human mammary epithelial cells
LOH	loss of heterozygosity
bp	base - pair

1. INTRODUCTION, PURPOSE, HYPOTHESIS AND SPECIFIC GOALS

1.1 Introduction

Breast cancer, a heterogeneous disease and one of the most frequently diagnosed cancers among women, accounts for more than 30% of all cancers and is the leading cause of death in women ages 40 - 55 years old (1). According to data reported by the National Cancer Institute's Surveillance, Epidemiology and End Results program (SEER), the estimated cases and deaths from this disease in the US for 2001 were 192,000 and 40,000 respectively (1). However, there is an enormous variation of these rates internationally suggesting possible genetic, socio-cultural and/or other lifestyle risk factors (2). For instance, the 1990 mortality rates varied more than six-fold internationally with the lowest rates occurring in China and the highest occurring in Northern Europe (rates were per 100,000 women-years, age adjusted to world standard). It is true that some of these differences may be due to incomplete reporting, inconsistent diagnostic patterns, and treatment styles. But, consistently higher rates in certain regions persist, suggesting a true difference in breast cancer risk. There is convincing evidence indicating that genetic, environmental and lifestyle factors may offer an explanation for this difference. Migrant studies, used to determine whether genetic or lifestyle and environment factors could explain rate differences between countries, show that an immigrant's risk may be lower in her home country, but her risk quickly catches up to her host country's risk (3). A study by Parkin et al.(4) reported that Chinese women living in Shanghai have one half the incidence rates of those living in North America and two thirds the rate of those living in Hong Kong and Singapore.

The large variation in breast cancer rates among countries and the change of incidence rates among migrant populations have brought diet to the attention of many scientists. Many studies suggest that a diet high in fruit and vegetables is protective against breast cancer; however the factor/factors in these foods that would explain that association are unknown. There is an increasing interest in folate, B₆ and B₁₂. Fruits and vegetables, including dark greens and dried bean are an excellent source of folate (5). Whole grains, which are a superior source of vitamin B₆ to fruits and vegetables, have been associated with a decreased in risk in several cancers (6). A meta-analysis of case-control studies showed that whole grains consumption was associated with a 40% decreased risk of developing stomach cancer and a 20% decreased risk of developing cancers of the rectum and colon (7). Vitamin B₁₂, a micronutrient found in meat, is less studied than folate and vitamin B₆; however, it is an important micronutrient, considering its involvement in the one-carbon cycle with vitamin B₆ and folic acid. Vitamin B₁₂ transfers a methyl group to homocysteine, converting it to methionine (8).

Folate deficiency results in decreased methionine availability which interferes with DNA methylation, and allows for aberrant DNA synthesis (5). The mechanism whereby folic acid is protective against breast cancer is not known, but it may be related to its role in DNA methylation and synthesis. In support of this hypothesis, several studies have shown evidence of increased DNA damage with diets low in nutrients involved in this pathway (9). More than a few studies have investigated the association between folate consumption and breast cancer risk, but few of those studies have taken into consideration the genetic polymorphisms of genes coding for key enzymes involved in one carbon metabolism. This pathway plays a very important role in the maintenance

of DNA methylation and nucleotide synthesis. There is evidence indicating that polymorphisms in genes coding for enzymes involved in folic acid metabolism, are associated with some cancers (10, 11). If in fact polymorphisms in genes coding for key enzymes involved in one carbon metabolism render those enzymes labile, then this finding corroborates the inverse association of folic acid and breast cancer risk. If a genetic variant of an enzyme with reduced activity is associated with the risk of a disease, then the reduced availability of the substrate of that enzyme should also be related to the risk of that disease.

Alcohol is not involved in one-carbon metabolism but some studies have found an increased risk of breast cancer with high alcohol use (12). It is known that alcohol increases endogenous estrogen levels, but what is of most interest to this study is its ability to interfere with the bioavailability of folic acid, since there is data to suggest that low folic acid intake is a breast cancer risk factor (13). Moderate alcohol consumption has been associated with an inverse effect on several health conditions including cardiovascular disease. In one study, data suggested that moderate alcohol intake increases serum high density lipoprotein (HDL) cholesterol levels, stimulating reverse cholesterol transport (14). The epidemiological data on alcohol and cancer is controversial; however some studies have shown that metabolism of alcohol leads to the generation of acetaldehyde and free radicals. Acetaldehyde is predominantly responsible for the alcohol associated carcinogenesis particularly the liver (15). Thus, there are positive aspects of moderate alcohol drinking relating to cholesterol levels and heart disease; on the other hand alcohol drinking is associated with carcinogenesis. What is unknown is the balance between modest drinking and protection, versus more drinking

and cancer, or whether there functional genetic polymorphisms that influence the human physiological response to alcohol consumption.

In addition to diet, it is a well established fact that different types of somatic genetic alterations are associated with breast cancer. *p53* is a tumor suppressor gene that is involved in several cellular processes such as induction of apoptosis, and cell cycle control (16). With approximately 30% of tumors having some type of *p53* mutation, this gene is the most frequent site for gene mutations in breast cancer (17). Studies have found increased *p53* mutations and expression in tumors of persons who consumed more than one alcoholic beverage per day (11). However, very few studies have examined fruit and vegetable consumption in relation to *p53* mutation frequency, and even less have examined the modifying effect of polymorphisms found in one-carbon genes on *p53* mutations. It is also known that half of the *p53* mutations are G:C > A:T transitions and most of these mutations occur at CpG dinucleotides; cytosines at this site are often methylated and prone to deamination if not efficiently repaired (18).

Finally, breast cancers are classified as estrogen receptor (ER) positive or negative, and this classification is often used to determine the prognosis for patients. Since the biology of this cancer and the role of risk factors may vary by ER status, it is important to consider the receptor status when evaluating risk factors for breast cancer.

1.2 The Purpose of this Study

There is significant evidence indicating that adequate vegetables and fruit in one's diet may be protective against the risk of breast cancer, and separately, that increased alcohol intake may increase that risk. It is quite possible that one carbon metabolism may explain the protective value of fruits, vegetables and the adverse consequences of alcohol drinking. The assessment of diet and genetic variation in enzymes that are involved in the one-carbon metabolism will help to determine if these pathways explain variations of breast cancer risk. The study of susceptible subgroups would help to highlight the importance of folic acid, B₆ and B₁₂ in diets, particularly in persons with genetic variants that increase risk and those who consume more than one alcoholic beverage daily. It should be noted that, since 1998, folic acid has been added to the US food supply. The quantity of folic acid added to flour is estimated to increase the mean intake by roughly 100 micrograms per day. This fortification has been helpful to some extent, but in spite of this change most people do not achieve the recommended daily amounts of 400 micrograms per day (19). Also this fortification does not include vitamin B₆ and B₁₂. Thus, the issue of folic acid, B₆ and B₁₂ as a risk factors for breast cancer remains important.

The relationship of risk factors for dysregulated methylation on breast carcinogenesis has received little attention, but the occurrence of this epigenetic change is common. It is quite likely that some women are genetically predisposed to such dysregulation; if empirically this is the case, then this study will help to identify and understand those risk factors in relation to diet and alcohol drinking as it affects breast carcinogenesis. We will also be able identify new pathways of importance, separate from

genetic pathways more frequently studied, such as carcinogen and estrogen metabolism and DNA repair. The identification of susceptible groups would allow us to better focus chemoprevention studies.

The design of this study will also allow consideration of overall risk; insight would be gained into the association of alcohol consumption and increased cancer risk, and if there are susceptible subgroups of the population based on their genotypes. The incorporation of both a case control analysis and the use of molecular markers in tumors that corroborate the case-control analysis would provide strong evidence for identified associations.

This project has the potential to identify altered methylation pathways of susceptible subpopulations, compounded by diets low in folate, B₆ and B₁₂ intake. By using new molecular makers, with cancer as the end point, it would also validate the mechanistic relationship to cancer by examining intermediate molecular markers (*p53* and hypermethylation).

1.3 Hypotheses and Specific Goals

1.3.1 Hypotheses:

1) Breast cancer risk is increased by inadequate folate, B₆ and B₁₂ intake, and moderate to excessive alcohol consumption. This increased risk is further modified by genes involved in one carbon metabolism.

2) The importance of the genes within one carbon metabolism pathways can be corroborated by the examination of molecular markers in tumors that reflect methylation status. The analysis of tumor tissue will identify women as "hypermethylator

phenotypes" who will have had a lower nutrient risk and a greater frequency of genetic polymorphisms in related genes.

3) Women who are prone to p53 mutations would also be prone to ER negative tumors, since both phenotypes may be the indirect consequence of inadequate folate, B₆ and B₁₂ nutrients and polymorphic variants of genes involved in the metabolism of these nutrients.

1.3.2 Specific Goals:

1) Genotype cases and controls, using DNA extracted from blood clots, for *MTHFR C677T*, *MTHFR A1298C*, *MS A2756G*, and *CBS 844ins68* identifying them as homozygous wild-type, heterozygote, or homozygous variant. Using unconditional logistic regression, calculate odds ratio, and 95% confidence intervals to determine the degree of relationship between these markers and folate, B₆ and B₁₂. Also this study will assess whether breast cancer risk is modified by these polymorphisms in *MTHFR*, *MS* and *CBS*.

2) Extract DNA from tumor tissue and treat it with sodium metabisulphate to modify all unmethylated cytosines. Use modified DNA to determine the methylation status of the promoter regions of *p16*, *BRCA1*, *ER*, and *Ecadherin*. Use unconditional logistic regression as a model to calculate odds ratio and 95% confidence interval to determine the effect of genetic polymorphisms on gene hypermethylation.

3) Determine the estrogen receptor status of tumors by immunohistochemistry and use this information along with previously determined *p53* mutations to identify any association between ER^{+/-} tumors and *p53* mutations. Use the estrogen receptor status

along with dietary factors and genotypes to determine if there is any relationship between

one-carbon nutrients, polymorphic one-carbon genes and estrogen receptors.

Unconditional logistic regression would be used to calculate odds ratios and 95% confidence intervals in these analyses.

2. BACKGROUND

2.1 One-carbon Metabolism and Methylation

One-carbon metabolism is a term used to describe a group of reactions that involve the transfer of one-carbon groups. This pathway is critical for the biological methylation of numerous compounds including DNA. It is also essential to the synthesis of purines, pyrimidine and thymidine. The mechanism whereby one-carbon metabolism affects cancer risk has not been elucidated, but two mechanisms have been suggested. The first mechanism involves the transfer of one-carbon compounds in the synthesis of S-adenosylmethionine (SAM). Folate in the form 5'-methyltetrahydrofolate (5'-methyl-THF) is essential in converting methionine to its activated form (SAM) by donating a methyl group to homocystine. SAM is the principal methyl donor in a number of biochemical reactions including DNA methylation, which is an epigenetic alteration that determines gene expression (8) (Figure1). Genes that are methylated at their promoters are either not transcribed or transcribed at a reduced rate; on the other hand, genes that are hypomethylated are up-regulated or transcribed at an increased rate. If SAM is depleted as a result of folate deficiency, then there may be a dysregulation of methylation, but rebound responses may increase methylation in certain regions. Separately, cytosines that are methylated are more unstable, and prone to spontaneously deaminate which can ultimately lead to base substitutions (8).

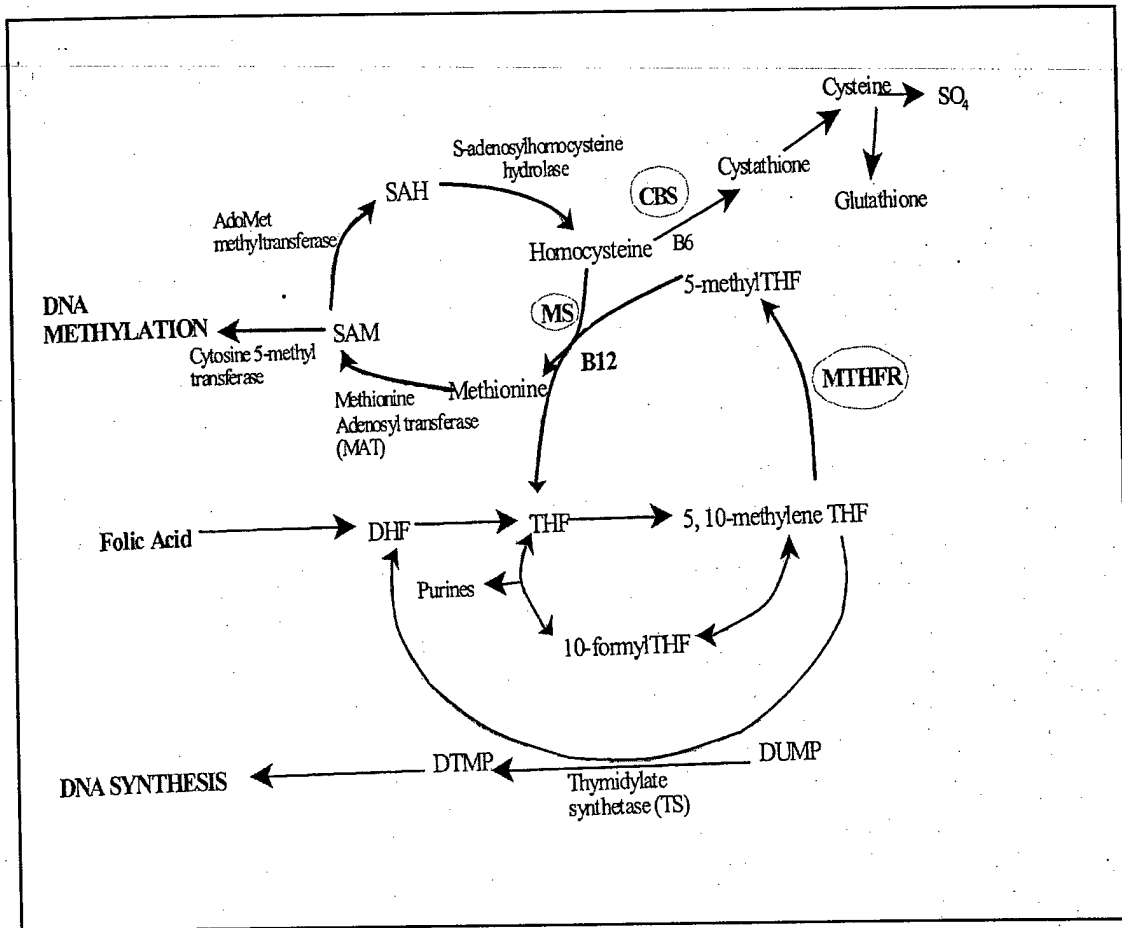


FIGURE 2.1 One-carbon metabolism pathway

Disruption, or alterations of DNA methylation, can lead to malignant transformation. There are several studies indicating that specific genes isolated from tumor tissue are either hypomethylated or hypermethylated when compared to genes found in adjacent normal tissue (20, 21). One study done by Wainfan et al.(22) suggests that gene methylation is decreased with a methyl deficient diet in experimental animals. In that study, rats were fed a methyl deficient diet (deficient in methionine, choline, folic acid, and vitamin B₁₂) that resulted in a significantly reduced level of SAM, and

hypomethylated DNA within one week; when fed this diet for a prolonged period of time, there was an overall decrease in methylation accompanied by increased gene expression. Nevertheless, these conditions were gradually reversed when their diets were restored to normal. Another study by Pogribny et al.(23) showed that an accumulation of DNA strand breaks is associated with progressive hypomethylation, and increased DNA methyltransferase activity, when rats were fed a diet deficient in methyl donors, such as choline, folic acid and methionine. A similar study was also done by Jacob et al.(24): eight postmenopausal women were given a low folate diet and their plasma homocysteine levels and lymphocyte DNA methylation later determined. The women's plasma folate levels were decreased, homocysteine levels increased, and their DNA hypomethylated; but these findings were reversed with folate repletion. This data indicates that short-term folate deficiency may alter DNA methylation composition in postmenopausal women.

While DNA methylation can lead to malignant transformation by alteration of gene expression, it is also a contributor to DNA mutation, which is also a significant factor in the etiology of breast cancer. Most CpG cytosines in the human genome are methylated to form 5-methylcytosine; this cytosine can experience spontaneous hydrolytic deamination to thymine. Cytosines can also deaminate to form uracil by DNA methyltransferase, during failed attempts to methylate the cytosine of CpG under conditions of low S-adenosylmethionine (25). Either mechanism results in a G to A transition mutation. The resulting T:G or U:G mismatch is normally recognized as a mismatch and repaired by thymine-DNA glycosylase, or uracil DNA glycosylase respectively. If the mismatch is not repaired, an adenine is paired to the thymine or uracil. During replication of the original DNA strand, a T replaces the original C of the CpG. It

has been reported by Schmutte et al.(26) that mismatched U, which occurs 6000-fold less than the mismatched T, is excised much more efficiently than T; this finding would suggest that the difference in repair efficiencies are the major source of these mutations.

Several types of mutations have been identified in tumor suppressor genes; however base substitutions are of special interest to us, particularly in *p53*. *p53* cannot be hypermethylated because it does not have a CpG rich promoter region. As such, there is no evidence of *p53* promoter region hypermethylation in breast cancer, but mutations in cytosines suggestive of deamination can be a marker for dysregulated methylation and defective DNA repair. Methylation of cytosines within the *p53* is quite common; approximately 42 CpG dinucleotides in all tissues are methylated and believed to be a promutagenic lesion (27). Most mutations in the *p53* gene produce proteins that fail to bind DNA, and, they do not activate the transcription of genes that are responsive to wild-type *p53* (28). Therefore there is unchecked tumor activity and a gain in oncogenic function by changing the range of genes whose expression is controlled by this transcription factor.

2.2 One-carbon Metabolism and DNA Synthesis

The second mechanism whereby one-carbon metabolism may contribute to increased cancer risk involves DNA synthesis and repair. Folate in the form of 5'10'methylene tetrahydrofolate (5' 10'-methyl – THF) acts as a methyl donor for the enzyme thymidylate synthase; this enzyme then converts deoxyuridine monophosphate (dUMP) to thymidine monophosphate (TMP) (8). It has been hypothesized that in the

case of folate deficiency dUMP is not methylated to TMP; this leads to an imbalance of DNA precursors and accumulation of excess dUMP that could be misincorporated into DNA in the place of thymidine (Figure 1). It is common for this mistake to occur under normal circumstances, and it is corrected by the DNA repair enzyme uracil DNA glycosylase which removes any misincorporated uracil from the DNA molecule. However, if this process repeats excessively, strand breaks, which occur as intermediates, in excision repair may destabilize the DNA molecule (29). *In vitro* studies have indicated that deficient folic acid can induce uracil misincorporation into DNA. In one study done by Wickramasinghe et al.(30) bone marrow cells were taken from 29 patients and examined for misincorporation of uracil into DNA. The results showed that there was marked increase in uracil misincorporation into DNA of patients with vitamin B₁₂ or folate deficient bone marrow cells. Another *in vitro* study using human lymphocytes cultured in decreasing amounts of folic acid showed that DNA strand breakage and uracil misincorporation increased in a time and concentration dependant manner (8).

3. KEY PLAYERS IN ONE-CARBON METABOLISM AND METHYLATION

3.1 Folate and the Risk of Cancer

Most cells require methyl groups for a range of biochemical reactions. The demand for methyl groups is not adequately met by normal dietary supply; therefore additional methyl groups are generated by *de novo* synthesis from the one-carbon folate pool. Folic acid is a water soluble B vitamin that plays a central role in one-carbon metabolism. Folic acid's function in one-carbon metabolism is considered to be very important since aberrations in DNA methylation, and disruptions of DNA synthesis and repair play major roles in carcinogenesis (31). Mammals cannot synthesize this vitamin; therefore they must obtain it from their diets. The daily requirement for folic acid in man 400µg, and the best food sources are dark green leafy vegetables, whole grain cereals, fortified grain products and animal products(5). Despite the fact that this vitamin is obtainable from a wide variety of foods, folate deficiency remains the most common vitamin deficiency reported in the US, affecting some 10 % of the general population, especially children and the elderly that live in poverty (32, 33). This deficiency is also compounded by high levels of alcohol intake. Chronic alcohol intake interferes with the absorption and subsequent metabolism of folate and vitamin B6, resulting in impaired methyl group synthesis and transfer. It also reduces the activity of methionine synthetase which remethylates homocysteine to methionine (5).

Severe folate deficiency has been associated with several conditions including megaloblastic anemia, neural tube defects in newborns (34), and coronary heart disease

by way of increased homocysteine (35). Recently, this area has been an emerging area of research for carcinogenesis, and has been implicated in the development of cancers of the cervix, lung, brain, colorectal and breast. There have been reports indicating that increased folate consumption can improve or reverse cervical dysplasia in women taking oral contraceptives (36). It has also been hypothesized that oral contraceptives cause a localized folate deficiency in the cervical epithelium which increases cancer risk; however large-scale placebo-controlled randomized trials have not confirmed this (36). Cigarette smoke has also been implicated in folate deficiency of the bronchial epithelium; it has been shown that serum folate concentrations are lower in smokers than non-smokers (37). The most convincing data so far is data linking folate deficiency and colorectal cancer. Data from several questionnaire-based trials indicate an inverse relationship between folate intake and colorectal cancer incidence (38, 39). Of the ten case-controls and eight nested case-control or cohort studies done, six case – control and six prospective studies found a statistically significant inverse relationship between dietary supplements intake, or serum folate levels and colorectal cancer (40, 41). The remaining studies showed no statistically significant relationship; however those studies were based on small numbers of cancer cases (42, 43). Furthermore, women who used multivitamins with folate for 15 or more years had a 75% reduction in colorectal cancer risk (10). Similarly, men who consumed folate by way of multivitamins for longer than 10 years had a 25% reduction in colon cancer risk (41). The inverse relationship between folic acid and cancer is further compounded by high (15g/d) alcohol intake (41) and lower methionine/protein intake (41, 44).

3.1.1 Folate and Breast Cancer

Unlike colorectal cancer, fewer studies have evaluated the relationship of folate consumption and breast cancer risk. There were eight case-control studies done(45-48), and all except two found a statistically significant inverse relationship between folate consumption and breast cancer risk for both pre- and postmenopausal women. A summary of published studies examining folate and breast cancer risk appears in Table 3.1

The largest study was done by Negri et al. (48) and consisted of 2569 pre and postmenopausal cases. They found a statistically significant reduced risk for breast cancer when women were stratified by menopausal status. A reduced risk was also found among those who consumed more than 25 grams of alcohol per day, approximately two drinks per day. There were however, a number of variables that were not adjusted for in this study such as age of first pregnancy, menarche, history of breast disease, relatives with breast cancer, body mass index and alcohol consumption.

The second largest study was done by Potischman et al. (47). That study consisted of 569 premenopausal women with *in situ* and localized disease, and 1,451 population-based controls. They noted a slightly reduced risk with high intake of vegetables, grains, or beans (OR = 0.86, 95% CI; 0.6-1.1). However, the confidence interval included 1 and no trends were seen across quartiles of increasing intake. The inclusion of vitamin supplements or dietary constituents related to these food groups such as folate, did not alter their results much. Several variables, such as a first degree relative with breast

TABLE 3.1 Summary of studies examining breast cancer risk associated with folate intake

Reference/ Design	Study	Menopausal Status	No. of cases	Folate measure	association	Relative risk/Odds ratio ^a (95% CI)
Case-control						
Graham et al., 1991		post	439	Diet	↓ ptrend 0.03	0.70 (0.48 – 1.02)
Freudenheim et al., 1996		Pre	297	Dietary Supplements	↓ NS	0.50 (0.31 – 0.82) 0.97 (0.67 – 1.42)
Potischman et al., 1999		pre	568	Diet plus supplements	NS	1.11 (0.8 – 1.5)
Ronco et al., 1999		Pre and post	400	Diet	↓ ptrend 0.01	0.41(0.26 – 0.65)
Negri et al., 2000		Pre and post	2569	Diet	↓ ↓ ↓	Pre 0.57 (0.41 – 0.78) Post 0.79 (0.62 – 0.99) ≥25 g alcohol/d 0.49 (0.32 – 0.74)
Sharp et al., 2002		Pre and post	62	Diet	NS	0.49 (0.20 – 1.20)
Beilby et al., 2004		Pre and post	141	serum	↓	0.23 (0.09 – 0.54)
Adzersen KH et al 2003		Pre and post	310	Diet	↓	0.47 (0.25 – 0.88)
Nested case-control						
Zhang et al., 2003		Pre and post	712	Diet	↓ ↓ ↓	Pre 0.65 (0.26 – 1.65) Post 0.75 (0.49 – 1.15) ≥ 15g alcohol: 0.11 (0.02 – 0.59)
Wu et al., 1999		Pre and post	195	serum	NS	0.79 (0.033 – 1.90)
Cohort						
Zhang et al., 1999		Pre and post	3483	Diet plus Supplements Multi- vitamins	NS ↓ ↓	0.93 (0.83 – 1.03) ≥ 15g alcohol: 0.56 (0.41 – 0.79) ≥ 14g alcohol: 0.74 (0.59 – 0.93)
Rohan et al., 2000		Pre and post	1469	Diet	NS ↓ ↓	All 0.99 (0.79 – 1.25) ≥ 14g alcohol: 0.34 (0.18 – 0.61) Post ≥14g alcohol: 0.28 (0.14 – 0.55)
Cho et al., 2003		pre	714	Diet	NS	1.03 (0.81 – 1.32)
Sellers et al., 2001		post	1586	Diet	NS ↑	1.33 (0.86 – 2.05) ≥ 4g alcohol ^b 1.59 (1.05 – 2.41)
Feigelson et al 2003		post	1303	Diet	NS	1.10 (0.94 – 1.29)

^a high vs. low, ^b low vs. high, ↓↑statistically significant inverse association

NS, statistically non-significant association

disease, body mass index, total energy intake, and other factors known to increase the risk of breast cancer were not matched or adjusted for.

One of the previous studies conducted on this study set by Graham et al.(45) reported that for 439 postmenopausal breast cancer cases and 494 controls, a statistically significant reduced breast cancer risk was associated with folate intake (adjusted OR = 0.70, 95% CI; 0.48– 1.02). Alcohol consumption was not considered in this analysis. One of the limitations of this study was low participation rate of both cases and controls (< 50%) which may have introduced some selection bias. However, a comparison of a fraction of those women who refused to participate to some who participated showed that there was no difference between the controls who participated and those who refused. The association of folate intake and breast cancer risk in the premenopausal women of this group was later reported by Freudenheim et al. (46). There were 297 cases and 311 controls, and there was a statistically significant reduced risk associated with folic acid (OR= 0.50; 95% CI; 0.31 - 0.82). There was no association between breast cancer risk and intake of folic acid, as supplements, and the association was no longer significant when adjusted for vegetable intake. Diet in this study was assessed by a very detailed questionnaire with questions regarding frequency and quantity of 172 foods.

The next study by Ronca et al.(49) consisted of both pre and postmenopausal women. There were 400 cases and 405 controls involved in this study and diet was determined by a food frequency questionnaire of 64 food items. An inversely associated risk was seen with high folate intake (OR = 0.70, 95%CI; 0.46 – 1.07), but the confidence interval included one. When total vegetable intake was considered, a statistically significant inverse association was seen in the highest fourth quartile when compared to

the lowest (OR= 0.41, 95% CI: 0.26 – 0.65); this inverse association remain significant when total vegetable intake was adjusted for dietary folate intake.

A smaller study by Sharp et al.(50) involved 62 cases with invasive breast cancer and 66 cancer free controls. A non-significant inverse association was seen in women reporting the highest dietary folate intake (OR = 0.24, 95% CI: 0.20 – 1.20). Limited details on the questionnaire used to collect dietary information were provided, and no information was collected on alcohol consumption.

A recent case-control study by Beilby et al. (51) consisted of 141 cases and 109 age-matched controls. Unlike the previous case-control studies, folate levels were determined by a competitive immunoassay, using folate binding protein. In this study, like most mentioned so far, folate showed a statistically significant inverse association with breast cancer risk when the highest quartiles of serum folate was compared to the lowest (OR = 0.23, 95% CI: 0.09 – 0.54) (51). One limitation of this study is the time at which blood samples were collected for folate analysis. Blood was collected immediately after diagnosis, where the presence of cancer could have influenced the eating habits of cases. This study also suffered from low participation rates; therefore it may have been biased by recruitment of controls that have a special interest in their diet and higher folate concentrations than the average community concentrations.

Prospective studies are recognized as studies that are community based and may lack some of the potential biases of case-control studies (particularly recall bias). Of the two nested case-control studies within cohorts, one reported no association between higher concentrations of folate and reduced breast cancer risk (52). The second more recent study by Zhang et al.(53) reported a statistically significant inverse association

with higher plasma levels of folate among pre- and postmenopausal women (OR = 0.72, 95% CI: 0.49 – 1.05), particularly those that consumed more than 15 g/day (OR = 0.11, 95% CI: 0.02 – 0.59).

Of the five cohort studies, three reported no direct association between folate and breast cancer risk, however when alcohol consumption was taken into consideration there was an inverse association between folate consumption and breast cancer risk. In a large cohort study of 3483 cases, no relationship was seen between total folate intake and the overall risk of breast cancer; but when folate associated with multivitamin use was considered, along with alcohol consumption in amounts greater than or equal to 15 grams per day, there was an inverse association noted for women who regularly consumed alcohol (OR= 0.56, 95% CI: 0.41 – 0.79) (54).

A second large cohort study of 1469 cases observed a statistically non-significant inverse association with dietary folate and breast cancer among pre and postmenopausal women (OR = 0.99, 95% CI; 0.79 – 1.25). That inverse association became significant when women consuming 14 or more grams of alcohol per day were considered (OR= 0.56, 95%CI; 0.41 – 0.79), particularly among postmenopausal women (OR = 0.28, 95% CI; 0.14 – 0.55)(55).

Like the previous cohort studies, Sellers et al.(12) did not detect an inverse association with low folate among postmenopausal women with breast cancer who were non-drinkers. However among drinkers there was a significant increased risk for those who consumed more than 4 grams of alcohol per day (OR = 1.59, 95% CI: 1.05 – 2.41).

Feigelson et al.(56) reported an increased breast cancer risk among women consuming 15 or more grams of alcohol per day, but found no association between risk of

breast cancer and dietary folate, total folate, and multivitamin use. Another study by Cho et al. (57) did not find any evidence indicating that higher intakes of folate reduces breast cancer risk.

In summary, six case-control studies and four prospective studies provided evidence of a statistically significant inverse association of folate and breast cancer risk. Five of those studies (one case-control and four prospective) considered alcohol use and reported an increased breast cancer risk with high alcohol intake (15g/day) and low folate use. These data suggest that higher intake of folate lowers breast cancer risk, and moderate alcohol intake in excess of 14 g/day increases breast cancer risk particularly in cases on low folate intake.

3.2 Vitamin B₁₂

Vitamin B₁₂, like folate, is one of the one-carbon nutrients important in DNA methylation and synthesis; it is water soluble and only found in animal products such as meat, poultry, fish, eggs and milk. It is noteworthy that these are very different food sources than the food sources for folic acid. This vitamin serves as a co-enzyme in the metabolism of fat and carbohydrates and in protein synthesis (5). Since vitamin B₁₂, like 5-methylTHF, is required for the methylation of homocysteine to methionine (Figure 1) one would expect that a deficiency in vitamin B₁₂ would also result in chromosome breaks by the same uracil misincorporation mechanism found in folate deficiency (58). In fact when cells are deficient in vitamin B₁₂, homocysteine levels are increased, and tetrahydrofolate remains methyl-THF. Stagnant methyl-THF results in a reduced methylene-THF pool, which is required for methylation of dUMP to dTMP, hence the

accumulation of uracil in DNA. Several studies of healthy elderly men and young adults showed that increased chromosome breakage was associated with low dietary intake of either B₁₂ or folate, and increased homocysteine levels (59, 60). Data from two studies show that increased chromosome breakage was associated with a B₁₂ and folate deficiency or elevated homocysteine levels (59, 60). These findings suggest that vitamin B₁₂ and folate may be working synergistically.

3.2.1 Vitamin B₁₂ and Breast Cancer

The association of vitamin B₁₂ and cancer risk has not been extensively investigated. There are two well known studies. The first is a nested case-control study of 32,826 women conducted between 1989 and 1990 and followed through 1996 for the development of breast cancer. In this study Zhang et al. (61) found that higher plasma vitamin B₁₂ was associated with reduced breast cancer risk in premenopausal women who were in the highest quartile of vitamin B₁₂ levels (RR = 0.36, 95% CI; 0.15 – 0.86). This observed association was not seen in postmenopausal women (RR = 1.08, 95% CI; 0.70 – 1.67) and did not differ substantially for those women who consumed at least 12g /day alcohol.

The second study was also a nested case-control study by Wu et al.(52) and consisted of 195 cases and controls selected from another study on environmental risk factors and breast cancer. An increase in breast cancer risk was observed among postmenopausal women in the lowest fifth of distribution of vitamin B₁₂ compared to the higher four-fifths, but the confidence interval included one (OR = 2.25, 95% CI; 0.86 – 5.91). Alcohol consumption was not considered in this study.

3.3 Vitamin B₆

Vitamin B₆ is a water-soluble vitamin found in a variety of plant and animal products. Whole grain bread and cereals, bananas, liver, fortified breakfast cereal and green beans are also a rich source of B₆ and have been associated with reduced breast cancer risk (58). Since there is no convincing evidence pointing to the specific constituents of these food items that is responsible for reduced breast cancer risk, and vitamin B₆ has several biological roles that are important in cancer, it might be important to examine the joint association of this nutrient and polymorphisms of genes involved in the folate metabolism pathway to breast cancer risk. Vitamin B₆ is an important one-carbon nutrient that works intricately with folic acid. A B₆ deficiency results in a decreased enzyme activity of serine hydroxymethyl transferase. This enzyme supplies the methylene group for methylene-THF. If the methylene-THF pool is disrupted by a B₆ deficiency, there is uracil misincorporation during DNA synthesis with associated chromosome breaks (62). Vitamin B₆ is also involved in the transsulfuration pathway where homocysteine is combined with serine to form cystathionine. This reaction is catalyzed by cystathionine β -synthase, a vitamin B₆ dependant enzyme (63). One would expect that a deficiency in this vitamin would have the same effect as a deficiency in B₁₂ or folate. A vitamin B₆ deficiency results in reduced DNA synthesis and impaired DNA repair (64). Further, a disruption of any of these reactions could lead to an imbalance of methyl groups and ultimately to impaired DNA methylation. Vitamin B₆ is also involved in the catabolism of homocysteine to cysteine, which is a main component of glutathione. And glutathione is a main cofactor of glutathione S-

transferases and glutathione peroxidases which have important roles in the detoxification of carcinogenic compounds (65).

There are several studies indicating an inverse association between vitamin B₆ deficiency and cancer risk, but some results are conflicting (52, 66, 67). Vitamin B₆ has shown inverse associations with pancreatic and prostate cancer (68-72). Another study reported a significantly lower risk of lung cancer in men with higher serum B₆ levels (73). Finally a large case-control study showed that serum homocysteine levels could predict the risk of developing invasive cervical cancer (72).

3.3.1 Vitamin B₆ and Breast Cancer

Like vitamin B₁₂, studies investigating the association of this nutrient to breast cancer risk are limited. Zhang et al.(53) considered this association in the same study that investigated the association of B₁₂ and found a stronger inverse association between vitamin B₆ and breast cancer risk in postmenopausal women (RR = 0.66, 95% CI; 0.43 – 1.01) compared to premenopausal women (RR = 0.91, 95% CI; 0.39 – 2.14). Both confidence intervals included unity. The inverse association seen for B₆ and breast cancer risk was stronger among women consuming at less than 15g/day alcohol (OR = 0.64, 95% CI: 0.44 – 0.93).

Vitamin B₆ association to breast cancer risk was also examined by Wu et al.(52). who examined vitamin B₁₂ and found no evidence of an association of B₆ and breast cancer risk. However, another study evaluating the association between dietary folate and breast cancer risk noted a striking inverse association for dietary folate and breast cancer, but only under circumstances where levels of vitamin B₆ and B₁₂ were high. Odds ratio

for the highest level of B₆ and the highest quintile of folate was 0.41, (95% CI: 0.23 – 0.73) (74). These data are suggestive of some interaction between folate and the cofactors vitamin B₆ and B₁₂.

3.4 Alcohol and Breast Cancer

Several studies have consistently showed that moderate to heavy alcohol consumption is associated to a moderate and statistically significant increase risk of breast cancer (75). Evidence indicates that alcohol as little as one or two drinks per day can increase risk. An analysis of data from around the world suggests that the relative risk (RR) for breast cancer increased 7% for each additional 10g of alcohol consumed daily (76). This association was observed among pre- and postmenopausal women, regardless of the type of alcohol consumed (77). Additionally, most of the prospective and one of the case-control studies, reviewed above, show that the inverse association seen with breast cancer risk is heavily influenced by alcohol consumption in excess of 14g/day (Table 1). No specific mechanism for the association alcohol and breast cancer has been established, but several have been proposed. One proposed mechanism is that alcohol increases endogenous estrogen levels; in fact a few studies have observed that pre and postmenopausal women taking oral hormone replacement therapy have higher circulating estrogens if they consume alcohol, compared with women who did not drink alcohol (78), (79, 80). Another mechanism is through the metabolism of alcohol to its metabolite, acetaldehyde which is a known carcinogen (81).

The mechanism that we are most interested in is the ability for alcohol beverages to interfere with or decrease the absorption of nutrients such as folate, vitamin B₆ and

B₁₂. Excessive alcohol drinking may also increase excretion, or interfere with the metabolism of these nutrients (82). Data produced by numerous epidemiological studies have consistently showed that cancer risk is increased in those persons who consumed inadequate amounts of folate, vitamin B₆, B₁₂ and alcoholic beverages equal to or exceeding 15g/day (52-54) (Table3.1). Lending more credibility to these findings is data that shows that polymorphic forms of genes coding for enzymes involved in the folate metabolism pathway interact with folate, vitamin B₆, B₁₂ and alcohol influencing risk. Data from two studies, Health Professionals' Follow-Up Study, and the Physicians Health Study, indicate that individuals with the methylenetetrahydrofolate reductase (*MTHFR*) 677TT variant are especially sensitive to the carcinogenic effect of alcohol (42, 83).

4. GENETIC VARIATION OF MAJOR ENZYMES INVOLVED IN ONE-CARBON METABOLISM

4.1 Gene-Environment Interactions

Breast cancer is a disease that is not purely genetic or environmental in nature, but it is a disease caused by both genetic and environmental factors. The data of several studies reviewed above suggests that diet is related to breast cancer risk. Consuming a diet rich in folate and B vitamins may be protective, but the mechanism by which it is protective is not known. Examining the metabolism of these nutrients may offer some insight into the mechanism by which these nutrients are protective. The human metabolism pathways vary with genetic makeup which is influenced by naturally occurring DNA sequence variations called genetic polymorphisms.

A genetic polymorphism is an inherited naturally occurring change in nucleotide sequence that is responsible for individual differences and is found in at least 1% of the general population. Ninety percent of these changes are a single nucleotide polymorphism or SNP. This inherited variant gene might be responsible for individual differences in susceptibility to disease. Even though the individual difference seen in risk may be attributable to heritable traits, these traits may not independently influence risk; instead they may act synergistically with environmental exposures, such as diet, modifying the effects of those exposures, hence gene-environment interactions. As seen in information provided above, some dietary exposures such as fruits and vegetables can influence breast cancer risk; however the outcome of such exposures may vary among individuals because of their genetic traits. This phenomenon is referred to as inter-individual variation. Our aim is to take this a step further, by looking at polymorphic

variants of one-carbon genes, and the interaction of those variants with one-carbon nutrients and alcohol to influence breast cancer risk. The genes responsible for these heritable traits are low-penetrance, contributing to common sporadic cancers and affecting a larger segment of the population than high penetrance genes (84). Single nucleotide polymorphisms provides a powerful tool for the investigating the role of nutrition in breast cancer risk and their integration into epidemiologic studies can contribute enormously to the definition of most advantageous diets.

Genetic mutations are different from genetic polymorphisms in that there is a change in the DNA sequence and it occurs in less than 1% of the general population. Mutations can occur within a gene preventing the synthesis of a normal protein, or they may occur within the promoter regions changing the expression levels of that protein. Either way they are rare events and affect a smaller faction of the general population than genetic polymorphisms.

Genetic susceptibility can be evaluated genotypically by assessing the genetic code or phenotypically by measuring gene expression and enzymatic function. We are measuring the *ER* expression by determining the estrogen receptor status of tumors. In this study *p53* mutations, and *p16* gene hypermethylation, which are phenotypes, can be used to determine the inter-individual inherited susceptibility to the epigenetic processes, leading to these phenotypes. The study of phenotypes such as *p53* mutations and gene promoter region hypermethylation can be very informative in the study of breast cancer etiology, exposure and genetic susceptibility because phenotypic assays have the advantage of providing information on the combined effect of genes, or genes and diet.

4.2 Enzymes Involved in One-Carbon Metabolism

One-carbon metabolism, as mentioned earlier, is a term used to describe a group of reactions that are involved in the transfer of one-carbon groups (Figure 2.1). These reactions are essential for several reasons; they are involved in the synthesis of DNA and other nucleotides, and methylation of DNA mainly at cytosine-guanine sites or CpG islands. One of our goals in this project is to examine the association of genetic variation in genes which code for key enzymes in the one-carbon metabolism pathway, to the risk of developing breast cancer. There are three important enzymes with genetic variants that are important in one-carbon metabolism: methylenetetrahydrofolate reductase (MTHFR), methionine synthase (MS) and cystathione β -synthase (CBS).

4.3 The Role of MTHFR in One-Carbon Metabolism

Several enzymatic reactions are involved in the metabolism of folate. MTHFR is a key enzyme involved in one-carbon metabolism, and it catalyzes the unidirectional reduction of 5, 10-methylenetetrahydrofolate (5, 10-methylene THF) to 5-methyl THF. This enzyme therefore determines the balance between 5, 10-methylenetetrahydrofolate and 5-methyl THF (Figure1). 5, 10-methylene THF is mainly found intracellularly; while 5-methyl THF is the predominant form of folate found in the plasma. As mentioned earlier, 5-methyl THF provides the methyl group for *de novo* synthesis of methionine. It also provides the methyl group for DNA methylation by way of methionine (40). The biochemical 5, 10-methylene THF is required for conversion of deoxyuridylate to thymidylate, and can be oxidized to 10-formylTHF for *de novo* purine synthesis (Figure

2.1). This biochemical, 5, 10-methylene- THF, is therefore critical to DNA biosynthesis and maintenance of the deoxynucleotide pool.

4.4 Polymorphisms in *MTHFR*

4.4.1. *MTHFR C677T*

The gene that codes for the MTHFR enzyme is located on chromosome 1. The complementary DNA sequence is 2.2 kilobases long and consists of 11 exons (85) and the major product of this gene is a catalytically active protein (86). Several polymorphisms exist in the gene that codes the MTHFR enzyme, and some are believed to be modifiers in the relationship between folate and breast cancer. One common polymorphism is the ⁶⁷⁷C → T, alanine → valine transition; the T variant, compared with the C results in a thermolabile protein with decreased MTHFR activity. Reduced MTHFR activity consequently increases the methylene-THF pool, and reduces the methyl-THF pool. Plasma homocysteine levels are also increased; in fact hyperhomocysteinemia is an indicator of impaired one-carbon metabolism (87). This polymorphic variant is fairly common in the North American population with an allele frequency of 35% (88) (89); it has been implicated as a risk factor for cardiovascular disease (90), Down's syndrome (91), and neural tube defects (89, 92). This polymorphism appears to be protective against the development of colorectal cancer (42, 83, 93) and acute lymphocytic leukemia (94).

4.4.2 MTHFR C677T and Carcinogenesis

Data from several studies suggest that persons with the homozygous variant (*MTHFR TT*) had a significantly decreased risk of colorectal cancer compared to those with the homozygous wild type (*MTHFR CC*) or heterozygous genotypes; however this protection appears to be extended only to those persons with adequate plasma folate levels, and low alcohol consumption. Persons with a low folate, vitamin B₆ and vitamin B₁₂, and high alcohol consumption had a marginally significant increased risk for colorectal cancer (42, 83, 93, 95). Another study demonstrated that low blood folate, vitamin B₆ and B₁₂ and methionine increased the risk of colorectal adenomas in persons with the *MTHFR 677TT* genotype (96). This mutation also increases the risk of endometrial cancers. Data analysis of a case-control study suggests a 2.9 fold increase in endometrial cancer risk in those persons with the variant genotype when compared to the wild type (97).

4.4.3 MTHFR C677T and Breast Cancer

A total of 8 case-control studies and one study of women at risk for developing breast cancer have investigated the influence of *MTHFR C677T* polymorphism on breast cancer risk. Six of those studies reported that the 677T allele is associated with an increased risk of breast cancer. Two studies reported a decreased risk associated with the 677TT genotype and one found no association. Table 4.1 provides a summary of all studies

TABLE 4.1. Summary of studies examining the relationship of *MTHFR* with breast cancer risk

Reference/ Study design	Total number of cases	Menopausal Status	Genotype	Ass.	Relative risk/ Odds ratio(95% CI)
Baruch et al. (2000)	491	Pre and post	677TT	↑	P= 0.0026
Case-control					
Sharp et al. (2002)	62	Pre- and post	1298CC 677TT and/or 1298CC	↓ ↓	0.24 (0.06 – 0.97) 0.26 (0.07 – 0.96)
Beilby et al. (2004)	141	Pre and post	677TT 677CC 677CT	NS ↓ ↓	1.37 (0.10 – 18.75) >9.0µg/L folate: 0.27 (0.09 – 0.80) >9.0 µg/L folate: 0.80 (0.01 – 0.52)
Langsenlehner et al. (2003)	500	Pre and post	677TT 677CT	NS NS	0.99 (0.68 – 1.43) 1.06 (0.82 – 1.36)
Ergul et al. (2003)	118	Pre	677TT 1298CC	↑ ↑	2.5 (1.1 – 5.5) 1.9 (1.002 – 3.9)
Lee et al. (2004)	189	Pre and post	677TT 677TT	NS ↑	1.7 (0.8 – 3.2) <1/week green veg. 5.6 (1.2 – 26.3)
Shrubsole et al. (2004)	1144	Pre and post	677TT 677CT 677CC 1298CC	↑ ↑ ↑ NS	2.51 (1.37 – 4.60) 2.17 (1.34 – 3.51) 1.94 (1.15 – 3.26) 1.94 (1.23 – 3.05) *P trend 0.18
Semenza et al. (2003)	105	Pre Post	677TT 677TT	↑ NS	2.8 (1.02 – 7.51) 0.8 (0.4 – 1.4)
Campbell et al. (2002)	335	Pre	677TT	↑	1.66 (1.12 – 2.41)

NS non- significant association, ↑ statistically significant increased risk
↓statistically significant decreased risk

examining the relationship of *MTHFR* with Breast cancer risk and following is a detailed review of those studies. The first study, conducted in Southampton UK, consisted of 233 healthy women as controls, and 235 women diagnosed with breast cancer before the age of 40, with bilateral breast cancer or a family history of breast cancer. No epidemiological data regarding descriptive characteristics and breast cancer risk factors such as reproductive factors, smoking habits, and body mass index were collected; however the mean age of both cases and controls was approximately 38 years. Instead of using the regression model, odds ratios were calculated using 2x 2 tables without adjustment for possible confounders. The *MTHFR* genotypes were determined by PCR and RFLP, and dietary folate, alcohol, vitamin B₆ or B₁₂ were not considered in this study. This study was quite limited in that they did not collect any epidemiological data regarding diet or descriptive characteristics. Therefore, no adjustments were made for possible confounders. It also focuses on young women with breast cancer, which is not common and might be different from sporadic breast cancer. Despite a few limitations, this study found that the 677T allele was more commonly observed among breast cancer cases diagnosed before age 40 than among controls (OR = 1.64, 95% CI: 1.12 – 2.41) (98).

The second study was a case-control study conducted in Turkey by Ergul et al.(99). This group examined the role of *MTHFR* C677T and A1298C polymorphism in breast cancer patients and found that the *MTHFR* 677T genotype had a 2.5 fold increased risk for breast cancer (OR = 2.5, 95% CI: 1.1 – 5.5). This was a small study with 118 premenopausal women with sporadic breast cancer as cases and 193 controls recruited from the same geographical region. DNA for genotyping was obtained from whole blood

and *MTHFR* polymorphisms were determined by polymerase chain reaction (PCR) and Restriction fragment length polymorphism (RFLP). No descriptive characteristics were presented, but the odds ratio and 95% confidence interval were computed using conditional logistic regression. However, the authors did not give much statistical detail of their findings and they did not, based on the information presented in the paper, compare the descriptive characteristics of their cases and controls. The joint association of folate or any other one-carbon nutrients, and *MTHFR C677T* with breast cancer risk was not examined (100).

The next study was a hospital based case-control study conducted in South Korea by Lee et al.(101). In that study there was a 1.7 fold increased breast cancer risk associated with the 677TT genotype, but the confidence interval included one (OR = 1.7, 95% CI: 0.8 – 3.2). When green vegetable consumption was taken into consideration, that risk went up to 5.6-fold (OR = 5.6, 95%CI: 1.2 – 26.3), but a test for interaction proved non-significant (p for interaction = 0.96). The cases in this study (189) were described as women with a first diagnosis of histopathologically confirmed, incident breast cancer, and for whom a blood sample was available. The control subjects (189) were individuals with no previous history of breast cancer and were recruited from the same hospital. Cases were frequency matched by age to controls. Epidemiological data was collected on demographic and several descriptive characteristics of participants. A short food frequency questionnaire administered by trained interviewers, who also collected information for alcohol consumption and consistency of five food groups. It is important to note however that the authors of this paper did not convert the diet information collected into specific nutrient consumption. Odds ratios were calculated by

unconditional logistic regression, and adjusted for possible confounding variables(102).

One limitation of this study is that folate, vitamin B₆ and B₁₂ levels were not analyzed. The authors could not evaluate the interacting effect of these nutrients and *MTHFR* on breast cancer risk. Further, even though information in alcohol consumption was collected, the overall deleterious effects of alcohol could not be fully explored. Other limitations are that this study was hospital-based, and there was no mention of quality control measures to test the accuracy of their genotyping results.

The selection of cases and controls in the next study was different from the studies mentioned so far. The cases (N = 105) were women older than 39 years of age that presented with a suspicious breast mass that was later confirmed as breast cancer. Histopathological criteria were used to stratify the controls (N= 247) into two groups of women with high or low risk of developing future breast cancer. Those women with atypical hyperplasia and proliferative disease without atypia were classified as high risk, while women without proliferative disease were defined as low risk. *MTHFR* genotypes were determined by PCR and RFLP assays, and unconditional logistic regression was used to determined risk of breast cancer for subjects with at least one variant of the *MTHFR*. The overall risk of breast cancer in this groups was not elevated (OR = 1.1; P = 0.65). But when women were stratified by menopausal status, premenopausal women had a three-fold increased breast cancer risk (OR = 2.8; P < 0.05). To confirm these findings with clinic-based controls, another group of population-based controls (61) with no history of cancer were randomly selected from county residents. This group of controls was smaller than the number of cases, nevertheless the logistic regression model with population-based controls yielded a marginally elevated odds ratio for *MTHFR* variants

(OR = 2.1; 95% CI: 1.1 – 4.1). The use of a population-based control group significantly increased breast cancer risk associated with *MTHFR* variant for premenopausal women (OR= 5.3, 95% CI: 1.2 – 22.2) and minimally for postmenopausal women (OR = 1.6, 95% CI: 0.7 – 3.4). There were substantial limitations in the study design; however the main limitation of this study is that it is a clinic-based population and not representative of the general population from which the population-based controls were selected. Another limitation is the small population-based control group, with a total of only 61 controls. Even though they were randomly selected, there is no information on if and how the controls were matched to cases. Finally, there seem to be no quality control measures in place to test the accuracy of the genotyping results.

The largest case control study was conducted by Shrubsole et al.(103) in urban Shanghai, China. This population based study consisted of 1144 breast cancer cases and 1236 controls and evaluated the two common polymorphisms in *MTHFR*, *C677T* and *A1298C*, and their effects on folate intake and breast cancer risk. An overall relationship between *MTHFR* genotypes and breast cancer risk was not detected in this study; however women with a low folate intake and who were homozygous for the *677T* polymorphism were at a substantially increased breast cancer risk (OR = 2.51, 95% CI: 1.37 – 4.60). Cases for this study were defined as women between the ages of 25 – 65 with no history of breast cancer and of the 1602 cases identified, 1459 were eligible. Controls were similar to cases except they were not diagnosed with breast cancer. They were randomly selected and frequency matched to cases by age. There were 1724 controls selected, but only 1556 were eligible. A 76-item food frequency questionnaire was used to assess dietary intakes, and blood samples collected on 1192 (82%) cases and

1310 (84%) controls. Alcohol consumption and vitamin supplements were both excluded from this study because very few women in the study consumed alcohol and data on folate content of vitamins was not available. The analysis was limited to cases and controls who were not known to consume alcohol or vitamin supplements. Genotyping for the *MTHFR C677T* and *A1298C* polymorphisms was performed using PCR-RFLP methods, and quality control samples were included in various batches of samples assayed. There was 98.5% concordance between repeated genotyping assays. Unconditional logistic regression was used to determine risk and 95% confidence interval after adjusting for all potential confounding variables. Overall this was a well designed population based case-control study.

The study by Sharp et al.(50) reported a non-significant reduced breast cancer risk among women reporting the highest dietary folate intake (OR = 0.49, 95%CI: 0.20 – 1.20) in a very small study. Risk was also reduced for women with the *1298CC* genotype compared to AA (OR = 0.24, 95% CI: 0.06 – 0.97) and compound heterozygote and homozygote variants (OR = 0.47, 95% CI: 0.11-1.92, and OR = 0.26, 95% CI: 0.07 – 0.96 respectively). This was a hospital-based case-control study of 62 cases with confirmed invasive breast cancer. The controls were women without breast cancer that were randomly selected from the registers of a hospital. Limited epidemiological data was collected on established breast cancer risk factors and a semi-quantitative food frequency questionnaire was used to collect dietary information. No information was provided on the number of items on the questionnaire. This was a poorly designed study for several reasons. First, with less than a hundred cases and controls, this was an extremely small study, especially considering that analysis was done for gene-gene

interactions. No mention was made of the statistical power, but there is a chance it was very low. Next, no information was collected on potential risk factors for breast cancer; consequently, no adjustments were made for possible confounders. There was no mention of quality control procedures to ensure accuracy of their genotyping results and no report on success rate of genotyping. DNA was extracted from mouth wash used by participants.

Baruch et al.(104) examined the frequency of the *MTHFR* polymorphism and its association with disease pattern in 491 Jewish women with sporadic or hereditary breast and or ovarian cancer and 69 asymptomatic *BRCA1* or 2 mutation carriers. They found that the 677T homozygous genotype was more commonly found among women with bilateral breast cancer and those with both breast and ovarian cancer than those with unilateral breast cancer. This study is quite different from the previous mentioned studies in that the cases were not compared to controls without breast cancer. Since this was not a population based study, the findings cannot be extended to the general population. However, given that increased risks for bilateral breast cancer and both ovarian and breast cancer were detected in persons with the 677TT genotype, these findings serve to corroborate findings of the case-control studies mentioned thus far.

In an Austrian study, Langsenlehner et al.(105) recruited 500 women with histologically confirmed breast cancer. Healthy controls were selected from two Austrian population-based screening studies and age matched to cases. There was no information on how the controls were selected. *MTHFR* genotypes were determined by PCR-RFLP methods, and even though negative controls were set up with each assay, there was no mention of any other quality control measures. The odds ratio for women with one 677-T

allele was 1.06 (95% CI; 0.82 – 1.36), and 0.99 (95% CI; 0.68 – 1.43) for women

homozygous for the 677-T allele. No epidemiological data was collected on diet or other descriptive characteristics for this study set, and statistical analysis did not adjust for possible confounders. Odds ratio was calculated, but the authors did not mention the models used in its calculation.

Unlike previous studies reviewed, a case-control study by Beilby et al.(51) found that in the highest quartiles of folate, persons with 677CC and 677CT genotype had a reduced risk of breast cancer. The odds ratio for the highest quartiles of folate ($>9 \mu\text{g/L}$) were 0.27 (95% CI: 0.09 – 0.80) and 0.08 (95% CI: 0.01 – 0.52) respectively. The variant homozygous genotype was not associated with breast cancer. Confirmed breast cancer cases (N = 141) between the ages of 30 and 84 were recruited from Perth, Western Australia and age matched controls (N = 109) that were randomly selected from the same postal code area. The overall participation rate for both cases and controls were poor, 41% and 25% respectively, and no statistical analysis was done to determine if participants were different from non-participants. Unlike most studies, the serum folate levels were determined by competitive immunoassay using folate binding protein. The *MTHFR* genotypes were determined by PCR and RFLP assays and the interpretation of the electrophoretic gels were checked separately by two scientists. The authors collected epidemiological data on several descriptive characteristics that are potential confounders, including alcohol consumption, and found that there was no significant difference between cases and controls. Nevertheless these variables were adjusted for and the odds ratio calculated by multivariate logistic regression. In addition to poor participation rate, several limitations were evident in this study. Most case-control studies assess dietary

folate intake instead of serum folate concentrations. Granted the direct folate measure is an advantage; but it is influenced by the timing of collection of blood samples. In this case blood was collected immediately following diagnosis of breast cancer, which may have influenced the diets of affected women(106).

In summary, studies on *MTHFR C677T* polymorphism and its effect on folate intake and breast cancer risk were unique and of varying quality. Few of the studies were population based; many relied on convenience samples. Selection and participation biases may explain some variations in findings. Nevertheless, most of those studies reported an increased breast cancer risk with the 677TT genotype; the remainder reported conflicting findings. One study considered folate, vitamin B₆ and B₁₂ but not alcohol in their analyses; in fact even though alcohol was mentioned it was not a part of any of the analysis.

4.4.4 Theories of *MTHFR C677T* and Carcinogenesis

It has been hypothesized that the C677T variant in the *MTHFR* gene, which results in reduced enzyme activity, extends its cancer-protective effect in folate-replete conditions by increasing the availability of 5,10-methylene-THF. Hence there is increased ease of nucleotide synthesis. However, the increased risk of colorectal cancer seen in those persons with the TT genotype and low folate status may be explained by the compromised biological methylation due to low levels of methyl-THF. Compromised methylation could in turn be a critical determinant of whether a cell becomes neoplastic or not (107) . Consistent with this hypothesis, one study by Stern et al. (107) determined genomic DNA methylation by using an established enzymatic assay that measured the

capacity of DNA to accept methyl groups *in vitro*, which is inversely related to endogenous methylation. They found that persons with the *MTHFR TT* genotype had a higher methyl group acceptance capacity when compared to the wild type (107), which suggests DNA hypomethylation in the *TT* genotype. Another study reported a strong association between *MTHFR TT* and *CT* genotypes with a low content of 5-methylcytosine in the DNA of normal tissue of cancer patients (108). It is rather clear that data collected from several studies indicate an interaction between the *MTHFR C677T* polymorphisms, alcohol consumption, plasma folate levels, vitamin B₁₂ and B₆ to modify cancer risk. These findings are new and need to be confirmed, and extended to assess the joint effects of these factors.

4.4.5 *MTHFR A1298C*

A second common polymorphism in the *MTHFR* gene is a 1298 A → C substitution which results in an alanine to glutamate substitution (109, 110). This polymorphism has a 10-33% allele frequency. A decreased *MTHFR* enzyme activity has been detected in those individuals homozygous for the C variant, but to a lesser extent than the *C677T* variant. Unlike the *C677T*, this variant does not result in a thermolabile protein, or increase plasma homocysteine levels. There is no evidence of this mutation influencing plasma folate concentrations (111, 112).

4.4.6 MTHFR A1298C and Carcinogenesis

Only one study has evaluated the role *MTHFR C677T* and *A1298C* polymorphisms play in breast cancer, and those data indicated a reduced risk of breast cancer with the *1298 CC* genotype and with compound heterozygosity (CT/AC) (OR = 0.07, 95% CI; 0.04 – 0.75) and homozygosity(TT/CC) (OR = 0.84, 95% CI; 0.43 – 1.62) (50). There are a few studies in other cancers. A study by Chen et al.(42) indicated that *MTHFR 1298 A-C* polymorphism is a less substantial independent risk factor for colorectal cancer compared to the *C677T* polymorphism. In another study, a decreased risk of acute lymphocytic leukemia was associated with the *MTHFR 1298 CC* genotype (OR = 0.07, 95% CI; 0.00 – 1.77); however a decreased risk was found in those persons who were double heterozygotes (*677CT/1298AC*) compared with *677CC/1298AA* individuals (94, 113). Additionally a case control study by Song et al. (114) revealed some association with the *1298CC* genotype and an elevated risk of esophageal squamous cell carcinoma compared with the *1298AA* genotype.

4.4.7 MTHFR A1298C and Breast Cancer

Only two studies have investigated the role of *MTHFR A1298C* polymorphism in breast cancer and they report conflicting findings. One case-control study by Ergul et al.(115), reviewed above, examined the role of both *MTHFR C677T* and *A1298C* in breast cancer patients and they found that the *C1298C* genotype had a 1.9-fold increased risk for breast cancer (95% CI; 1.002 – 3.929). The compound genotypes *T677T/A1298A*

and *C677C/C1298C* also showed an increased risk for breast cancer (OR= 4.472, p = 0.001, and OR = 2.301, p = 0.024) respectively. The second study, also reviewed above, was by Shrubsole et al.(103) and that group found no modifying effect of *A1298C* genotypes on the association of folate intake with breast cancer risk. This was the only study that examined the association of vitamin B₆ and B₁₂. No study considered alcohol consumption in the analysis.

4.5 Methionine Synthase (MS) and One-carbon Metabolism

Methionine synthase is another mammalian enzyme involved in one carbon-metabolism. This enzyme is dependent on vitamin B₁₂ and catalyzes the remethylation of homocysteine to methionine, and the concurrent demethylation of 5-methyltetrahydrofolate to tetrahydrofolate, used in nucleotide synthesis (116). Methionine synthase activity can be lost as a result of depleted B₁₂, and under such circumstance there is a build up of 5-methyl –THF, and homocysteine along with a consequential decrease in methionine. Methionine is an essential amino acid and the precursor of SAM (Figure1), and a decrease in SAM levels is expected to result in decreased DNA methylation (117). MS is also essential for maintaining adequate intracellular folate pools, and a deficiency can result in megaloblastic anemia along with neuronal dysfunction and mental retardation, hyperhomocysteinemia, homocystinuria and cardiovascular disease (118, 119).

4.6 Modifying Effect of *MS A2756G* on the Risk of Carcinogenesis

A 2756 A to G (glycine – aspartic acid) polymorphism has been reported in the *MS* resulting in lower enzyme activity (119), and elevated homocysteine (120, 121). Paz et al. (108) were able to demonstrate that persons with the homozygous genotype, *MS 2756GG*, had tumors with a lower number of hypermethylated CpG islands of tumor suppressor genes. The presence of this variant has also been associated with a lower colorectal cancer risk (108), and there was some interaction noted between the *MS 2756 GG* genotype and alcohol intake. Even though this variant allele appears to be protective against cancer, in one study of 10 cases and 21 controls, persons with the *GG* genotype who consumed one or more alcohol drinks per day had a 10 fold higher cancer risk than those who drank less (120). These findings were consistent with what was observed for the *MTHFR* genotype. Unlike the lower colorectal cancer risk seen in persons with the *MS2756 GG* genotype, one group found a higher susceptibility to malignant lymphoma (122); the reason for this discrepancy is unclear, but it has been suggested that the pattern of methylation and cell transformation may be different for different tumors. There are no reports on the effect of this polymorphism on breast cancer.

4.7 Cystathionine β -synthase (CBS) and One-carbon Metabolism

Cystathionine β -synthase along with its cofactor, vitamin B₆, catalyses the condensation of homocysteine to cystathionine; cystathionine, in turn, is a precursor for

cysteine (Figure 1). It has been reported that impaired CBS enzyme activity leads to elevated homocysteine and methionine along with decreased cystathionine and cysteine levels in both plasma and urine (123). This enzyme is the major competitor to the remethylation of homocysteine by *MS* and the production of methionine and vitamin B₆ is a necessary cofactor in this reaction.

The entire *CBS* has been sequenced and several polymorphisms have been identified (124, 125). Most of the polymorphisms identified so far are too rare to conduct epidemiologic studies with adequate power (126, 127). However a 68-bp insertion is relatively common, being present in the heterozygous state in approximately 12 % of the population (128, 129). Persons who are heterozygous or homozygous for this polymorphism have a lower fasting total homocysteine level, and decreased homocysteine responsiveness to methionine loading (128). This effect seems to be enhanced by low vitamin B₆ concentrations which fuels some speculation that the 68bp insertion is associated with higher levels of CBS enzyme activity (130).

4.8 Effects of *CBS 844ins68* on Carcinogenesis

Very few studies have investigated the role of this polymorphism in carcinogenesis. One such study done by Kimura et al. (131) looked at the polymorphisms in the methyl metabolism genes in an attempt to identify any association with transitional cell carcinoma (TCC) of the urinary bladder. They found the *CBS* insertion allele was slightly less frequent among TCC patients than controls; however there was no significant difference for any of the combined genotypes. Another study by

Paz et al. (108) analyzed polymorphisms in these genes and their association with DNA methylation; they did this by comparing all the different haplotypes that were generated by combining each separate genotype of the four alleles. Considering the polymorphisms mentioned thus far, for example the double genotype *MTHFR 677 CT + MS 2756GG* and the tetra-genotype *MTHFR-677CT + MS-2756AG + MTHFR-1298AA + CBS-no* insertion, there was some gene-gene interaction associated with low percentage of CpG island hypermethylation, however this association was only seen with risk alleles; the contribution of the haplotype was minimal. There are no reports on the effect of this polymorphism on breast cancer risk. Table 4.2 summarizes the polymorphisms selected to determine the modifying effect on one-carbon nutrients.

TABLE 4.2. Genes and Polymorphisms Selected to Examine Gene-environment Interactions

Gene	SNP/ Polymorphism	Chromosome Location	Genotype Frequency	Effect
<i>MTHFR</i>	C677T	1p36.3	1 – 20%	T/T - ↓ enzyme activity, ↓ folate levels, and ↓ colon cancer with low alcohol consumption
<i>MTHFR</i>	A1298C	1p36.2	7 – 12%	C/C and C/T – ↓ enzyme activity
<i>CBS</i>	844ins68	21q22.3	1- 3%	1- 2 insertions; ↓ homocysteine level
<i>MS</i>	A2756G	1q43	1 – 5%	Associated with ↑ blood homocysteine, G/G may ↓ colon cancer with low alcohol consumption

5. ETIOLOGICAL MARKERS IN CARCINOGENESIS

5.1 DNA Methylation

Carcinogenesis, as mentioned earlier, is a multifactorial disease with several contributing factors such as gene amplifications, gene deletions, and loss of heterozygosity, chromosomal rearrangements, epigenetic alteration and overall aneuploidy. Epigenetic alteration is a vastly common event in cancers and has attracted a significant amount of attention from researchers lately (111, 132). These changes are somewhat different from other genetic alterations in that they occur at a higher frequency in defined regions of the genome, and are experimentally reversible after treatment with certain pharmacological agents. As a result of these changes some genes are silenced or transcribed at a reduced rate (111). There are several mechanisms by which abnormal DNA methylation may be involved in the carcinogenic process. The mechanisms of most interest to this study is the C to T mutation by deamination of 5-methylcytosine to thymine, particularly in p53 genes(133). A second mechanism of interest to us is the transcriptional inactivation of tumor suppressor genes due to *de novo* methylation of CpG islands in the promoter region (111).

CpG islands are about 500 – 2000 base pair in length and are located around transcription start sites of most human genes (134, 135). Over the past several years, researchers have noted that the CpG islands of a large number of genes were unmethylated in normal tissues but methylated to varying degrees in human cancers (136). The regions most commonly affected were those that span the promoters of house-

keeping genes, and tumor suppressor genes. Under normal circumstances fully methylated CpG islands are only found in the promoter regions of silenced alleles such as imprinted genes or genes found on the inactivated X chromosome of females (137). Genes involved in cell cycle regulation, DNA repair, drug resistance, detoxification, differentiation, apoptosis, angiogenesis and metastasis have all been methylated in different cancers (138). The mechanism responsible for eliciting hypermethylation is not well understood, however because of the consequences, this is a significant alteration in the cancer genome.

5.2 Methylation Patterns in Cancer

Unlike the normal cell, the methylation pattern of the cancer cell goes through major changes; the bulk of the genome, including areas that were hypermethylated and silent regions with repetitive sequences, becomes hypomethylated while the promoter regions of certain genes become hypermethylated (139). The mechanism responsible for genome wide hypomethylation seen in carcinogenesis is unknown; however there have been several proposed possibilities, one of which is insufficient dietary folate or genetic lesions in the folate metabolic pathway. As mentioned earlier, folate deficient diets can result in genome hypomethylation and increased DNA strand breaks. Hypomethylation may contribute to malignancy by activating oncogenes (20), and latent retrotransposons (140), or causing chromosome instability (139). Even though there is limited convincing evidence for the activation of oncogenes by specific gene demethylation in cancer, hypomethylation has been reported in the body of *cMYC* and *H-RAS* oncogenes (141). An important research question is how the genome goes from total genome

hypomethylation to hypermethylation of some CpG islands. The DNA methyltransferases, DNMT1, DNMT3a, DNMT3b, DNMT3L and DNMT2, are enzymes involved in DNA methylation. However only DNMT1, which is involved in methylation maintenance, and DNMT3b, involved in *de novo* methylation, has increased enzyme activity in solid and hematological malignancies (142). One study showed that the over expression of DNMT1 was accompanied by increased *de novo* methylation of endogenous genes, however only a subset of genes succumb to aberrant methylation. This suggests that some CpG islands may be prone to more *de novo* methylation than others(143, 144).

Results obtained through studies done on genome-wide and candidate gene methylation indicates that each tumor type has a specific set of genes that is susceptible to methylation (145). In breast cancer CpG hypermethylation is implicated in the loss of a variety of critical genes expression. These genes fall into several broad categories including cell cycle regulation, steroid receptors, tumor susceptibility, carcinogen detoxification, and cell adhesion (146). For this study we had planned to determine the methylation status of four genes involved in breast cancer, however because of technical difficulties we choose to only include the assay that could be validated. Although these genes are involved in several different pathways, their selection was not influenced by their roles. Instead, genes were selected to reflect a wide spectrum of methylation incidence in breast cancer; for instance we wanted genes that represented the lowest, intermediate and the highest incidence of methylation. Table 5.1 summarizes the genes for which promoter region methylation status were determined.

TABLE 5.1. Genes selected for the determination of methylation status

Gene	Function	Chromosome Location	Methylation Frequency (%)
<i>P16</i>	Cyclin-dependant kinase inhibitor	9p21	17 - 48
<i>BRCA1</i>	DNA damage repair	17q12-21	15
<i>ERα</i>	Steroid receptor	6q25.1	15 - 60
<i>E - cadherin</i>	Epithelial cell-cell adhesion	16q24	50

5.3 Genes Selected for Promoter Region Hypermethylation Determination

5.3.1 *p16*

This is a tumor suppressor gene whose protein is a cyclin-dependent kinase inhibitor (147). It regulates the transition from G₁- to S-phase by blocking the transcription of important cell-cycle regulatory proteins in G₁ phase. The lack of p16 protein activity prevents cell-cycle arrest giving cells a selective clonal advantage. Loss of p16 function can result from homozygous deletion, point mutation and methylation of the promoter region of that gene. Homozygous deletion and methylation are common features of many cancers (148, 149), however in breast cancer *p16* mutations are uncommon (150, 151). Methylation of the 5' region has been reported in several human breast cancer cell lines as well as 20-30% of primary breast cancers (152). The

methylation of the *p16* is associated with loss of expression, and decreased p16 protein levels (153). An interesting finding by Foster et al, (154) indicated that inactivation of p16 in human mammary epithelial cells (HMEC) is associated with progressive methylation of the *p16* promoter CpG island, which in turn allows HMECs to escape the M0 check point. These findings would indicate that CpG methylation together with *p16* silencing is a possible contributor to breast tumorigenesis. Data collected from a study done by Hui et al. (153) indicate that high expression of *p16^{INK4a}* and reduced expression due to *de novo* INK4a methylation are common events in primary breast cancers. Also, a high *p16^{INK4a}* mRNA expression was associated with high tumor grade, axillary lymph node involvement, ER negativity, and an increased risk of relapse.

Loss of p16 expression is one of the most common abnormalities observed in human cancers, and, as mentioned above, is normally related to *de novo* methylation of the CpG island in the promoter region. Several studies (reviewed above) have provided data suggesting that a number of cancers are inversely related to dietary folate consumption by way of the one-carbon pathway. We know that inadequate one-carbon nutrients such as folate can interfere with the production of SAM, resulting in impaired DNA methylation. Yet, few studies have explored the effects of dietary folate, B vitamins and alcohol on promoter hypermethylation of the p16 gene.

There is one study by Engeland et al.(155) that looked at the association of DNA hypermethylation of several genes, including *p16*, with folate and alcohol intake in colorectal cancer patients. Even though their findings did reach statistical significance ($p = 0.085$), the prevalence of promoter hypermethylation was higher in patients with low methyl donor intake (folate = 187 – 215 $\mu\text{g/day}$) when compared with patients with high

methyl donor intake (folate = 215 – 255 µg/day). Low methyl donor patients also had a better chance of having at least one gene methylated compared to high methyl donor patients (OR = 2.13, 95% CI; 0.89 – 5.11). Remarkably, a study by Jacob et al. (24) showed that when eight postmenopausal women were fed a diet deficient in folate to create a subclinical folate deficiency with decreased plasma folate, genome-wide DNA hypomethylation was related to dietary folate depletion. Still, another study by Stern et al. (107) investigating whether a common C677T mutation in the MTHFR gene affects genomic DNA methylation found that as red blood cell folate levels decreased, DNA methylation also significantly decreased in those with the T/T genotype ($P < 0.02$). These findings support the theory of genome-wide hypomethylation and rebound promoter region hypermethylation as a result of inadequate SAM production due to folate depletion. Another study by Paz et al. (108) addressed this matter by genotyping a group of 233 patients with colorectal, breast and lung cancer, for variants in *MTHFR*, *MS*, *CBS* and analyzed their association with DNA methylation parameters. They found that carriers of the *MTHFR* 677T allele had low levels of 5-methylcytosine in their genomes ($p = 0.002$) and their tumors were not severely hypomethylated. They also found that persons homozygous for the *MS* 2756G allele had a lower number of hypermethylated CpG islands of tumor suppressor genes ($p = 0.029$).

It is noteworthy that methylation of *p16* promoter sequences occur in normal mammary tissue of healthy cancer free women as well. One study by Holst et al. (156) used a sensitive MSP assay to determine the p16 promoter methylation status in DNA isolated from histologically normal mammary tissue sections, and detected methylated p16 promoter sequences in 7 out of 15 women. The significance of this finding is not

known, but it is quite possible that *p16* hypermethylation is an early event in sporadic breast cancer.

5.3.2 *BRCA1*

BRCA1 codes for a protein that is expressed in numerous tissues. The murine equivalent to the human, *brca1*, is also expressed in a wide variety of tissues, including breast, during embryogenesis; but its expression becomes more tissue specific after birth (157). The mRNA levels of *brca1* increases during puberty, but it decreases during lactation. This expression pattern of *brca1* suggests a link to the regulation of cellular proliferation (158), and is supported by observations that the human *BRCA1* mRNA expression is low in cells arrested in G₀ or early G₁ phase and highest at the G₁-S phase transition (159, 160). *BRCA1* may also play a role in repairing oxidative DNA damage, hence maintaining integrity of genetic material (158). The protein that this gene codes for interacts with several proteins including p53 (158), which increases in response to DNA damage (161). This finding supports the idea that *BRCA1* may be involved in repairing DNA damage. In addition it has been reported that p53 point mutations and LOH are more commonly seen in breast cancers from *BRCA1* mutation carriers (162, 163). Inhibition of *BRCA1* expression increases the proliferation of normal and malignant cells, while over-expression of wild type *BRCA1* suppresses MCF-7 breast cancer cell tumorigenesis in mice (146). Furthermore, the expression of *BRCA1* is reduced or undetected in the majority of high-grade ductal carcinomas which suggests that the absence of this gene product contributes to the pathogenesis of sporadic breast cancers (164).

There is a high degree of loss of heterozygosity (LOH) at the *BRCA1* locus in sporadic breast and ovarian cancer; however somatic mutations of this gene have not been noted (165). DNA methylation has been proposed as the alternative mechanism to inactivate this gene; in fact *BRCA1* promoter region methylation was found in about 13% of sporadic breast cancers and, as expected, the promoter region of *BRCA1* in all normal tissues examined as well as 21 breast cancer cell lines were all unmethylated (166, 167). In one study done by Esteller et al. (167) RT-PCR revealed that unmethylated breast cancer cell lines and breast cancer xenografts all expressed BRCA1; however, expression was abolished in breast cancer xenografts that were completely methylated at the *BRCA1* promoter region. Furthermore, *BRCA1* methylation only occurs in breast and ovarian cancer, which suggests a tissue-specific event (146).

5.3.3 Estrogen Receptor α (ER)

ER is a member of the of the steroid hormone super-family. These proteins bind their ligands (estrogens) with high affinity and specificity. Tumors that lack ER also lack ER gene expression, however this is not a result of mutations within the *ER* (168, 169); in fact deletions, mutations or polymorphisms are never seen within the *ER* (170, 171). One mechanism that has been proposed and examined by numerous researchers is methylation of the cytosine-guanine-rich areas or CpG islands in the 5' regulatory region, and first exon of this gene (172). One group found that cultured ER negative cell lines have demonstrated extensive methylation of that gene's CpG island, (148) and treatment with the demethylation agent 5-aza-2'deoxyctidine resulted in the production of functional ER protein (173). Methylation of *ER* CpG islands was also demonstrated in

25% of ER negative primary human breast cancers, but not in normal tissue (168, 174),(175) ; indicating that methylation does result in loss of ER receptor expression. Nass et al. (176) found that CpG island methylation was evident in all tumor stages and showed similar increases during progression from DCIS to metastatic tumors. The loss of ER receptors has also been associated with poorly differentiated tumors and poor prognosis (171, 176).

5.3.4 *E-cadherin (CDH1)*

E-cadherin is a large glycoprotein with a large extracellular domain that interacts with E-cadherin molecules on adjacent cells, and in so doing establishes adhesion between epithelial cells. This protein also has a short conserved cytoplasmic domain, which interacts with a group of proteins called catinins; these proteins are responsible for anchoring E-cadherin to the actin cytoskeleton of cells. It is believed that E-cadherin suppresses the invasive quality of transformed tumor cells, from tumor cell lines, in *in vivo* tumor model systems (177). Changes in normal expression pattern of *E-cadherin* have been found in several human cancers. In breast cancer this change results in loss of differentiation characteristics, cells become invasive, there is increased tumor grade, metastatic behavior and poor prognosis (178). The mechanism by which E-cadherin is lost is unclear, however several studies have indicated that classical mutation and deletions play a role in loss of gene expression (179, 180). Loss of *E-cadherin* expression has also been associated with aberrant CpG island methylation in primary human breast

tumors (181) and there is some indication that aberrant methylation of *E-cadherin* begins before invasion and increases with metastatic progression (182).

5.4 *p53* Mutations

5.4.1 Normal Function of *p53*

So far, genetic variation as a tool for elucidating the mechanisms of effect of dietary exposures on cancer risk has been the focus; however added clarification of these mechanisms can come from studying etiological markers in carcinogenesis. Therefore the mutational spectra of *p53* are of particular interest. *P53* is a tumor suppressor gene that codes for a phosphoprotein that is expressed at low levels in normal cells (183).

Whenever there is physical or chemical DNA damage, *p53* responds by arresting cell cycle progression in the late G1 phase of the cell cycle so that DNA can be repaired before replication, or it induces apoptosis, leading to cell death (184, 185). If *p53* becomes nonfunctional due to mutation or other means, the DNA repair or apoptosis pathway also becomes nonfunctional, and as a result there is inefficient DNA repair and the emergence of cells that are genetically unstable (186, 187).

5.4.2 *P53* Mutations and Carcinogenesis

The *p53* gene is mutated in most human cancers (183). The major mechanism for *p53* inactivation is either deletion or point mutation, but there is no evidence of promoter region hypermethylation in breast cancer (188). Interestingly, methylation of cytosines within the gene is quite common and, as mentioned earlier, is considered to be a

promutagenic lesion. Of the over 1500 published reports of *p53* mutations in various types of human cancers, most occur between exons 5-8 (189). This region is highly conserved in vertebrates and contains the DNA binding domain that is essential for *p53* functional activity (190). Exons 5-8 are also very sensitive to point mutations which change its highly ordered three-dimensional conformation, and interfere with its DNA binding specificity.

5.4.3 Mechanisms of P53 Mutations

P53 mutations can develop as a result of endogenous mutagenic mechanisms or exogenous mutagenic agents. There is strong evidence linking certain environmental exposures to particular mutations in *p53*. For instance, mutations in codon 249 have been linked to aflatoxin exposure in liver cancer (191, 192). There is also strong evidence linking ultra violet light to CC to TT double base changes mutations in dipyrimidine sites (193), and cigarette smoke to G:C to TA transversions (194). Although *p53* is mutated in only 25% of breast cancers, no environmental exposure has been conclusively linked to *p53* mutations in breast carcinoma.

Another mechanism of *p53* mutations is the spontaneous deamination of cytosines and 5-methylcytosine residue to uracil and thymine; if this deamination is not repaired it results in a G: C → A: T transition. Most of these deamination mutations occur at CpG dinucleotides that are frequently methylated (195). Interestingly, most *p53* mutations in breast cancers occur at the cytosine and 5-methylcytosine site and they are G: C → A: T transitions.

5.4.4 Diet and p53 Mutations

Few population-based studies have looked at diet as a factor related to p53 mutations. One breast cancer study has examined the p53 mutational spectra in relation to several risk factors including alcohol consumption(196). Detailed information on the participant's alcohol consumption was not provided, yet this study found no association between p53 mutations and alcohol consumption. Another case-control study evaluated associations between p53 mutations in colon cancer with diet and lifestyle factors(197). Detailed diet and lifestyle information was collected by trained certified interviewers. Participants were asked about their diet two years before diagnosis (cases) or selection (controls). The authors reported that cases with p53 mutations were more likely to consume a Western diet compared to controls (OR = 2.03, 95% CI: 1.53 – 2.69) than were cases with wild type p53 (OR = 1.57, 95% CI: 1.20 – 2.06). Specific components of the Western diet that was most strongly associated with p53 mutations were diets high in sugar, red meat and trans-fatty acid. Other dietary factors such as folate were not associated with overall p53 mutations. Vegetables, fruits and alcohol consumption were not considered in this study.

5.4.5 Diet and p53 Mutations in Breast Cancer

To date no study has examined genes coding for enzymes related to one-carbon metabolism to determine if these factors are related to p53 mutations in breast cancer. We will take the previous analysis a step further to determine if polymorphic genes coding

for key enzymes in the one-carbon metabolism pathway have a modifying effect on *p53* mutations.

5.5 Estrogen Receptor

The estrogen receptor is a member of the steroid hormone super-family. It is a transcription factor, and in the presence of estrogens binds DNA and regulates the expression of estrogen-responsive genes. This receptor exists in ER α and ER β isoforms, and they are both described as ligand-dependant nuclear transcription factors. ER β was only recently discovered in 1995, and initial studies indicate that it is expressed in luminal epithelial and myoepithelial cells (198) as well as in fibroblasts and stromal cells in normal breast tissue (199). There is some suggestion that ER β might interact with and negatively modulate the actions of ER α (200), however the function of this receptor remains unclear and further studies are needed to establish a more distinct role. ER β is not being considered in this study.

5.6 Estrogen Receptors in Breast Cancer

The ER status is one of the most widely used factors in the evaluation of breast cancer prognosis, which essentially represents ER alpha. It plays a very important role in breast cancer because its presence in primary tumors indicates a potential for response to endocrine therapy, although 40-50% of ER positive patients do not respond to therapy (201). These tumors usually metastasize and become resistant to anti-estrogen therapy,

which often occurs in the clinical course of cancer progression. ER is expressed in a minority of cells in most normal breast tissue. (202) Also, pre-menopausal women have a lower proportion of ER positive cells (20%) than postmenopausal women (50%). Research data indicate that nearly all pre-malignant breast lesions express high levels of ER, (203, 204) and there has been some suggestion that this may in fact contribute to their increased proliferation by allowing them to respond more effectively to estrogen (202). Still, the mechanisms responsible for the change in proportion of ER remain unclear.

Two hypotheses have been proposed for the relationship between ER-positive and ER-negative breast cancers. One hypothesis suggests that ER-negative breast cancers results from the lost ability to produce estrophilin during clonal evolution of estrogen receptors in ER-positive cancers (205, 206). The second hypothesis considers ER-positive and ER-negative breast cancers different, with different risk factors profiles. We believe the second hypothesis is true and that ER expression is lost due to DNA methylation of the gene.

5.6.1 Diet and ER in Breast Cancer

Some evidence indicates that folate plays a role in DNA methylation, and that hypermethylation of the promoter region of *ER* and is associated with reduced ER expression (175). As mentioned above, several studies have also reported an increased risk for breast cancer associated with low folate and high alcohol consumption. What is interesting is that acetaldehyde, a primary metabolite of alcohol, has been shown to

inhibit methionine synthetase activity *in vitro* (207). It is quite possible that the inhibition of methionine synthetase in addition to low folate could lead to hypermethylation of the estrogen receptor gene. Even though alcohol has been reported as a risk factor for tumors lacking receptors for ER and progesterone receptors (PR) (208), studies examining interaction by hormone receptors are largely lacking.

There was one study by Hislop et al. (209) involving pre and postmenopausal women with ER negative and positive breast cancers and pre and postmenopausal controls. Participants completed self-administered questionnaires that collected epidemiological data in breast cancer risk factors and diet. The information collected on diet included information on frequency of consumption of 31 specific food items and usual eating habits in the past year. Specific nutrient content of specific foods were not determined and alcohol consumption was not taken into consideration. Limited information was provided on the methods for determining estrogen receptor status. Women were analyzed separately in three groups: all women, premenopausal and postmenopausal women. Multivariate analysis with binary and polychotomous logistic regression were used to analyze each group. Several known risk factors such as age, family history of breast cancer, age at first birth, age at menarche and history of benign breast disease, were entered into the regression. This group of authors found that consumption of green vegetables and carrots was significantly associated with the risk of ER positive ($p = 0.01$) and ER negative ($p = 0.02$) tumors, but no trends were observed across categories of these foods. They also found a strong inverse association among postmenopausal women between carrots, green vegetables and ER positive tumors ($p = 0.05$). However, none of these dietary factors were clearly associated with ER negative or

ER positive tumors and trends in risk were not observed. Also, the results for most comparisons were statistically nonsignificant.

A more recent study by Sellers et al. (210) examined the interaction of alcohol and low folate and its effect on the risk of postmenopausal breast cancer stratified by receptors for estrogen and progesterone. This was a cohort study conducted in Iowa and consisting of 41,836 licensed drivers between ages 55-69 years. A 127-item semi-quantitative food frequency questionnaire gathered information on known or suspected breast cancer risk factors, alcohol, multivitamins and supplements use. Even though the authors mentioned collecting information on folate consumption, there was no information on the source of that folate. Cancer incidence and ER/PR status were determined through annual record linkage to the Iowa cancer registry. Therefore, there was no available information on staining and scoring methods used to determine ER and PR status. This group found that alcohol was not associated with risk of any receptor defined category of breast cancer if folate intake was adequate; also folate and alcohol consumption was not associated with estrogen receptor positive tumors. Nevertheless, the most striking observation was made among women with low folate and increased alcohol consumption where there was a 2-fold increased relative risk for ER negative tumors (RR = 2.14, 95% CI; 1.18 – 3.85).

5.7 Estrogen Receptors and P53 Mutations

As mentioned earlier, the *p53* acts as a tumor suppressor and its inactivation is the most common genetic alteration in human carcinogenesis. The p53 protein maintains

genetic integrity by blocking cell replication after DNA damage until the damage is repaired or initiating apoptosis if the damage is too extensive for repair (211). Since loss of p53 function eliminates growth arrest in response to certain DNA damaging agents and in so doing gives cells a selective growth advantage, cells lacking p53 function are more resistant to ionizing radiation and some anticancer drugs. Therefore they are expected to be more aggressive clinically than cells with normal p53 function (212). Several studies have examined the prognostic and/or therapeutic implications of *p53* mutations; however the clinical value of this mutation as a prognostic tool remains controversial. Some studies indicate that *p53* mutations cannot be used to predict survival (213) while some studies indicate the contrary (214, 215). The association of *p53* mutations and loss of ER were examined in several studies suggesting that these events appear to have distinct meaning as determinants of overall survival and early recurrence. Takahashi et al. (216) investigated the additive effects of both factors on overall survival by comparing tumors with *p53* mutations and loss of ER versus those without mutations and ER. They found that *p53* mutations and ER loss act cooperatively as a strong risk factor for disease free survival; however they both have distinct roles in the prognosis of individual breast cancer patients. Another study by Zheng et al. (217) found that the presence of ER or ER mRNA was inversely correlated with the mutant p53 expression ($p < 0.05$). Several additional studies noted the same observation (213, 218), (219). These results lend support to the hypothesis that mutated p53 allows cells with damaged DNA to grow and divide, and rapidly growing cells eventually accumulate a lower amount of ER (220).

6. RESEARCH METHODS

6.1 Research Design

6.1.1. Study Population

This study utilized previously-collected data and biological samples from a case-control study of the epidemiology of breast cancer in Erie and Niagara counties in Western New York (1986-1991). Data were collected on 617 premenopausal and 933 postmenopausal women. Extensive details of the participants and the interview were reported elsewhere (46). Cases were ascertained by nurse-case finders who visited hospitals at regular intervals. They examined pathology department records to obtain names of individuals aged 40 to 85 with histologically confirmed diagnosis of primary breast cancer who lived in Erie and Niagara counties and had no previous history of breast cancer. Once the cases were identified a letter was mailed to the physician seeking confirmation of diagnosis and permission to interview the patient. After receiving the physician's approval, the patient was invited to participate in the study and to give informed consent. This procedure was approved by the Institutional Review Board of the University at Buffalo and by the IRB at each cooperating hospital.

Controls under age 65 were randomly selected from a list of New York State licensed drivers. Those 65 and over were selected from rolls of the Health Care Finance Administration. Women with a history of cancer were excluded. Controls were frequency-matched to cases on age, race, and county of residence. Women who were currently menstruating were considered premenopausal; those women who were not

menstruating because of hysterectomy or other medical intervention, or if they had at least one of their ovaries removed and were less than age 50 were also considered premenopausal. All other women were considered postmenopausal. Of all the premenopausal women contacted, 66% of eligible cases and 62% of eligible controls participated. For postmenopausal women contacted, 54% of cases and 44% of controls participated. There were no statistically significant differences ($p < .05$) in socioeconomic, hormonal, reproductive, or dietary factors between premenopausal women who participated and those who did not. However, there were slight differences among postmenopausal subjects; in controls there was a greater mean number of pregnancies (3.5 vs. 2.9, $p < .01$) for those providing blood compared to those who did not (46).

6.1.2 The Interview

The interview was conducted using a questionnaire that took approximately 2 hours to complete. The questionnaire was developed by our collaborators who have extensive experience with a number of pretests, and have conducted similar detailed interviews with over 5,000 subjects since 1975. The questionnaires were completed by participants before they came to the clinic. At the clinic, clinic staff checked the questionnaires for completeness and queried participants regarding any questionable responses or missing information.

The interview was based on portions of interviews that were developed in a number of careful pretests and in several studies. It was designed to study frequency and amount of various foods ingested over a one year period starting 2 years before the

interview. The amount ingested was determined by respondents referring to pictures of portion sizes, reference to standard measures of volume or the numbers of items or pieces (e.g. eggs, pieces of chicken, ears of corn). At the clinic, participants completed informed consent procedures, had their blood drawn and physical measurements taken.

Approximately 45% of premenopausal and 63% of postmenopausal women consented to phlebotomy. The interview collected demographic information, medical history, medications used in the last 30 days, lifetime physical activity, and diet history.

Participants completed self-administered and interviewer-administered questionnaires. Most of the questions about current exposures in the interview, for both cases and controls, refer to exposures one year in the past so that any changes in habits by cases because of illness did not influence their responses. For questions pertaining to lifetime exposures, questions referred to lifetime up to one prior year. The interview consisted of questions on lifetime alcohol consumption, diet history, history of physical activity, reproductive history, weight history, use of non-steroidal anti-inflammatory agents, residential history, family history of disease specifically cancer, occupational history, lifetime history of social support and social network, employment history, lifetime smoking history, and lifetime passive smoking history. Family history of cancer was defined as having at least one first degree relative with breast cancer. Interviews for cases with cancer were conducted approximately 2 months after diagnosis. No interviews were conducted more than one year after diagnosis.

6.1.3 Diet History

The National Cancer Institute diet history questionnaire was used in collecting epidemiological data on diet; this questionnaire has been shown to be efficient, reliable and valid in collecting such data (221, 222). This in-depth food frequency questionnaire was geared to gather information on the intake of 172 foods in the two years before the interview, and was designed to include adequate measurement of intake of those nutrients of interest to this study. The information collected included frequency and portion size as well as consumption of food in and out of season, and cooking methods. Participants indicated the frequency of food intake from nine frequency categories; they also indicated how their usual portion size corresponded to standard portion size (small, larger, the same). The nutrient composition of foods was calculated by use of data from the U.S. Department of Agriculture data tapes published food composition tables (223, 224) with updates and associated data tapes, along with tables from Paul and Southgate(225) and Pennington(226, 227). Food composition data from individual nutrients were based on data for more than 2300 fruits, vegetables and multi-ingredient foods from the U.S. Department of Agriculture (228, 229). These values were limited to nutrients found in fruits and vegetables and did not include those in animal products.

For any values that were missing, composition values were used from similar foods. An index of total vegetable intake, in grams, was calculated from each participant's interview based on questions regarding the usual intake of 31 vegetables, and total fruit intake was based on questions regarding usual intake of 21 fruits (46).

The index of vegetables did not include mixed foods that contained items such as spaghetti, lasagna and pizza.

This study also focused on life time alcohol consumption. For the assessment of alcohol intake, information on quantity and frequency of consumption of individual alcoholic beverages 2, 10 and 20 years ago and at the age of 16 was collected. Questions were asked about the usual frequency of intake and the number of drinks per occasion for wine, beer and hard liquor during those years. The total alcohol intake was calculated as the sum of the reported number of beer, wine and hard liquor. The alcohol content of one glass of beer or wine or one shot of hard liquor was assumed to be approximately the same.

There has been reasonable concern about the reliability of retrospective dietary measures; however, a number of assessments of the reliability of this specific interview process have been conducted. These assessments include concurrent interviews of spouses in regard to the subject dietary behavior as observed by the spouse. Marshall et al. (230) have shown that when interviews of 158 males in a Western New York study of cancer epidemiology were compared to their spouses estimates of their dietary history taken in separate interviews, 60-80% of the respondent pairs agreed exactly on the frequency of consumption for individual food items. Cases and controls were also re-interviewed by telephone subsequent to the face to face interview, and 3-5 years after the initial interview. Studies aimed at determining whether the retrospective report or the report of a current diet is the better indicator of past diet have shown that dietary histories as recalled from the distant past give a better estimate of a persons diet from several years in the past (231, 232). We concede that it would be risky to make point estimates based

on retrospective dietary measures; however it allows us to compare groups of cases and controls on their relative level of ingestion of various foods. Besides, cancer is mostly related to habitual dietary patterns and food frequency questionnaires are very efficient in determining habitual intakes (31).

6.1.4 Clinical Measurements

Physical measurements were made on participants in light clothes and no shoes. Measurements included height, weight, abdominal girth, waist circumference hip circumference, arm circumference, blood pressure and pulse rate. A fasting blood sample was collected from all participants who agreed to the blood draw. Samples were processed immediately and were stored in our biological specimen bank. The specimens were separated into serum, plasma, buffy-coat, and packed red blood cells and they were stored in liquid nitrogen at -196°C and at -80°C in mechanical freezers.

6.1.5 Medical History and Medication

Detailed information was collected on the participants' medical history, with date of diagnosis, for a large number of chronic conditions. In addition, information was collected on all medications, vitamins and dietary supplements that participants were currently taking.

6.1.6 DNA Extractions from Tumor Sections

Slides prepared from 418 archived tumor blocks were available for this study. The slides were cut from blocks with replacement of blades between each block. Each block holder was cleaned with xylene, between blocks, to prevent contamination of tissue from one block to another. Glass slides were treated to prevent contamination with DNAases and RNAases.

DNA was extracted from tumor tissue that was microdissected from slides, and then extracted with standard phenol methods. One five micron slide from each case was stained by the Hematoxylin and Eosin method and the tumor circled with a permanent marker by a pathologist. Those slides were then used as templates to allow for microdissection. Using the circled slide as a guide, adjacent slides were scraped with a needle to remove tumor tissue. The tumor tissue was added to 270 microliter (μl) of sterile TE buffer and digested with 0.5 mg/ml Proteinase K in SDS for 4 days at 56° C. After complete digestion of tissue, 300 μl of 1:1 phenol/chloroform was added and the mixture vortexed for 15 seconds and centrifuged for 5 minutes. This step was repeated once after transferring the clear aqueous layer to a new tube. One hundred microliters (μl) of 10M NH₄OAc was then added to the clear aqueous layer along with 5 μl of freshly thawed glycogen and 900 μl of cold 100% ethanol. After incubating this mixture at -20° C overnight it was centrifuged for 15 minutes at high speed. The liquid was poured off, 1 ml of 70% alcohol was added to the pellet and the samples were centrifuged for 5 minutes at high speed, and alcohol discarded. The pellet was dried and reconstituted in 30-50 μl of TE buffer. We were able to extract DNA from approximately 95% of all

samples and the average concentration of the DNA after reconstitution was 75ng/ul and total yields ranged from 2.25 – 3.75µg. Figure 2 gives an overview of the extraction methods used for DNA extraction from tissue.

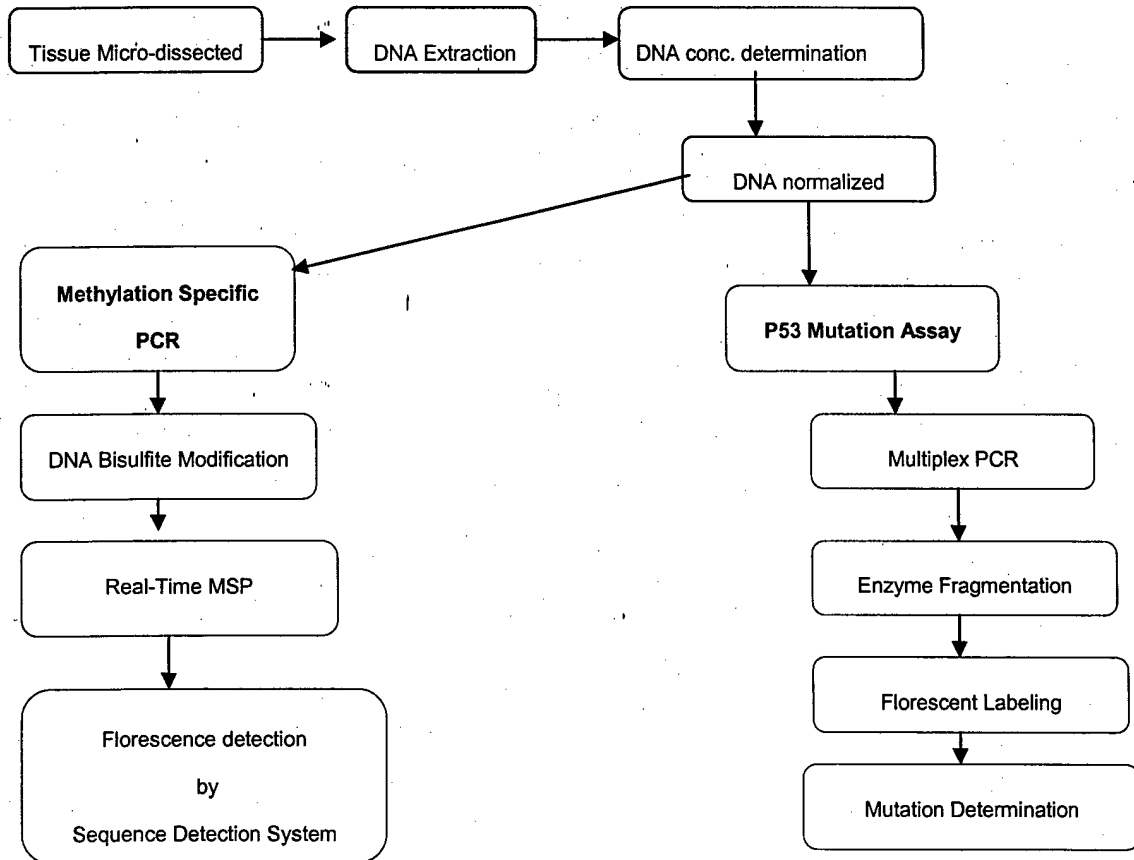


Figure 6.1 Schematic for DNA extraction for P53 mutations and methylation

6.2 Genetic Polymorphism Analysis

We obtained 810 frozen blood clots that were collected from cases and controls in 1986-91 and stored at -80°. DNA was extracted from all clots using a modified protocol provided with the GeneQuick DNA extraction kit (BBL). Briefly, blood clots were thawed at 37° C and all red blood cells lysed by incubating samples for 10 min with a blood lysis solution. Samples were then centrifuged for 10 min and the supernatant discarded. Sample lysis solution and proteinase K were then added and samples incubated at 55° for 1 hour to overnight to lyse white and all remaining cells. Protein was precipitated with a solution to remove the protein (Protein-out Solution) and DNA precipitated with 100% isopropanol. All samples were re-constituted in TE buffer. The average yield was 750 – 1250ng.

Previously established PCR/RFLP protocols for *MTHFR C677T*, *CBS 844ins68* and *MS 2756G* polymorphisms were utilized (see Table 6.1 for primer sequence and conditions) (108). Fifty nanograms of DNA were used to do 50 µl reactions. The *MTHFR C677T* Polymorphisms were identified by digesting the PCR product with *HinfI* enzyme (New England Biolabs) at 37°C overnight. The homozygote wild-type was identified by a 198-basepair (bp) band, and the variant allele was identified by 175 and 23-basepairs. *MS-A2756G* polymorphisms were identified by digestion of PCR product with *HaeIII* enzyme (New England Biolabs) at 37°C for 24 hours or overnight. The homozygote wild-type allele was identified by 189-bp fragment whereas the variant allele yields 159 and 30-bp fragments. The *CBS 844ins68* PCR assay was somewhat different in that it did not involve a digestion step. This is an insertion deletion assay where the wild type allele

or deletion allele yielded a 184-bp band and the variant allele yielded a 252-bp band. All PCR fragment were resolved on a 2% agarose gel stained with ethidium bromide.

Real-time quantitative PCR technology was used to determine the *MTHFR A1298C* polymorphism. Previously published primers, probes and conditions on SNP500 Cancer Database were closely followed for this assay (see Table 6.1). Briefly, 20ng of DNA was used to do 15ul reactions. Assay specific concentrations of primers and probes (200nM probes and 900nM primers) were used along with 7.5 µl of the 2X Universal Master Mix (Applied Biosystems). The assay specific cycling conditions published on the SNP500 web site was closely followed. In step 1 there was AmpErase UNG activation (AppliedBiosystems) for 2 minutes at 50°C followed, in step 2, by enzyme activation at 95°C for 10 minutes. The template was denatured in step 3 at 92°C for 30 seconds (if using 3'MGB quencher) followed, in step 4, by annealing at 60°C for 1 minute (this is assay specific). Finally, step 3 is repeated 49 times before holding at 4°C. At the end of thermo-cycling the plate was read by the ABI 7900 sequence detection system. The different genotypes are displayed in an allelic plot; which contained 4 distinct clusters representing Allele 1 or 2 homozygotes, allele 1 and 2 heterozygotes and all negative controls.

TABLE 6.1. PCR primers/probes used for detecting genetic polymorphisms

Gene	Polymorphism	Primers	Magnesium Conc.	Cycles #	Annealing Temp.(°C)
<i>CBS</i>	68bpIns	5'- GCCTTGAGCCCTGAAGCC (F) 5'- CGGGCTCTGGACTCGAC (R)	0.4mM	40	61
<i>MS</i>	2756A → C	5'-GAACTAGAAGACAGAAATCTCTA (F) 5'- CATGGAAGAATATCAAGATATTAGA(R)	0.4mM	35	54
<i>MTHFR</i>	677C → T	5'- TGAAGGAGAAGGTGTCTGCGGGA (F) 5'- AGGACGGTGCGGTGAGAGTG (R)	0.2mM	35	60
<i>MTHFR</i>	1298 A → G rs 801131	5'- GGAGGAGCTGCTGAAGATGTG (F) 5'- CCCGAGAGGTAAAGAACAAAGACTT (R) 5'- AGACACTTGCTTCACT (PROBE 1), FAM 5'- CAAAGACACTTTCTTC (PROBE 2), VIC	N/A	49	60

Homozygotes and heterozygotes selected from our human cell lines (Coriell Cell Repositories) were used as controls. Three positive controls and one negative control were used for PCR and enzyme restriction digests. The positive controls, one representative of each genotype, were selected from our human cell line. The negative control was the PCR reaction excluding template DNA. All assays were subjected to standing quality control and quality assurance procedures. To test the reproducibility and accuracy of our assays, a random selection 20% of our samples were repeated with each test. Further, we validated all assays by confirming polymorphic Mendelian inheritance patterns in seven human family cell lines consisting of 134 family members. Each family consisted of at least three generations. We also validated our *CBS* assay by direct DNA

sequencing. All results are read twice and verified by 2 independent researchers. Figure 3 outlines a schematic for our genotyping procedure.

6.3 Methylation Specific Real – Time Quantitative PCR for *p16*, *BRCA1*, *ERα*, and *Ecadherin*

6.3.1 DNA Modification

The DNA methylation patterns in the CpG islands of 4 genes, *p16*, *BRCA1*, *ERα*, and *Ecadherin*, were determined by real-time quantitative PCR. Before doing a PCR, the extracted DNA was subjected to chemical conversion of the unmethylated cytosines to uracil according to a previously described method (see Figure 4) (233). Briefly, 1- 2 micorgrams (μg) of DNA was denatured by adding 3M NaOH to make a final concentration of 0.3M. Samples were then incubated for 20 min at 50° C. A volume of 500 ul freshly made bisulfite/hydroquinone (2.5M sodium-metabisulfite and 125 mM hydroquinone [ph 5.0]) solution was added to each denatured DNA sample and samples were incubated, at 70° C for 1 – 3 hours in the dark.

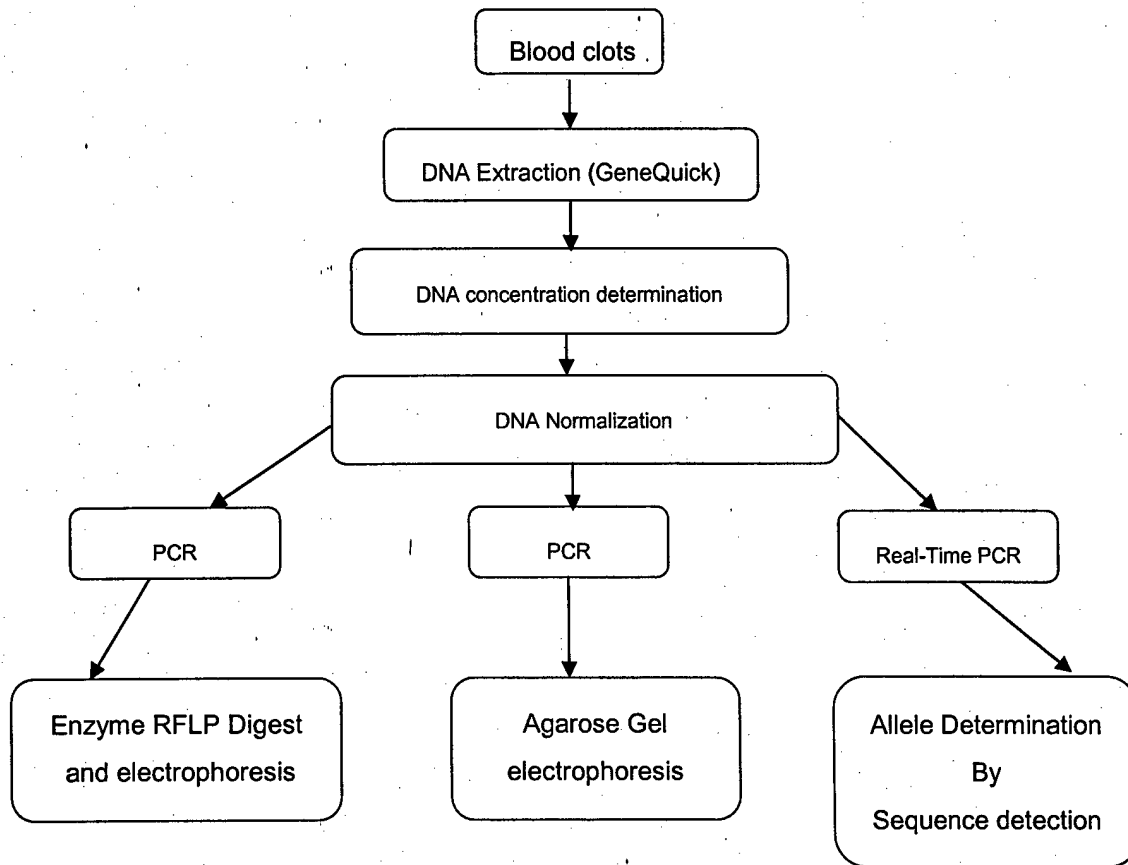


Figure 6.2 Schematic of genetic polymorphism analysis

The bisulfite modified DNA was then purified by using the Wizard DNA Clean-Up Kit (Promega Corp., Madison, WI) according to the manufacturer's protocol. A volume of 1ml resin was added to each sample and mixed thoroughly before adding to the column and applying vacuum. Approximately 2 ml of 80% isopropanol was added to samples and vacuum applied, this process was repeated twice. Samples were then eluted in 45 μ l of water preheated to 80°C. The eluted DNA was denatured for a second time in 3M NaOH for 10 minutes at room temperature; complete deamination was achieved by

adding ammonium acetate (final concentration, 1mM) and incubating at room temperature for 5 minutes. The DNA was then precipitated by adding glycogen and 2.5

UNMETHYLATED DNA

TGGACGAAATGGGCGACCCTCGCCGGCGCT

**Sodium bisulfite
modification**



TGGAUGAAATGGGUGAUUUTUGUUGGUGUT

METHYLATED DNA

TGGACGAAATGGGC^mGACCCTC^mGCCGGC^mGCT

**Sodium bisulfite
modification**



TGGAUGAAATGGGCGAUUUTCGUUGGCGUT

Figure 6.3 Principle involved in sodium bisulfite modification process

volumes of 100% ethanol. Samples were then spun at maximum speed for 15 minutes, and pellets washed with 70% alcohol, dried and resuspended in 30ul water.

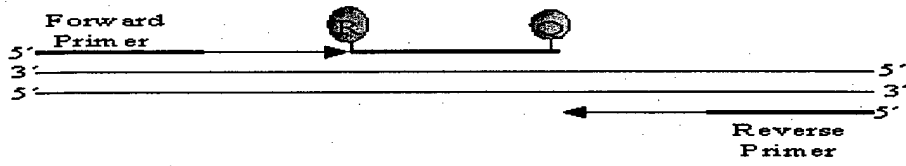
6.3.2 Real-Time Quantitative MSP

A previously described fluorescence based real-time MSP was used to amplify our sodium bisulfite modified DNA (233). All MSPs were done in 96-well plates using a PerkinElmer Applied Biosystems 7700 Sequence Detector. (PerkinElmer Corp.,

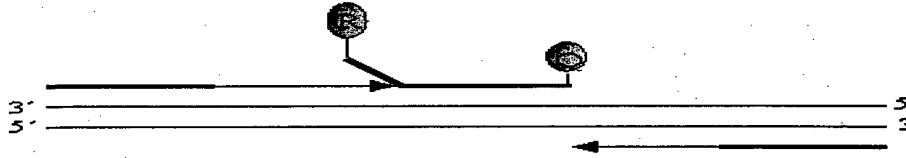
FosterCity, CA). The PCR is performed using two primers (forward and reverse) and an amplicon-specific fluorogenic hybridization probe. The probe is labeled with a 6-carboxy-fluorescein dye at the 5' end which serves as a reporter and a 6-carboxytetramethyl-rhodamine, located at the 3' end which serves as a quencher. During amplification, the 5' → 3' exonuclease activity of the Taq polymerase cleaves the reporter from the probe thus releasing it from the quencher, and the increase in fluorescence emission of the reporter dye is monitored. The increased fluorescence emission represents the number of DNA fragments generated (Figure 6.4). All primers were designed to specifically amplify the bisulfite-converted DNA within the promoter region. Probes on the other hand were specifically designed to anneal within the amplicon.

Amplification of the β -actin gene was used as an internal control and those primers and probes were designed to amplify a region of the gene lacking CpG nucleotides. Therefore, unlike the other genes, β -actin would amplify despite its methylation status. It should be kept in mind that the methylated cytosines are not deaminated to uracils after DNA modification. Rather, wild type sequence is maintained and amplification is proportional to the degree of cytosine methylation within the promoter. All probes and primers were synthesized by PE applied Biosystems (PerkinElmer Corp.) All assays were done in a 15 μ l reaction volume consisting of 600nM of each primer (forward and reverse), 200nM of probes, 2 μ l bisulfite treated DNA and 1X master mix (PE Applied Biosystems). Reactions were carried out in a 96 well plate in a 7700 Sequence Detector (PE Applied Biosystems) under the following conditions: 50°C for 2 minutes, and 95° C for 10 minutes followed by 55 cycles of 95° for 15 seconds and 60°C for 1 minute. See Table 6.2 for all primer and probe sequences.

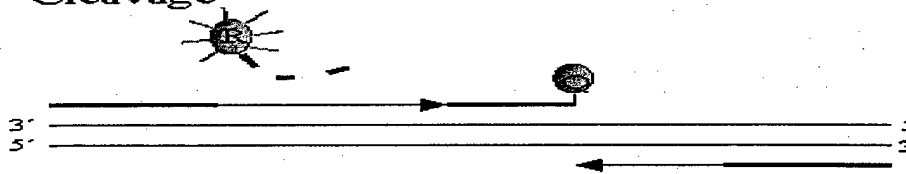
Polymerization



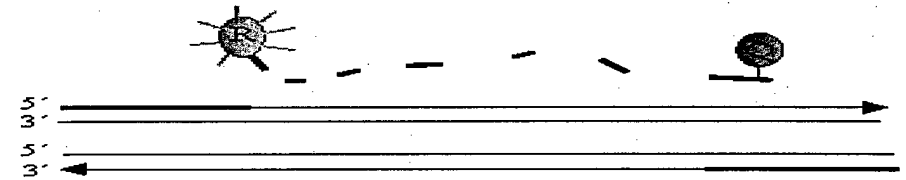
Strand Displacement



Cleavage



Polymerization Completed



R- reporter, Q – quencher

Figure 6.4 Principle involved in real-time PCR by Taq-Man

TABLE 6.2. Sequence of primers and probes for Real-time PCR

Gene	Primer 5'-3'(forward and reverse)/Probes 5'-3'	Genbank Accession	Amplicon size
<i>P16</i>	TTA TTA GAG GGT GGG GCG GAT CGC (F) AGT AGT ATG GAG TCG GCG GCG GG (P) GAC CCC GAA CCG CGA CCG TAA (R)	U12818	150
<i>BRCA1</i>	AGT CGG GTG TGG TGT CGT TT (F) FAM-CTC CAC CTC CCG AAT TCT AAC GAT TCT CCT-TAMRA ACG CGA TCT CGA CTC ACT ACA A (R)	NA	NA
<i>ER</i>	TTG TAA TGT ATA TGA GTT CGG GAG ATT AG (F) FAM-CCC TCC GCC AAC TCC TAA ACT CCC A - TAMRA ACC GAC AAC CCG ACG AAA C (R)	NA	NA
<i>Ecad</i>	AAT TTT AGG TTA AGA GGG TTA TCG CGT (F) FAM-CGC CCA CCC GAC CTC GCA T - TAMRA TCC CCA AAA CGA AAC TAA CGA C (R)	L34545	NA

6.3.3 Quality Control Procedures

As part of our quality control and quality assurance procedures all assays included a positive and negative control. One well of each reaction plate contained modified pedigree DNA which served as a negative controls. Another well contained modified CpGenome universal methylated DNA which served as a positive control (Chemicon International, Temecula CA) and several wells contained water instead of DNA (No template controls). Additionally, a few of our samples (16) were tested for *p16* methylation by traditional Methylation Specific Polymerase Chain Reaction (MSP) after being tested by real-time MSP and the results showed 100% concordance. Twenty

percent of all assays were repeated to ensure accuracy and reproducibility and also showed 100% concordance.

6.4 P53 Mutations

P53 mutation analysis on DNA extracted from archived tumor blocks for exons 2-11 was previously completed by a technician in our laboratory. The mutational spectra were identified using the Affymetrix Gene Chip System (Santa Clara, California) which analyzed nucleic acid sequences and identified nucleotide base changes. The technique involves a single multiplex PCR reaction that amplifies all exons, followed by enzymatic fragmentation and fluorescent labeling of the fragmented PCR products. These products are then hybridized onto the oligonucleotide probe array. The array contains oligonucleotide probes with the wild type p53 sequence and the most common p53 mutations. The binding of template DNA to the probe is determined with a laser scanner and evaluated with software that uses algorithmic analysis to give a valid numerical score for p53 mutations. This information was used in a prior study, along with epidemiological data to examine the association of diet and alcohol consumption in relation to p53 mutations. We used this information to determine the modifying effect of polymorphic one-carbon genes on p53 mutations in pre and postmenopausal women.

6.5 Immunohistochemistry for Estrogen Receptors

Five mm tissue sections were previously cut and mounted on glass slides. The sections were deparaffinized in xylene, and hydrated in graded alcohols (absolute, 95% and 70% ethanol for 2 min each) before rinsing in deionized water. Antigen retrieval was performed by heating slides in a citrate buffer (pH 6) at 96° C for 10 min, the slides were then cooled and rinse in phosphate buffered saline (PBS). The endogenous peroxidase activity was reduced by incubating slides with a 3% hydrogen peroxide solution for 10 min; slides were then rinsed with PBS and incubated with 1% goat serum for 10 min to reduce background staining. Subsequently, slides were incubated with 1:50 dilution of the primary anti-estrogen receptor mouse monoclonal antibody, clone ER1D5 (Immunotech, France).

The primary antibody detection was accomplished using Biotin-Streptavidin Horseradish Peroxidase detection kit (Biogenex). Slides were incubated with secondary antibody (LINK) for 20 min, and enzyme conjugate (LABEL) for 20 min with PBS washes between steps. The diaminobenzidine tetrachloride substrate (DAB) kit (Biogenex) was used to visualize the antibody-antigen complex. Slides were incubated with DAB for 3 minutes. Positive and negative controls were included with each batch stained. After immunostaining, slides were stained in hematoxylin, rinsed in deionized water, dehydrated in graded alcohols and washed in zylene before cover-slipping.

6.5.1. Assigning Scores to Tumors

The scoring method used in this study was outlined by Allred et al. (234) and was shown to have a 90 % intra- and inter-observer reproducibility. All slides were examined and scored by myself and a pathologist (Dr. Defa Tian) in our laboratory and reviewed by the a second pathologist (Balgit Singh) from the Georgetown University Hospital. A proportion score (PS), which represents the proportion of positive tumor cells on the entire slide (range 0-5), was assigned along with an intensity score (IS) that estimates the average staining intensity of positive tumor cells (range 0-3). The proportion and intensity scores for each slide were added to obtain a total score (0-8) and the average of the two sets of scores (assigned by myself and a second person) calculated to determine the final score of each slide. Tumors with a total score of 3 or more were reported as positive. This value was used in separating 800 breast cancer patients into low risk ($TS \geq 3$) and high risk ($TS \leq 3$) subsets with a 30% difference in disease free survival (DFS) at 5 years. This finding accentuates the strong predictive power of assessing ER by this method (235).

7. METHODS FOR STATISTICAL ANALYSIS

7.1 Descriptive Characteristics of Cases and Controls

The differences between cases and controls were determined by comparing the means of risk and dietary factors, for breast cancer. This was done for the entire study set as well as the subset of participants for whom we obtained blood clots. Since we only obtained blood clots on a subset of participants (25% of premenopausal and 63% postmenopausal women) in this study we wanted to ensure that the two groups were similar as far as the established breast cancer risk factors we adjusted for. We used the student *t*-test to determine the differences in mean between cases and controls with continuous variables. The chi-square test was used for categorical variables, and the *p*-value was calculated based on these test statistics.

7.2 Distribution of Genotypes

7.2.1 Calculation of Genotype and Allele Frequency

Chi-square (X^2) analysis will be used to determine the distribution of all possible genotypes of each gene (*MTHFR C677T*, *MTHFR A1298C*, *CBS 844ins68*, and *MS A2756G*) tested between cases and controls, stratified by menopausal status. Before doing this the allele frequencies will be estimated. The allele frequency refers to that specific allele of a gene in a population. Each individual has two copies of each gene, one inherited from each parent,

therefore the allele frequency of C allele in *MTHFR* C677T polymorphism would be: $C = \frac{N_{CT} + 2 N_{CC}}{2 N_{TOTAL}}$.

7.2.2 Hardy-Weinberg

The distribution of genotypes in the general population will be compared to that calculated from the allele frequencies, to determine if our study set is in Hardy-Weinberg equilibrium. If the probability of obtaining a single allele is independent of the probability of obtaining a second allele, then the population is in Hardy-Weinberg equilibrium. However, if the two probabilities are not independent of each other then the population is not in Hardy-Weinberg equilibrium. The chi-square test with two degrees of freedom was used to test Hardy-Weinberg equilibrium.

7.3 **Methods for Evaluating Risks Associated with Polymorphisms**

We will examine breast cancer risk in relation to polymorphisms found in genes coding for three enzymes involved in the one-carbon metabolism pathway. To measure the association of breast cancer risk with polymorphisms, we used unconditional logistic regression with breast cancer as the outcome. Using this model we calculated odds ratios (OR) and 95% confidence intervals (CI). Because of differences in risk factors for pre- and postmenopausal breast cancer, and the possibility that the disease may be different in the two groups, analyses were done on all cases and controls stratified by menopausal status (236). We also repeated the analyses by removing the stratification by menopausal status and analyzing women as a whole. Genotypes will be examined in association with

risk separating the homozygotes from the heterozygote. Some genotypes will be collapsed into dichotomous variables to prevent extremely small cells in stratified analysis. If the variant genotype affects function only in the homozygote, we will compare risk in the wild type homozygote to risk in the heterozygotes combined with the variant homozygotes. Additionally, we will combine the variant group of homozygotes with the heterozygotes for additional analysis. If function in the heterozygote is intermediate between the homozygous genotypes, we will examine function in the three strata separately. We will also analyze our data for interaction of C677T and A1298C polymorphisms found in the MTHFR gene. Two models will be used for all analysis; one, a crude analysis where no adjustment will be made for risk factors, and the other analyses will be adjusted for potential risk factors.

7.4 Methods for Evaluating Risks Associated with Polymorphisms and Diet.

To evaluate breast cancer risk associated with the combined effect of polymorphisms and diet, we will measure the joint association of polymorphisms and dietary factors with breast cancer risk. Participants will be classified into categories defined by genotype and dietary factors related to one-carbon metabolism. For example, we will examine the association of risk in categories defined by MTHFR genotype and by categories of folate, vitamins B₁₂, B₆ and alcohol consumption. Each dietary exposure, folate, B₁₂, and B₆, will be grouped as a continuous variable into three categories, based on the even distribution of our control subjects and with the low intake group as the referent group. We will then divide the exposure variables at the median to categorize

women into high and low consumption of each factor. Again analyses were done on all cases and controls stratified by menopausal status. We will also repeat the analyses by removing the stratification by menopausal status and analyzing women as a whole. Unconditional logistic regression with breast cancer as the outcome will be used for this analysis. Odds ratios (OR) and 95% confidence intervals (CI) will be calculated.

7.5 Methods for Determining Interaction of Polymorphisms and p53 Mutations

For this analysis we will examine risk of having a tumor with p53 mutation based on the classification of participants into categories defined by genotypes (*MTHFR C677T*, *MTHFR A1298C*, *CBS 844ins68* and *MS A2756G*). In order to determine the risk of having a p53 positive or negative tumor, based on these polymorphisms, in relation to cancer free controls, we will analyze risk of having a p53 positive tumor referent to controls. We will also analyze risk of having p53 negative tumor referent to controls. Further, we will do a case-case comparison of p53 positive to p53 negative cases to determine the relative prevalence of mutations by categories defined by genotypes. Logistic regression and odds ratio estimates of relative risk with 95% confidence intervals will be calculated using unconditional logistic regression. Women will be stratified by menopausal status as in prior analysis, and as a whole without menopausal stratification. Each genotype for the four polymorphisms will be tested separately except in cases where genotypes were collapsed into dichotomous variables to prevent extremely small cells in stratified analysis.

7.7 Methods for Determining the Interaction of ER with Diet, Polymorphism and p53 Mutations

Finally, we will examine the interaction between dietary factors, and genes related to the one carbon pathway and ER negative tumor. In order to determine the risk of having an ER negative tumor in relation to cancer-free controls, we will make comparisons of ER positive and negative cases to controls. To achieve this we will use unconditional logistic regression to calculate odds ratios (OR) and 95% confidence intervals (CI). Using regression analysis with *p53* mutation as the dependant variable, we will stratify our cases by ER status to determine if women prone to *p53* mutations are also prone to ER negative tumors. We will also assess risk of the nutrient intake to ER positive and negative tumors by comparing only women with positive or negative tumors to controls and then repeated this analysis to include *p53* mutations.

7.8 Statistical Power Analysis

Power for this study is based on the method of Schlesselman for two-sided comparisons ($\alpha=0.05$) (237). Power analyses are stratified by menopausal status and are based on 134 pre and 181 postmenopausal cases, 126 pre- and 230 postmenopausal controls. We have estimated power for an odds ratio of 2, and we assumed that the proportion of control at-risk genotypes for *MTHFR*, *MS* and *CBS* would be similar to those reported in the literature. The proportion of controls with the "at risk" genotype

would be: 15% for the *MTHFR C667T* (42), (238), 12 % for the *MTHFR C1298A* (238), 4% for the *MS A2756G* and 3% for *CBS 844in68* (239).

For power analysis of genotype and risk there was 61% power to detect an OR of 2 for the *MTHFR C677T* and the *A1298C* genotypes in pre-menopausal women. For postmenopausal women, the power is 74%. For the *MS* analysis, there was 25% power to detect an OR of 2 in pre- and 32% in postmenopausal women. For the *CBS* analysis, there was 61% power to detect an OR of 2 in pre- and 74% in postmenopausal women. For hypermethylation, based on the literature reported methylation frequencies, we expected 30% of the tumors to be methylated for the *p16* gene. Based on those expectations there was 68% power for pre- and a 71% power for postmenopausal women to detect an odds ratio of 2.

7.9 Choice of Covariates for Models

To maintain consistency throughout the analysis, we did two sets of analysis. Phase one was a crude analysis where we did not adjust for possible confounders, and the second phase of analysis was adjusted for possible confounders such as age, education, number of pregnancies, body mass index, age at first birth, age at first menarche, monthly alcohol intake, monthly folate intake, history of benign breast disease (defined as having breast lumps, cysts or fibrocystic disease), family history of breast cancer, total caloric intake (without alcohol) and age at menopause (for postmenopausal women only). The body mass index was calculated from as reported height and weight, as weight (kg)/height² (m²) reported for 2 years prior to the interview. A family history of breast

cancer was defined as having at least one first degree relative (mother, sister, and daughter) with breast cancer and categorical variables were used for adjustment (yes/no). Categorical variables were also used for adjustment of previous benign breast disease (yes/no) and age at first birth (four dummy variables: never, age 20-21 years, age 22-25 years, and age 26-39 years). For categorical analysis, cutoffs for quartiles were determined by the even distribution of control subjects to four categories. The total alcohol was calculated as the reported number of drinks of beer, wine and hard liquor, assuming that the alcohol content of a glass of beer, wine or one shot of hard liquor was approximately the same. Kilocalorie-adjusted nutrients were calculated by the method of regression residuals described by Willet and Stampfer (240).

All statistical analysis will be performed using SAS; release 8.1 (copyrighted by the SAS Institute, Inc., Cary NC, USA,).

8. PRILIMINARY RESULTS

8.1 Descriptive Characteristics of Cases and Controls

Table 8.1 shows the comparison of descriptive characteristics for all of our premenopausal breast cancer cases and controls compared to the subset on which blood clots were received.

TABLE 8.1. Characteristics of Premenopausal women with and without blood clot samples

	All data		Blood clots only	
	Case	Control	Case	Control
Premenopausal	301	316	149	130
Age (years)	45.8(3.9)	46.1 (3.5)	45.8 (4.1)*	46.7 (3.6)
Education (years)	13.8(2.8)	14.1 (2.7)	14.0 (2.8)	13.9 (2.6)
Age at menarche (years)	12.5 (1.6)	12.8 (1.7)	12.6 (1.7)*	13.0 (1.7)
Age first pregnant (years)	23.6 (4.9)*	22.4 (3.9)	23.6 (4.8)*	22.2 (4.3)
Number of pregnancies	2.7 (1.9)	2.9 (1.9)	2.6 (1.8)*	3.4 (2.1)
Body mass index (kg/m ²)	25.1 (5.7)	25.9 (5.2)	24.8 (5.3)	25.8 (4.7)
Family history of breast cancer	13%	7%	-	-
Alcohol, monthly gm	230.2 (372.7)	214.5 (367.3)	224.4 (349.2)	212.1 (343.4)
Folate, monthly mcg	8696.9 (3330.2)*	9403.7 (3473.9)	9346.4 (3557.6)	9307.2 (3091.9)
B6 monthly mg	58.3 (22.2)*	61.9 (21.0)	61.5 (23.9)	60.8 (19.1)
B12 monthly mcg	241.7 (162.8)	255.9 (168.2)	290.5(232.8)	270.4 (180.1)

*P < 0.05 for case control differences, *t* test for continuous variables, X² test for categorical variables. Mean (SD)

For comparisons between cases and controls of the entire study, cases were in general older at first live birth, and had a lower average monthly intake of folate and vitamin B₆. Cases were also slightly younger at age at menarche ($p = .09$). For the most part, comparisons between risk factors for breast cancer (risk factors for which logistic models were adjusted) were similar within the larger study set and the subset for which we have genotyping results. However there was one difference: the premenopausal cases had significantly less number of pregnancies. The premenopausal cases also had lower folate and higher alcohol intake, but these differences were not statistically significant. For the most part there were no differences between cases and controls for both groups (total subjects and those with blood clots).

Comparisons between postmenopausal cases and controls for the entire study set and the subset for which we had blood clots are shown in Table 8.1 Postmenopausal women had a higher body mass index, and were older at their first live birth. They also had a lower monthly folate intake. Overall the entire study set was not much different from the subset with blood clots. The body mass index was somewhat higher in cases than controls; however this difference was not statistically significant.

TABLE 8.2. Characteristics of postmenopausal women with and without blood clot samples

	All data		With blood clots	
	Case	Control	Case	Control
Postmenopausal	439	494	248	288
Age (years)	62.8(7.6)	63.5(7.7)	62.4 (7.6)	62.1 (7.6)
Education (years)	12.4 (2.8)	12.2 (2.6)	12.5 (2.9)	12.0 (2.5)
Age at menarche (years)	12.8 (1.6)	12.9 (1.6)	12.8 (1.6)	12.8 (1.6)
Age first pregnant (years)	24.4 (4.8)*	23.5 (4.5)	24.5 (5.1)*	23.4 (4.4)
Number of pregnancies	3.2 (2.4)	3.3 (2.3)	3.3 (2.5)	3.5 (2.5)
Body mass index (kg/m ²)	26.5 (5.4)*	25.7 (5.2)	26.3 (5.1)	25.6 (5.0)
Family history of breast cancer	16%	8%		
Alcohol, daily grams	253.8 (491.9)	222.6 (403.4)	239.0(502.1)	208.0(256.2)
Folate, daily mcg	9190.0 (3456.5) ^a	9684.6 (447.5)	9276.1 (3113.4)	9803.3 (4501.4)
B6 monthly mg	59.2 (21.7)	61.2 (24.9)	59.5 (19.1)	60.9 (25.4)
B12 monthly mcg	242.0 (176.8)	248.7 (190.7)	240.4 (176.7)	256.8(203.1)

*P < 0.05 for case control differences, *t* test for continuous variables, X² test for categorical variables
Mean (SD)

8.2 Allele and Genotype Frequencies for Cases and Controls

Values for the allele frequencies of cases and controls within each group, by menopausal status were calculate (see TableA.1). For the MTHFR C677T polymorphism, the C allele was the most common allele within the entire study set.

Among the controls the C allele was present in 70% of chromosomes evaluated; however it was lower in both pre and postmenopausal women with breast cancer (67% and 66% respectively). The T allele, on the other hand, was less common in all groups, but overall it was higher in cases than controls (30% and 27% respectively). Using the X^2 test with two degrees of freedom we tested our genotypic frequencies to determine if they were in Hardy-Weinberg equilibrium, and found that, among controls, the *MTHFR C677T* did not vary from the expected frequencies ($p = 0.41$).

8.3 Genotype Distributions

8.3.1 MTHFR C677T

This case control study consisted of a total of 736 cases and 805 controls. But we were only able to obtain blood clots on a total of 397 cases and 418 controls because many subjects did not provide blood specimens. We successfully genotyped 351 cases and 390 controls for the *MTHFR C677T* polymorphism (failure rate = 9%). The frequencies of *MTHFR C677T* genotypes, by case control status, and association between *MTHFR* genotypes and breast cancer risk are presented in Table A.2, for both pre and postmenopausal women.

Among the premenopausal women, risk for breast cancer did not differ statistically between the *C677T* genotypes. Using the homozygous C as the reference genotype, those with one T allele appeared to be at increased risk of breast cancer compared to those homozygous for the C allele (adjusted OR = 1.2; 95% CI, 0.6-2.2). However, this trend did not persist with premenopausal women homozygous for the T

allele when compared to the referent homozygous C allele (adjusted OR = 0.8; 95% CI, 0.3-2.2). There was no effect when genotypes were dichotomized (*MTHFR* CT and TT combined with CC as the referent).

The association between *MTHFR* C677T and breast cancer risk was much stronger among postmenopausal women. There was moderate increased risk with one T allele, but those women who had two T alleles had more than a 2.5 fold statistically significant increased risk for breast cancer (adjusted OR = 2.3; 95% CI 1.0 -5.2). This trend persisted when the CT and TT genotypes were combined and compared to CC (referent).

Next we evaluated the joint association of *MTHFR* genotype and alcohol intake in premenopausal women. Low or high alcohol intake was not associated with breast cancer risk in any of the *MTHFR* C677T genotypes in premenopausal women (Table A.3). Table A.4 shows the joint association of *MTHFR* C677T and alcohol intake in postmenopausal women. Low alcohol intake is mildly associated with increased breast cancer risk among all C677T genotypes in postmenopausal women with breast cancer. Women with one T allele showed a slight increase in risk (OR = 1.7; 95% CI: 0.9 – 3.2), however dose- effect was not very strong with two T alleles (OR = 1.4; 95% CI: 0.4 – 4.8) and both confidence intervals included unity. Unlike low alcohol intake, high alcohol intake showed a very strong association for breast cancer risk among all C677T genotypes. With CC as the reference, one T allele (CT) increased risk slightly (OR = 1.4; 95% CI: 0.7 – 2.6). This increased risk was not statistically significant and the confidence interval included 1. However there was a clear dose effect with two T alleles (TT), where there was more than a 3-fold increase in risk (OR = 3.5; 95% CI: 1.0 – 13.5). The

confidence interval did not include unity even though it was quite wide. When genotypes were dichotomized with *CC* as the referent genotype, women who had at least one *T* allele were at a significantly increased risk for breast cancer.

The joint effect of *MTHFR* genotype and dietary folate in premenopausal women is shown in Table A.5. Low intake of folate was associated with a 2-fold increased risk in persons with one *T* allele compared to persons homozygous for the *C* allele even though the confidence interval included unity (adjusted OR = 2.0; 95% CI, 0.7 – 5.2). Premenopausal women homozygous for the *T* allele appeared to have a slightly higher risk than those with one *T* allele however, the CI included one and was very wide (adjusted OR = 2.2; 95% CI, 0.3 – 15.5). There was no effect in premenopausal women with a high intake of folate.

Folate consumption and *MTHFR* was then examined in postmenopausal women (Table A.6). Like premenopausal women who had low folate consumption, postmenopausal women with low folate were at a 2-fold increased risk for breast cancer if they had one *T* allele (adjusted OR = 2.0; 95% CI, 1.0 – 3.9), and a 3-fold increased risk if they were homozygous for the *T* allele (adjusted OR = 3.1; 95% CI, 0.8 – 11.6). The confidence interval included 1 and was somewhat wide. But the *T* allele definitely showed a dose effect with this increasing trend. This effect was not as dramatic among women who consumed high amounts of folate, but it was nevertheless present. There was a slight increase with one *T* allele (adjusted OR = 1.2; 95% CI, 0.6 – 2.2) and in persons homozygous for the *T* allele (adjusted OR = 1.7; 95% CI, 0.5 – 6.3).

We next examined the joint association of *MTHFR* and dietary vitamin B₆ intake with breast cancer in pre and postmenopausal women.

The joint association of *MTHFR C677T* and vitamin B₆ consumption with breast cancer among premenopausal women is presented in Table A.7. Overall, there was no association between this polymorphisms, vitamin B₆ and breast cancer risk. There is some suggestion of an increased risk among high consumers of vitamin B₆ with one T allele; however there was no dose effect with the homozygous T allele and both confidence intervals included one. The effect was more dramatic among postmenopausal women.

Table A.8 shows the effect of *MTHFR* and dietary vitamin B₆ intake with breast cancer risk among postmenopausal women. Low intake of B₆ was associated with increased risk for those women with one T allele (adjusted OR = 2.1; 95% CI: 1.1-4.0). The association was much stronger for those with two T alleles (adjusted OR = 3.3; 95%CI: 0.9-11.7). However, a much weaker association was observed among women who consumed high amounts of Vitamin B₆. A suggested increased risk was seen for the homozygous T allele. (OR = 1.8; 95% CI: 0.4 – 7.3), but this was not statistically significant.

Finally we evaluated the association of *MTHFR C677T* and dietary vitamin B₁₂ with breast cancer risk for pre-and postmenopausal women. The association of *MTHFR* and dietary Vitamin B₁₂ with breast cancer risk for premenopausal women can be found in Table.9. There was no association with low or high consumers on vitamin B₁₂, and *MTHFR C677T* with breast cancer among premenopausal women. However for postmenopausal, as seen in Table A.10 there was a borderline increase risk for low consumers of dietary B₁₂ with one T allele and a 2 fold increased for those with two T alleles (OR = 2.7; 95% CI: 0.9 – 8.5) The *MTHFR C677T* polymorphism did not alter the

breast cancer risk of high consumers of dietary B₁₂. Table 8.3 provides a summary of all the positive findings for the joint association of MTHFR C677T, diet and breast cancer.

In summary, postmenopausal women with the *C677T* polymorphism, showed an increased risk for breast cancer, especially when their alcohol intake was high and their dietary folate, vitamin B₆ and B₁₂ was low. There was an overall gene-dose effect seen with the T allele. The *C677T* heterozygote showed some increase in risk, however that effect was more pronounced with the *C667T* homozygote. Despite the positive associations seen with the *C677T* polymorphisms in postmenopausal women who consumed low dietary folate and high alcohol, a test for interaction of this polymorphism with alcohol and folate was statistically non-significant ($p > 0.05$). Also, some of these findings were not statistically significant, perhaps because of small sample size in stratified analysis.

We analyzed the data for pre- and postmenopausal women together and the results remained the same (data not shown). *MTHFR C677T* in association with high alcohol, low folate, B₆ and B₁₂ was associated with increased risk of breast cancer.

TABLE 8.3. Odds ratio and 95% confidence intervals for risk of breast cancer with *MTHFR* C677T by diet in postmenopausal women

Diet/genotype	Cases(n)	Controls(n)	OR	95% CI	P trend
<i>MTHFR</i> 677					
CC	97	137	1.0		
CT	107	122	1.4	(1.0 - 2.1)	
TT	23	13	2.3	(1.0 - 5.2)	0.02
Alcohol - Low					
CC	55	68	1.0		
CT	57	60	1.7	(0.9 - 3.2)	
TT	10	8	1.4	0.4 - 4.8)	0.16
High					
CC	42	69	1.0		
CT	50	62	1.4	(0.7 - 2.6)	
TT	13	5	5.3	(1.0 - 13.5)	0.05
Folate - Low					
CC	45	64	1.0		
CT	57	58	2.0	(1.0 - 3.9)	
TT	13	7	3.1	(0.8 - 11.6)	0.02
High					
CC	52	73	1.0		
CT	50	64	1.2	(0.6 - 2.2)	
TT	10	6	1.7	(0.5 - 6.3)	0.38
Vitamin B6 -Low					
CC	44	75	1.0		
CT	58	63	2.1	(1.1 - 4.0)	
TT	15	8	3.3	(0.9 - 11.7)	0.01
High					
CC	53	62	1.0		
CT	49	59	1.1	(0.6 - 2.1)	
TT	8	5	1.8	0.4 - 7.3)	0.57
Vitamin B12 -Low					
CC	47	67	1.0		
CT	51	57	1.7	(0.9 - 3.3)	
TT	15	8	2.7	(0.9 - 8.5)	0.04
High					
CC	50	70	1.0		
CT	56	25	1.4	(0.8 - 2.5)	
TT	8	5	1.1	(0.2 - 4.9)	0.28

8.4 Research accomplishments

- Assigned intensity and proportion scores to ER stained slides for 465 cases.
- Extracted DNA from 810 blood clots
- Completed genotyping for *MTHFR* C677T, A1298C, *MS* A2756G, and *CBS* 488ins68 polymorphisms.
- Determined the methylation status of p16, *BRCA1*, *ERa*, and *Ecad* genes on a fraction of cases.

8.5 Future Plans

- Complete the methylation analysis of p16, *BRCA1*, *ERa*, and *Ecad* genes on all cases.
- Validate all methylation results
- Conduct statistical analyses for all genotyping, methylation and IHC data collected
- Write papers

8.6 Training

During the past year I attend two conferences. In April 2003 I attended the AACR

Special conference in Cancer Research held in Washington DC, and in September I
attended the AACR SNP's Haplotypes, and Cancer conference in Key Biscayne Florida.

8.7. APPENDICES

TABLE A.4 Allele frequencies among pre- and postmenopausal women

<i>MTHFR C677T</i>	Pre-menopausal		Postmenopausal	
	Case	Control	Case	Control
<i>Allele (C)</i>	.67	.68	.66	.72
<i>Allele (T)</i>	.32	.31	.33	.27

TABLE A.5 Odds ratios and 95% confidence intervals for risk of breast cancer with *MTHFR* C677T Genotypes, WNYDS

Genotype	Cases, n (%)	Controls, n (%)	OR* (95% CI)	OR** (95% CI)
Premenopausal				
CC	55 (44.3)	60 (50.8)	1.0	1.0
CT	57 (45.9)	43 (36.4)	1.4 (0.8-2.5)	1.2 (0.6-2.2)
TT	12 (9.6)	15 (12.7)	0.9 (0.4-2.0)	0.8 (0.3-2.2)
P for trend			0.7	0.78
CC	55	60	1.0	1.0
TT + CT	69	58	1.3 (0.8-2.2)	1.1 (0.6-2.2)
P for trend			0.3	0.98
Postmenopausal				
CC	97(42.7)	137(50.3)	1.0	1.0
CT	107(47.1)	122(44.8)	1.2 (0.9-1.8)	1.4 (1.0-2.1)
TT	23(10.1)	13(4.7)	2.5 (1.2-5.2)	2.3 (1.0-5.2)
P for trend			0.02	0.02
CC	97	137	1.0	1.0
TT + CT	130	133	1.4 (1.0-1.9)	1.5 (1.0-2.2)
P for trend			0.09	0.04

OR and 95% CI computed with unconditional logistic regression; *unadjusted; **adjusted for age, education, # pregnancies, body mass index, age 1st birth, age at menarche, monthly alcohol intake, monthly folate intake, monthly B6 intake, monthly B12 intake, history of benign breast disease, family history of breast cancer, and age at menopause (postmenopausal)

TABLE A.6 Odds ratios and 95% confidence intervals for risk of breast cancer with *MTHFR* C677T genotype by alcohol intake, WNYDS

	Cases, n (%)	Controls, n (%)	OR** (95% CI)	OR*** (95% CI)
Premenopausal				
Low alcohol*				
CC	25 (43.8)	27(49.0)	1.0	1.0
CT	27 (47.3)	22 (40)	1.3 (0.6-2.9)	1.1 (0.4-2.8)
TT	5(8.7)	6 (10.9)	0.9 (0.2-3.3)	0.5 (0.1-5.5)
P for trend			0.8	0.81
CC	25	27	1.0	1.0
TT + CT	32	28	1.2 (0.6-2.6)	1.0 (0.4-2.6)
P for trend			0.6	0.81
High alcohol				
CC	30 (44.7)	33 (52.3)	1.0	1.0
CT	30 (44.7)	21 (33.3)	1.6 (0.7-3.3)	1.1 (0.4-2.6)
TT	7 (10.4)	9 (14.2)	0.9 (0.3-2.6)	0.8 (0.2-3.3)
P for trend			0.8	0.7
CC	30	33	1.0	1.0
TT + CT	37	30	1.4 (0.7-2.7)	1.0 (0.4-2.5)
P for trend			0.4	0.7

*low and high alcohol defined as above and below the median intakes of the controls;
 ** Unadjusted ***adjusted for age, education, # pregnancies, body mass index, age 1st birth, age at menarche, monthly alcohol intake, monthly folate intake, monthly B6 intake, monthly B12 intake, history of benign breast disease, family history of breast cancer, and age at menopause (postmenopausal)

TABLE A.7 Odds ratios and 95% confidence intervals for risk of breast cancer with *MTHFR C677T* genotype by alcohol intake, WNYDS

	Cases, n (%)	Controls, n (%)	OR** (95% CI)	OR*** (95% CI)
Postmenopausal				
Low alcohol*				
CC	55 (45.0)	68 (50.0)	1.0	1.0
CT	57 (46.7)	60 (44.1)	1.2(0.7 – 2.0)	1.7 (0.9-3.2)
TT	10 (8.1)	8 (5.8)	1.5 (0.6 – 4.2)	1.4 (0.4-4.8)
P for trend			0.3	0.05
CC	55 (45.0)	68 (50.0)	1.0	1.0
TT + CT	67 (54.9)	68 (50.0)	1.2(0.7 – 2.0)	1.7 (0.9-3.1)
P for trend			0.4	0.1
High alcohol				
CC	42 (40.0)	69 (50.7)	1.0	1.0
CT	50 (47.6)	62 (45.5)	1.3 (0.8 – 2.3)	1.4 (0.7-2.6)
TT	13 (12.3)	5 (3.6)	4.3 (1.4 – 12.8)	3.8 (1.0-14.1)
P for trend			0.02	0.04
CC	42 (40.0)	69 (50.7)	1.0	1.0
TT + CT	63 (60.0)	67 (49.2)	1.5 (0.9 – 2.6)	1.6 (0.9-2.9)
P for trend			0.1	0.14

*low and high alcohol defined as above and below the median intakes of the controls;
 **adjusted for age, education, # pregnancies, body mass index, age 1st birth, age at menarche, monthly folate intake, monthly B6 intake, monthly B12 intake, history of benign breast disease, family history of breast cancer, and age at menopause (postmenopausal)

TABLE A.8 Odds ratios and 95% confidence intervals for risk of breast cancer with *MTHFR C677T* genotype by folate intake, WNYDS

	Cases, n (%)	Controls, n (%)	OR** (95%CI)	OR*** (95% CI)
Premenopausal				
Low folate*				
CC	29 (47.5)	33 (62.2)	1.0	1.0
CT	27 (44.2)	15 (28.3)	2.0(0.9 – 4.6)	2.0 (0.7-5.2)
TT	5 (8.1)	5 (9.4)	1.1 (0.3 – 4.3)	2.2 (0.3-15.5)
P for trend			0.3	0.28
CC	29 (47.5)	33 (62.2)	1.0	1.0
TT + CT	32 (52.4)	20 (37.7)	1.8 (0.9 – 3.9)	1.8(0.8-4.6)
P for trend			0.1	0.21
High folate				
CC	26 (41.2)	27 (41.5)	1.0	1.0
CT	30 (47.6)	28 (43.0)	1.1 (0.5 – 2.3)	0.6 (0.2-1.5)
TT	7 (11.1)	10 (15.3)	0.7 (0.2 – 2.2)	0.5 (0.1-2.2)
P for trend			0.7	0.18
CC	26 (41.2)	27 (41.5)	1.0	1.0
TT+CT	37 (58.7)	38 (58.4)	1.0 (0.5 – 2.0)	0.6 (0.3-1.5)
P for trend			0.9	0.18

*low and high folate defined as above and below the median intakes of the controls;
 Unadjusted *adjusted for age, education, # pregnancies, body mass index, age 1st birth, age at menarche, monthly alcohol intake, monthly folate intake, monthly B6 intake, monthly B12 intake, history of benign breast disease, family history of breast cancer, and age at menopause (postmenopausal)

TABLE A.9 Odds ratios and 95% confidence intervals for risk of breast cancer with *MTHFR* C677T genotype by folate intake, WNYDS

	Cases, n (%)	Controls, n (%)	OR** (95% CI)	OR*** (95% CI)
Postmenopausal				
Low folate*				
CC	45 (39.1)	64 (49.6)	1.0	1.0
CT	57 (49.5)	58 (44.9)	1.4 (0.8 – 2.4)	2.0 (1.0-3.9)
TT	13 (11.3)	7 (5.4)	2.6 (1.0 – 7.1)	3.1 (0.8-11.6)
P for trend			0.04	0.02
CC	45 (39.1)	64 (49.6)	1.0	1.0
TT+CT	70 (60.8)	65 (50.3)	1.5(0.9 – 2.5)	2.1 (1.1-3.9)
P for trend			0.1	0.02
High folate				
CC	52 (46.4)	73 (51.0)	1.0	1.0
CT	50 (44.6)	64 (44.7)	1.1 (0.7 – 1.8)	1.2 (0.6-2.2)
TT	10 (8.9)	6 (4.1)	2.3 (0.8 – 6.8)	1.7 (0.5-6.3)
P for trend			0.2	0.38
CC	51 (46.4)	73 (51.0)	1.0	1.0
TT+CT	60(53.5)	70(48.9)	1.2 (0.7 – 2.0)	1.6 (0.5-5.3)
P for trend			0.5	0.5

*low and high folate defined as above and below the median intakes of the controls;
 ** Unadjusted ***adjusted for age, education, # pregnancies, body mass index, age 1st birth, age at menarche, monthly alcohol intake, monthly folate intake, monthly B6 intake, monthly B12 intake, history of benign breast disease, family history of breast cancer, and age at menopause (postmenopausal)

TABLE A.10 Odds ratios and 95% confidence intervals for risk of breast cancer with *MTHFR* C677T genotype by B₆ intake, WNYDS

	Cases n, (%)	Controls n, (%)	OR** (95% CI)	OR*** (95% CI)
Premenopausal				
Low B₆*				
CC	30 (52.6)	26 (50.0)	1.0	1.0
CT	25 (43.8)	21 (40.3)	1.0 (0.5 – 2.3)	1.2 (0.4-3.3)
TT	2 (3.5)	5 (9.6)	0.3 (0.1 -1.9)	0.1 (0.04-3.3)
P for trend			0.5	0.56
CC	30 (52.6)	26 (50.0)	1.0	1.0
TT+CT	27(47.3)	26(50.0)	0.9 (0.4 – 1.9)	0.1 (0.4-2.5)
P for trend			0.8	0.93
High B₆				
CC	25 (37.3)	34 (51.5)	1.0	1.0
CT	32 (47.7)	22 (33.3)	2.0(0.9 – 4.2)	1.4 (0.6-3.3)
TT	10 (14.9)	10 (15.1)	1.4 (0.5 – 3.8)	1.2 (0.3-4.1)
P for trend			0.3	0.76
CC	25(37.3)	34 (51.5)	1.0	1.0
TT+CT	42(62.6)	10(48.4)	1.8(0.9 – 3.6)	1.3 (0.6-2.9)
P for trend			0.1	0.66

*low and high folate defined as above and below the median intakes of the controls;

**adjusted for age, education, # pregnancies, body mass index, age 1st birth, age at menarche, monthly alcohol intake, monthly folate intake, monthly B₆ intake, monthly B₁₂ intake, history of benign breast disease, family history of breast cancer, and age at menopause (postmenopausal)

TABLE A.11 Odds ratios and 95% confidence intervals for risk of breast cancer with *MTHFR* C677T genotype by B₆ intake, WNYDS

	Cases, n (%)	Controls, n (%)	OR** (95% CI)	OR*** (95% CI)
Postmenopausal				
Low B ₆ *				
CC	44 (37.6)	75 (51.3)	1.0	1.0
CT	58 (49.5)	63 (43.1)	1.6 (0.9 – 2.6)	2.1 (1.1-4.0)
TT	15 (12.8)	8 (5.4)	3.2 (1.3 – 8.1)	3.3 (0.9-11.7)
P for trend			0.008	0.01
CC	44 (37.6)	75 (51.3)	1.0	1.0
TT+CT	73 (62.3)	71 (48.6)	1.8 (1.1 – 2.9)	2.2 (1.2-4.1)
P for trend			0.03	0.008
High B ₆				
CC	53 (48.1)	62 (49.2)	1.0	1.0
CT	49 (44.5)	59 (46.8)	1.0 (0.6 – 1.6)	1.1 (0.6-2.1)
TT	8 (7.2)	5 (3.9)	1.9 (0.6 – 6.1)	1.8 (0.4-7.3)
P for trend			0.6	0.57
CC	53 (48.1)	62 (49.2)	1.0	1.0
TT+CT	57 (51.8)	64 (50.7)	1.0 (0.6 – 1.7)	1.1 (0.6-2.1)
P for trend			0.9	0.78

*low and high folate defined as above and below the median intakes of the controls;

** Unadjusted ***adjusted for age, education, # pregnancies, body mass index, age 1st birth, age at menarche, monthly alcohol intake, monthly folate intake, monthly B₆ intake, monthly B₁₂ intake, history of benign breast disease, family history of breast cancer, and age at menopause (postmenopausal)

TABLE A.12 Odds ratios and 95% confidence intervals for risk of breast cancer with *MTHFR* C677T genotype by B₁₂ intake, WNYDS

	Cases	Controls	OR** (95% CI)	OR*** (95% CI)
Premenopausal				
Low B12*				
CC	24 (48.9)	28 (53.8)	1.0	1.0
CT	20 (40.8)	18 (34.6)	1.3 (0.6 – 3.0)	1.4 (0.4-4.7)
TT	5 (10.2)	6 (11.5)	1.0 (0.3 – 3.6)	1.0 (0.1-7.3)
P for trend			0.8	0.97
CC	24	28	1.0	1.0
TT+CT	25	24	1.2(0.6 – 2.7)	1.2 (0.4-3.4)
P for trend			0.6	0.78
High B12				
CC	31 (41.3)	32 (48.4)	1.0	1.0
CT	37 (49.3)	25 (37.8)	1.5(0.8 – 3.1)	1.6 (0.7-3.7)
TT	7(9.3)	9 (13.6)	0.8 (0.3 – 2.4)	1.1 (0.3-4.1)
P for trend			0.8	0.89
CC	31	32	1.0	1.0
TT+CT	44	34	1.3 (0.7 – 2.6)	1.4 (0.6-3.0)
P for trend			0.4	0.76

*low and high folate defined as above and below the median intakes of the controls;
 ** Unadjusted ***adjusted for age, education, # pregnancies, body mass index, age 1st birth, age at menarche, monthly alcohol intake, monthly folate intake, monthly B6 intake, monthly B12 intake, history of benign breast disease, family history of breast cancer, and age at menopause (postmenopausal)

TABLE A.13 Odds ratios and 95% confidence intervals for risk of breast cancer with *MTHFR C677T* genotype by B₁₂ intake, WNYDS

	Cases, n (%)	Controls, n(%)	OR** (95% CI)	OR*** (95% CI)
Postmenopausal				
Low B12*				
CC	47 (41.5)	67 (50.7)	1.0	1.0
CT	51 (45.1)	57 (43.1)	1.3 (0.8 – 2.2)	1.7 (0.9-3.3)
TT	15 (13.2)	8 (6.0)	2.7 (1.0 – 6.8)	2.7 (0.9-8.5)
P for trend			0.05	0.04
CC	45 (41.5)	67 (50.7)	1.0	1.0
TT+CT	66 (58.4)	65 (49.2)	1.4(0.9 – 2.4)	1.8 (1.0-3.4)
P for trend			0.2	0.09
High B12				
CC	50 (43.8)	70 (70.0)	1.0	1.0
CT	56 (49.1)	25 (25.0)	1.2(0.7 – 2.0)	1.4 (0.8-2.5)
TT	8 (7.0)	5 (5.0)	2.2 (0.7 – 7.3)	1.1 (0.2-4.9)
P for trend			0.2	0.28
CC	50	70	1.0	1.0
TT+CT	64	30	1.3 (0.8 – 2.1)	1.4 (0.8-2.5)
P for trend			0.3	0.25

*low and high folate defined as above and below the median intakes of the controls;

** Unadjusted ***adjusted for age, education, # pregnancies, body mass index, age 1st birth, age at menarche, monthly alcohol intake, monthly folate intake, monthly B6 intake, monthly B12 intake, history of benign breast disease, family history of breast cancer, and age at menopause (postmenopausal)

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