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GENETIC POLYMORPHISMS IN THE FOLATE METABOLIZING PATHWAY
AND BREAST CANCER SURVIVAL

By

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M.A., Tezpur Central University, 2001
M.A., University of Louisville, 2006

A Dissertation
Submitted to the Faculty of the
School of Public Health and Information Sciences of the University of Louisville
In Partial Fulfillment of the Requirements
For the Degree of

Doctor of Philosophy

Department of Epidemiology and Population Health
University of Louisville
Louisville, Kentucky

May 2012

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A Dissertation Approved

April 26, 2012

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DEDICATION

This dissertation is dedicated to my parents
Late Lakshmi Prasad Lahkar and Mrs. Alaka Lahkar
and my beloved husband
Mr. Ratan Kumar Das
who has always been my source of inspiration.

ACKNOWLEDGEMENTS

First of all I would like to express my sincere gratitude to my mentor Dr. Richard N. Baumgartner, who has been an endless source of knowledge and guidance. He has given me the opportunity to be a graduate assistant for the department of Epidemiology and be a part of the Healthy Eating Activity and Life (HEAL) study which he along with other experts in the field initiated approximately fifteen years ago. He has not only guided me throughout this project, but also coached me every step of the way during my entire doctoral degree program. To me Dr. Rick Baumgartner is a true epitome of a “Guru”, a figure often associated with Brahma in Indian culture. The skills that I acquired from him during the course of the past few years have not only made me a better Epidemiologist but also shaped my personality in a way that will leave lasting impressions on my life.

If Dr. Rick Baumgartner reminds me of Lord Brahma, Dr. Kathy B. Baumgartner makes me think of Saraswati, the Hindu goddess of knowledge and wisdom. She along with Dr. Rick Baumgartner recruited me as a Susan G. Komen Trainee. Being a part of her Long Term Quality of Life (LTQOL) study, which is a fifteen years follow-up study of the New Mexico Women’s Health Study (NMWHS), she helped me acquire first-hand experience on various aspects of Epidemiological research. Throughout the program, she identified my strengths and coached me to improve upon them and pointed at my flaws and

suggested ways to rectify them. Being a woman, someday I aspire to walk on her footsteps and be a well-rounded individual and be a superb mentor like her.

I sincerely appreciate Dr. Richard A. Kerber for teaching me the ABCs of Genetic Epidemiology. He instilled in me a sense of confidence in exploring the highly complex and ever expanding world of Genetic Epidemiology. Without his guidance I would not have been able to initiate this project. I would also like to express my thankfulness to Dr. Cornelia Ulrich, who is a world renowned expert in the field of Nutritional Epidemiology and spent most of her career studying the folate pathway. Her expertise was extremely helpful for this project. I would like to extend my gratitude to Dr. Shesh N. Rai for helping me with the statistical aspects of this project.

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I also take this opportunity to thank my two brothers who always stood by my side in every step of my life. After my father passed away almost two decades ago, I realized that he left behind for me two fathers to follow my dreams and learn to be a better human being in the process. I am sure my father will continue to shower his blessings on me and the rest of the family. Words fall short while trying to express my gratitude to my mother who has always been a source of inspiration for me. She may live across the globe but always a phone call away from me when I need her. Last but not least I would like to express my thanks to my beloved husband, Ratan, for always lifting my spirits with his sense of humor. He brings out the best in me by keeping me focused and on schedule. I feel blessed to be his wife.

ABSTRACT

Genetic polymorphisms in the folate metabolizing pathway and breast cancer survival

Nandita Das

April 26, 2012

Genes in folate metabolizing pathway are responsible for modulating key enzymes in folate metabolisms necessary for DNA synthesis and repair and influence DNA methylation. Irregularity in DNA methylation may modify tumor phenotype response to chemotherapy and abnormal DNA synthesis, and repair may lead to carcinogenesis. This dissertation project tested the hypothesis that the key genes in the folate metabolizing pathway, namely Methylenetetrahydrofolate reductase (*MTHFR*), betaine-homocysteine-methyltransferase (*BHMT*), 5-methyltetrahydrofolate-homocysteine (*MTR*), 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (*MTRR*), and serine hydroxymethyltransferase (*SHMT1*) and folate receptor 1 (*FOLR1*) influences all- cause and breast cancer-specific mortality and the association between the genes in the folate metabolizing pathway and breast cancer-specific survival is modified by chemotherapy and estrogen receptor (ER) status. Gene-gene interactions among the genes in the folate metabolizing pathway were also tested in this study. Data for this study were obtained from the New Mexico site of the Health, Eating, Activity, and Lifestyle (HEAL) Study for 446 incident

primary breast cancer cases (stage 0 - IIIA). Over 11.46 years of follow-up, there were 67 deaths; 22 due to breast cancer. In the Cox proportional hazards analysis, women who carried at least one variant allele for *MTRR* G66A polymorphism had statistically significant inverse associations with all- cause (Hazard Ratio [HR]: 0.51; 95% confidence interval [CI]: 0.3-0.8; p-value <0.01) and breast cancer-specific mortality (HR: 0.40; 95% CI: 0.2-0.9; p-value <0.01). There were no statistically significant interactions with chemotherapy or ER status. Synergistic multiplicative interaction was observed between *BHMT* A742G and *FOLR1* G606A polymorphisms in predicting breast cancer-specific mortality (p for interaction: 0.03). In this sample, there was antagonistic multiplicative interaction between *MTRR* G66A and *MTHFR* C677T polymorphisms in predicting breast cancer-specific mortality (p for interaction: 0.08). These results should be further explored in larger cohorts and in clinical populations.

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CHAPTER I

INTRODUCTION

STUDY RATIONALE

According to the American Cancer Society's 2012 report¹, breast cancer is the second leading cause of cancer death among women in the US, with over forty thousand expected deaths in 2012 alone¹. Studies show that folate deficiency may be associated with breast cancer risk and may also influence breast cancer-specific survival²⁻⁴. Folate plays an important role in DNA methylation². It helps in the synthesis of purines, thymidylate, glycine, and methionine thus serving an important role in nucleotide synthesis². Its deficiency could, therefore, lead to abnormal DNA methylation. Abnormal DNA synthesis, and repair may lead to carcinogenesis and irregularity in DNA methylation may modify response to chemotherapy^{5 6}. Genes in folate- or one-carbon metabolizing pathway are responsible for modulating key enzymes in folate metabolisms necessary for DNA synthesis and repair and influence DNA methylation^{2 7 8}. Methylenetetrahydrofolate reductase (*MTHFR*), betaine-homocysteine-methyltransferase (*BHMT*), 5-methyltetrahydrofolate-homocysteine (*MTR*), 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (*MTRR*), and serine hydroxymethyltransferase (*SHMT1*) are some of the key genes in the folate metabolizing pathway responsible for the enzymes in folate metabolism. *MTHFR*, *MTR*, *MTRR*, *BHMT* and *SHMT1* act as catalysts in

the conversion of 5, 10-methylTHF to 5-methyleneTHF, a donor for the remethylation of homocysteine to methionine, the precursor for S-adenosylmethionine (SAM) ⁹. Folate receptor 1 (*FOLR1*), which lies outside the one-carbon metabolizing pathway, modulates cellular folate availability, which may indirectly influence intracellular metabolism and subsequently DNA methylation, and synthesis. Also taken together these genes regulate folate availability and metabolism. Polymorphic variations in these genes, in the presence of folate deficiency, could lead to abnormal DNA methylation, and synthesis ¹⁰.

Several studies have investigated the association of *MTHFR* polymorphisms, dietary folate, and breast cancer risk¹¹⁻¹⁷. Three studies have so far investigated the association between *MTHFR* polymorphisms and breast cancer survival^{12 18 19} of which only one study, the Long Island Breast Cancer Study¹⁹ has investigated the associations of the other genes in the folate metabolizing pathway with breast cancer survival. The Long Island Breast Cancer Study¹⁹ with an average follow-up of 5.6 years and a sample size of 1,508 women diagnosed with both *in situ* and invasive breast tumors, reported an association between the *MTHFR* (C677T) polymorphism and breast cancer mortality. In their sample, estrogen receptor (ER) status significantly modified the effect of the association between C677T breast cancer-specific survival. The Shanghai Breast Cancer Study²⁰ did not detect any significant association between *MTHFR* polymorphisms and five year survival. C677T genotype non-significantly increased breast cancer-specific mortality among women diagnosed

with late stage (stage III-IV) tumor. Martin et al¹⁸ observed similar inverse association between C677T and breast cancer-specific mortality. They also investigated the effects of ER status and race on the association between *MTHFR* polymorphisms and breast cancer-specific mortality. In their sample, women with ER negative tumors had worse survival for both C677T and A1298C and race (Caucasian vs. African American) modified the effect of *MTHFR* polymorphisms on breast cancer-specific mortality.

Study Objectives

The overall objective of this dissertation is to investigate the association between genetic variants in the folate metabolizing pathway and the genes responsible for maintaining blood folate level and breast cancer-specific survival and to examine the effects of chemotherapy use, ER status, and folate intake on these associations. Additionally, gene-gene interaction was evaluated.

The specific aims of this dissertation include:

- 1. To investigate the associations between genetic variants in the folate metabolizing pathway (*BHMT*, *SHMT1*, *MTR* and *MTRR*) and the gene responsible for maintaining blood folate level (*FOLR1*) in relation to breast cancer-specific survival, adjusted for age at diagnosis, ethnicity, stage, chemotherapy, ER-status, folate and B-complex vitamin, alcohol and energy intake.**

Genes in folate metabolizing pathway are responsible for modulating key enzymes in folate metabolisms necessary for DNA synthesis and repair. Several studies have investigated the association of *MTHFR* polymorphisms and breast

cancer risk¹¹⁻¹⁷ particularly with respect to the modifying effect of *MTHFR* polymorphisms on the association between folate intake and breast cancer risk. Associations between genes in the folate metabolizing pathway and risk of colorectal cancer and adenomas have also been investigated^{21 22}. Studies have also investigated the association between *MTHFR* polymorphisms and breast cancer survival^{12 18 19} but only one cohort study, the Long Island Breast Cancer Study¹⁹ has so far investigated the association between other genes in the folate metabolizing pathway and breast cancer mortality.

2. To investigate if breast cancer survival associated with each of these genes differs when other genes in the same pathway are present (gene- gene interaction).

Since the enzymes in a pathway may interact with each other, it is important to investigate the interactions among genes in the folate metabolizing pathway on breast cancer-specific survival. Studies have not yet investigated the effect of gene-gene interactions on folate metabolizing pathway in predicting breast cancer survival. Hence this dissertation project, sought to test if the presence of variants of one gene influences the associations of variants in other genes in the folate metabolizing pathway with breast cancer survival.

3. To investigate if breast cancer survival associated with each of these genes differs with use of (a) chemotherapy, and (b) ER status.

In breast cancer cell lines^{23 24}, mice models²⁴ and breast cancer patients^{20 25-27}, *MTHFR* polymorphisms are reported to alter the chemo-sensitivity of commonly used chemotherapeutic agents in breast cancer treatment but few studies have

investigated the effects of chemotherapy on the associations of genes in the folate metabolizing pathway with breast cancer-specific survival. ER status is a widely used prognostic marker and indicator of breast cancer treatment²⁸. Since the Martin et al¹⁸ and Xu et al¹⁹ have investigated the effects of ER status on the association of genes in the folate metabolizing and breast cancer survival, the current study is an attempt to replicate and extend their findings.

In short, this dissertation will help to enrich the current literature on the folate metabolizing pathway and breast cancer survival. Since ER status is a widely accepted prognostic marker in suggesting breast cancer treatment, its effects on the genes in the pathway in predicting breast cancer survival rates may help clinicians in future to decide the types of treatment suggested to women with a certain genetic make-up. The findings of current study may also help to clarify research to chemotherapy. Future studies should include cohorts and randomized clinical trials to further explore the findings of the current study.

CHAPTER II

LITERATURE REVIEW

INTRODUCTION

Among the U.S. women breast cancer is the second leading cause of cancer death, following lung cancer¹. Not including skin cancer, it is also the most commonly diagnosed form of cancer among women. According to the estimates of the American Cancer Society, the life time risk for being diagnosed with breast cancer in the United States is approximately 1 in 8 and the probability of dying from it is about 1 in 35²⁹. During 2012 alone, an estimated 226,870 new cases of invasive and 63,300 new cases of *in situ* breast cancer will be diagnosed in the US¹. In the past few decades the rates of survival following breast cancer diagnosis has significantly increased mostly due to improvement treatment and early detection²⁹.

PRIMARY INDEPENDENT VARIABLES

1. Folate metabolizing Pathway

Folate deficiency may be associated with breast cancer risk and breast cancer-specific survival²⁻⁴. Folate helps in the synthesis of purines, thymidylate, glycine, and methionine thus serving an important role in nucleotide synthesis and intracellular methylation⁴. Its deficiency could, therefore, lead to abnormal DNA methylation. Genes in the folate metabolizing pathway like *MTHFR* (C677T;

rs1801133 and A1298C; rs1801131), *BHMT* (A742G; rs3733890), *MTR* (A2756; rs1805087), *MTRR* (G66A; rs1801394), *SHMT1* (G1420A;rs1979277) and *FOLR1* (G606A; rs3796517) encode for key enzymes in folate metabolism, which is essential in the process of DNA methylation, synthesis and repair^{2 7 8}. DNA methylation involves addition of the methyl group to the 5th position of the cytosine pyrimidine ring which helps regulates cell development and differentiation. Methylation occurs in CpG (cytosine-phosphate-guanine) islands rich in C and G, upstream of the promoter region and is facilitated by a group of enzymes called the DNA methyltransferases. Figure 1 provides a diagrammatic representation of the pathway. To investigate the influence of these genes, one of their single nucleotide polymorphisms (SNP) which has been shown in the literature to be associated with other cancers in humans was selected. Table1 lists the genes in the folate metabolizing pathway along which are of interest in this dissertation project, their location, function, sequence showing nucleotide change, allele frequency estimates and the populations from which the frequency estimates were estimated.

Figure 1: Simplified version of the folate metabolizing pathway; figure modified from ref^{14, 15}

Table 1 Genes in the one-carbon metabolizing pathway along with their respective physical location and nucleotide change²⁴⁻²⁶

Gene/Polymorphs	Function	Gene Location	Sequence	Allele Freq (%) Population*	
MTHFR (Methylene tetrahydrofolate reductase) rs1801133 (C677T)	Catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which acts as a donor for the remethylation of homocysteine to methionine ⁵ .	1p36.22 (11,785,525-11,788,057 bp)	AGGTGTCTGCGGGAG[C/T] CGATTTTCATCATCAC	EU AS AA SSA	23-31 25-51 8-13 10-36
MTHFR (Methylene tetrahydrofolate reductase) rs1801131 (A1298C)	Catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which acts as a donor for the remethylation of homocysteine to methionine ⁵ .	1p36.22 (11,785,525-11,788,057 bp)	AGCTGACCAGTGAAG[A/C] AAGTGTCTTTGAAGT	EU AS AA SSA	34-50 14-25 12-13 10-12
MTR (5-methyltetrahydrofolate-homocysteine) rs1805087 (A2756G/D919G)	Catalyzes the final step in methionine synthesis ²⁷ .	1q43 (236958581-237067280 bp)	GGAAGAATATGAAGATA TTAGACAGG[A/G]CCATT ATGAGTCTCTCAAGGTA AGT	EU AS AA SSA	16-50 7-23 23-42 30-32

*EU (European); AS (Asian); SSA (sub Saharan African); AA (African American); ** Location type: Missense

Table 1 Continued: Genes in the one-carbon metabolizing pathway along with their respective physical location and nucleotide change²⁴⁻²⁸

Gene/Polymorphs	Function	Gene Location	Sequence	Allele Freq (%) Population*	
MTRR (5-methyltetrahydrofolate-homocysteine methyltransferase reductase) rs1801394 (A66G/I22M)	Generate functional methionine synthase which serves as catalyst during methionine synthesis, essential for protein synthesis and OCM ²⁴ .	5p15.31 (7869217-7901233 bp)	CAGGCAAAGGCCATCG CAGAAGAAAT[A/G]TGT GAGCAAGCTGTGGTACA TGGAT	EU AS SSA	45-50 25-30 23-50
BHMT (betaine-homocysteine methyltransferase) rs3733890 (G742A)	Catalyzes the conversion of betaine and homocysteine to dimethylglycine and methionine, respectively ²⁵	5q13.1-q15 (78,407,604-78,428,112 bp)	CATGAAGGAGGGCTTG GAGGCTGCCC[A/G]ACT GAAAGCTCACCTGATGA GCCAG	EU AS SSA AA	26-31 21-36 24-50 ~7
SHMT1 (serine hydroxymethyltransferase 1) rs1979277 (C1420T)	Catalyzes the reversible conversion of serine and tetrahydrofolate to glycine and 5,10-methylene tetrahydrofolate which donates one carbon units for methionine, thymidylate, and purines synthesis ^{30,31} .	17p11.2 (18243719-18266856 bp)	GTCAGGCAGGCCAGGC AGAGGGAAGA[A/G]AGA GGCGAAGCTCTCAACCT CCTCC	EU AS SSA AA	31-33 2-8 43-50 ~35
FOLR1 (folate receptor 1) rs3796517 (G606A)	Binds with folic acid and its derivatives and transport 5-methylTHF into cells ²² .	11q13.3-q14.1 (71903173-71907366 bp)	AAATTAAGTGGATGGAA GAGAACTA[A/G]ACATA GCACTTTTGCCCATTTAT GT	EU AS SSA AA	31-50 15-22 30-50 ~22

*EU (European); AS (Asian); SSA (sub Saharan African); AA (African American); ** Location type: Missense

Following is a brief overview of the genes in the folate metabolizing pathway that are investigated in this dissertation:

***MTHFR* (C677T and A1298C):**

MTHFR is an enzyme that is coded by the gene with the same name and symbol located at chromosome 1, p36.22 between 11,785,525 and 11,788,057 bp³⁰. It is a key enzyme in folate metabolism, which is essential in the process of DNA methylation, synthesis and repair²⁷⁸. Figure 2 below provides a diagrammatic representation of chromosome 1 showing the location of *MTHFR* gene.

Figure 2: Diagrammatic representation of chromosome 1 showing *MTHFR*

Studies on genetic polymorphisms of *MTHFR* have identified two common single nucleotide polymorphisms (SNP): C677T (rs1801133) and A1298C (rs1801131) with population minor allele frequencies of 32% and 23% respectively³¹.

Results from the Long Island Breast Cancer Study suggested significant reduction in breast cancer-specific mortality rates among C677T allele carriers compared to non-carriers (HR= 0.58; 95% CI: 0.38 - 0.89)¹⁹. They also reported that estrogen receptor-progesterone receptor (ER/PR) status modified the effect of C677T polymorphism breast cancer mortality (p = 0.05). No significant association was observed between A1298C genotype and breast cancer

mortality. The Shanghai BC Study²⁰ did not detect any significant association between *MTHFR* polymorphisms and five year survival. C677T genotype significantly increased the likelihood (HR= 2.97, 95% CI: 1.10, 7.98) of long term mortality among patients diagnosed with late stage (stage III-IV) disease. . Martin et al¹⁸ observed similar inverse association between C677T and breast cancer-specific mortality. They also investigated the effects of ER status and race on the association between *MTHFR* polymorphisms and breast cancer-specific mortality. In their sample, women with ER negative tumors had worse survival (C677T: HR: 0.36; 95% CI: 0.12-1.35; A1298C: HR: 2.70; 95% CI: 1.17-6.23) and there was significant interaction between race (Caucasian vs African American) and breast cancer mortality (C677T, p for interaction: 0.03; A1298C, p for interaction: 0.09). These studies, however, did not investigate potential effect modification of the association between *MTHFR* polymorphisms and breast cancer mortality by the use of chemotherapy. During the preliminary phases of this study, associations between *MTHFR* polymorphisms and breast cancer mortality, particularly with respect to the modifying effect of chemotherapy on these associations were investigated using data from all three sites of the Health, Eating, Activity and Lifestyle (HEAL) Study (Refer to chapter IV for details related to findings of the preliminary study; unpublished results).

MTR (A2756G):

MTR encodes for the enzyme 5-methyltetrahydrofolate-homocysteine methyltransferase, which is a Vitamin B₁₂ dependent enzyme³² (figure 1). It is also known as methionine synthase. It catalyzes the methylation of

homocysteine to methionine and simultaneously converting 5-methylTHF to THF required for nucleotide synthesis³³. *MTR* is located in chromosome 1(1q43) (figure 3).

Figure 3: Diagrammatic representation of chromosome 1 showing *MTR*

A2756G (rs1805087) is a common variant of the gene which substitutes the amino acid aspartic acid to glycine at codon 919^{34 35}. Although studies have reported the effects of *MTR* A2756G on plasma homocysteine in humans, they are inconsistent about the levels of homocysteine across genotypes of A2756³⁴⁻³⁶. Evidence of the association between folate and B complex vitamins and homocysteine levels is limited³⁷. The current literature is also inconsistent about the effects of *MTR* A2756G on breast^{35 38-41} and colon cancer^{22 42} risk, particularly with relation to established risk factors like menopausal status, alcohol intake, and folate intake. There is insufficient research to date on the association of *MTR* and breast cancer mortality.

***MTRR* (G66A):**

MTRR gene encodes for the enzyme methionine synthase reductase which acts as catalyst in the final step of the conversion of homocysteine to methionine. *MTRR* is located in chromosome 5 (5p15.31) (figure 4).

Figure 4: Diagrammatic representation of chromosome 5 showing *MTRR*

G66A (rs1801394) is a common variant of the gene which substitutes isoleucine with methionine at codon 22⁴³ and as the name suggests (reductase), activates methionine synthase, also known as *MTR* by acting as a carbon donor for homocysteine remethylation. Like the *MTR* polymorphism, studies have consistently observed an association between homocysteine levels and *MTRR* G66A but while some studies report elevated levels of homocysteine among carriers of AA genotype^{44 45} others report decrease in homocysteine levels⁴⁶. Studies also did not find significant associations between folate, B-complex vitamins, and alcohol intake and *MTRR* G66A genotypes. In order to better understand the impact of the variant of *MTRR* G66A in predicting breast cancer survival, research on this polymorphism is due.

***BHMT* (A742G):**

BHMT encodes for the protein betaine-homocysteine methyltransferase and is located in chromosome 5 (5q13.1-q15) (Figure 5). Unlike *MTR*, *MTRR*, and *MTHFR*, *BHMT* is not involved in the folate-dependent re-methylation process. Instead *BHMT* utilizes betaine, a metabolite of choline, as its carbon donor in the remethylation of homocysteine to methionine (figure 1)⁴⁷.

Figure 5: Diagrammatic representation of chromosome 5 showing *BHMT*

Studies have observed significant correlation between plasma homocysteine and plasma betaine levels and have suggested that plasma betaine is capable of lowering plasma homocysteine levels⁴⁷. So *BHMT* plays an important role in the re-methylation of homocysteine in the folate metabolizing pathway when the pathway is compromised due to mutations in the *MTRR* or *MTR* polymorphisms or deficiencies in vital nutrients^{47 48}. As for *MTHFR* polymorphisms, studies have investigated the association of *BHMT* A742G with risk for hyperhomocysteinemia⁴⁹, vascular diseases^{49 50} and congenital diseases like neural tube defect⁵¹, and Downs Syndrome⁵². Studies have yet to investigate the association between *BHMT* A742G and breast cancer survival.

***SHMT1* (C1420T/G1420A):**

SHMT1, sometimes called cytosolic serine hydroxymethyltransferase (*cSHMT1*), encodes for the protein of the same name and is one of the less frequently studied genes in the folate metabolizing pathway. It is located in chromosome 17 (17p11.2) (figure 5).

Figure 6: Diagrammatic representation of chromosome 17 showing *SHMT1*

It has a functional Leu > Phe polymorphism (C1420T/G1420A; rs1979277). It is vitamin-B₆ dependent enzyme that facilitates SAM synthesis and catalyzes the conversion from 5-methylTHF to 5-10-methyleneTHF. It has been suggested that carriers of the minor alleles of the *SHMT1* G1420A polymorphism may decrease production of the 5-10-methylTHF for pyrimidine synthesis. This may reduce levels of DNA methylation through negative feedback which may in turn result in decreased 5-methylTHF production and accumulation of THF, leading to carcinogenesis⁵³. It has been studied in relation to risk for acute lymphocytic leukemia⁵⁴, malignant lymphoma⁵⁵, and colorectal cancer^{53 56}. The current research is an attempt to contribute to the literature on the *SHMT1* polymorphism and breast cancer mortality.

***FOLR1* (A606G):**

As mentioned already, although outside of the folate metabolizing pathway, the folate receptor α enzyme encoded by the *FOLR1* gene is located in chromosome 11 (11q13.3-q14.1) (Figure 6) plays an important role in the uptake of folate in the cell⁵⁷

Figure 7: Diagrammatic representation of chromosome 11 showing *FOLR1*

While dietary folate is known to exist in a polyglutamated state, folic acid from fortified foods and supplements is in monoglutamated state and is taken up by the cell differently. Folic acid does not require hydrolysis before absorption. Once folate is inside the intestinal cells, regardless of poly or mono-glutamated states, various coenzymes convert it to 5-methyl THF prior to release into the bloodstream. Folate receptor α binds with folic acid and its derivatives and helps transport 5-methylTHF for SAM synthesis. Depending on the type of tissue, 5-methyl THF is also taken up by tissues via folate receptor α from the bloodstream⁵⁷. Like *SHMT1*, *FOLR1* G606A (rs3796517) is also one of the less frequently studied polymorphism associated with folate metabolism. Effects of *FOLR1* polymorphisms have been investigated along with genes in the folate metabolizing pathway with respect to renal cell⁵⁸ and prostate⁵⁹ cancer risk. But studies have not yet investigated its association with breast cancer survival, so in this dissertation the effects of the variant of the *FOLR1* G606A are explored.

2. Chemotherapy:

Biochemical studies have shown that besides being an anti-metabolite of folate, two metabolites of fluoropyrimidine (5-FU) a commonly used chemotherapeutic agent in breast cancer treatment, 5-fluoro-2'-deoxyuridine-5'-triphosphate and 5'-flurouridine-5'-triphosphate, can interfere with the level of

DNA stability and RNA functioning. Further, 5-fluoro-2'-deoxyuridine-5'-monophosphate (5FdUMP), an active metabolite of 5-FU, forms a complex with TS and 5,10-methyleneTHF, which inhibits the function of TS which in turn depletes intracellular thymidylate resulting in suppression of DNA synthesis⁶⁰. Again, 5'-formylTHF, the precursor of 5, 10-methyleneTHF potentiates the cytotoxic effects of 5-FU by stabilizing the inhibition of 5, 10-methyleneTHF-TS-5FdUMP complex. Studies have shown that *MTHFR* polymorphisms that increase the concentration of 5, 10-methyleneTHF may result in elevation of the cytotoxic levels of 5FU⁶⁰. Conversely, *MTHFR* polymorphisms that increase of accumulation of 5,10 –metheleneTHF may alter the cytotoxic effects of Methotrexate (MTX), another common chemotherapeutic agent⁶¹. MTX inhibits dihydrofolate reductase, decreases intracellular 5,10 – methyleneTHF levels for the synthesis of thymidylate and inhibits the synthesis of purine. Accumulation of dihydrofolate can inhibit the activities of TS and other enzyme⁶¹. These chemotherapeutic agents affect DNA synthesis and down-regulate cell replication. Experimental studies on breast cancer cell lines^{23 24}, mice models²⁴ and breast cancer patients^{20 25-27} have shown that *MTHFR* polymorphisms influence the concentration and cellular pathway of folate metabolism altering their chemosensitivity.

Some of the common chemotherapy medicines used currently for breast cancer treatment are abraxane, adriamycin, carboplatin, cytoxan, daunorubicin, doxil, ellence, fluorouracil also called 5-fluorouracil or 5-FU, gemzar, halaven, ixempra, methotrexate, mitomycin, navelbine, taxol, taxotere, thiotepa,

vincristine, and xeloda^{62 63}. These chemotherapy medicines are, however, commonly used in combinations. The type of chemotherapy regimen recommended to a patient is dependent upon the patient's general health, menopausal status and clinical characteristics like the stage at diagnosis, hormone receptor status, HER2 status, and lymph node status. Some of the current standard chemotherapy regimens^{62 63} used for breast cancer treatment include:

- ❖ Adriamycin and Taxotere (AT)
- ❖ Adriamycin and Cytosine with or without Taxol or Taxotere (AC± T)
- ❖ Cytosine, Methotrexate, and Fluorouracil (CMF)
- ❖ Cytosine, Epirubicin, and Fluorouracil (CEF)
- ❖ Fluorouracil, Adriamycin, and Cytosine (FAC)
- ❖ Cytosine, Adriamycin, and Fluorouracil (CAF)
- ❖ Taxotere, Adriamycin, and Cytosine (TAC)
- ❖ Gemzar, Epirubicin, and Taxol (GET)

Studies have not yet investigated the effect of various commonly used chemotherapy medicines on the association of *BHMT*, *MTR*, *MTRR*, *SHMT*, and *FOLR1* and breast cancer survival. In this study, therefore, it was investigated if chemotherapy modified the associations of these genes with breast cancer survival.

ER status:

ER is a group of receptors that are activated by estrogen hormone⁶⁴. The main function of ER is to serve as a DNA binding transcription factor that

regulates gene expression⁶⁵. There were two reasons for investigating the effects of ER status on the association between genes in the folate metabolizing pathway and breast cancer survival. First, DNA methylation has been suggested in the literature to be associated with silencing of ER expression⁶⁶⁻⁶⁹. The enzymes in the folate metabolizing pathway may influence ER status. Secondly, it is often hypothesized that tumorigenesis is triggered by binding of estrogen to the ER which stimulates proliferation of mammary cells decreasing the rate of cell division, DNA replication and mutation⁷⁰. Either way, normal cell cycle and DNA repair mechanisms are disrupted resulting in inhibition of apoptosis and tumor formation. ER-status is, therefore, a widely used prognostic marker and indicator of breast cancer treatment²⁸. ER expression is an important marker for deciding whether or not a patient receives endocrine therapy (for example tamoxifen) as part of breast cancer treatment regimen.

In the Long Island Breast cancer study¹⁹ a significant elevation of mortality rates was observed due primarily to breast cancer among C677T (*MTHFR*) allele carriers compared to non-carriers. Additionally, ER/PR status modified the association of C677T polymorphism with all-cause mortality after breast cancer treatment. Martin et al¹⁸ also observed the effects of ER status on the association between both the *MTHFR* polymorphisms and breast cancer-specific mortality. In their sample, women with ER negative tumors had worse survival.

COVARIATES OF INTEREST

1. Folate intake:

Folate is essential for DNA methylation and nucleotide synthesis. Folate deficiency could, therefore, lead to abnormal DNA methylation¹⁰. Epidemiological studies have shown that deficiency in folate could lead to breast¹¹⁻¹⁷, colorectal⁷¹⁻⁷³, and endometrial⁷⁴ cancer and the effect of the deficiency is modified by *MTHFR* polymorphisms^{11-15 71-74}. Recent studies on colorectal cancer have also shown that while folate in general is anti-neoplastic before the establishment of tumor foci, after the tumor is established it accelerates tumor proliferation^{75 76}. While high consumption of folate reduces risk for colorectal cancer by 30-40% compared to lowest consumption of folate, timing of intake is crucial because the literature suggests that progression of premalignant to cancerous lesions is accelerated with high folate intake^{77 78}. Since folate provides one carbon unit in the thymidylate synthase mediated conversion of dUMP to dTMP, its depletion could result in the accumulation of intracellular uracil resulting in the mis-incorporation of uracil into DNA and increase in DNA damage^{79 80}.

2. Stage at diagnosis:

Stage at diagnosis is another widely used prognostic marker that is an indicator for breast cancer treatment type⁸¹ and for survival. Shrubsole et al¹² in their study on *MTHFR* polymorphisms observed a non-significant elevation of risk (HR= 1.8; 95% CI: 0.79-4.14) for mortality due to breast cancer among TT genotype compared to carriers of CC genotype who were diagnosed at a later

stage (stage III-IV) and the risk was almost three fold higher (HR= 2.97; 95% CI: 1.10-7.98) among those that survived longer than two years following initial diagnosis. Results from both the Long Island Breast Cancer Study²⁶ and the Shanghai Breast Cancer Study²⁰, indicated that C677T genotype significantly elevated the risk for breast cancer-specific mortality among patients diagnosed with late stage (stage III-IV) disease. The study by Martin and colleagues did not stratify on disease stage¹⁸.

3. Treatments type:

Besides chemotherapy, tamoxifen therapy was a covariate of interest in this study. It is estimated that approximately 70% of breast cancer cases are ER positive⁷⁰ and ER status is a clinical deciding factor for prescription of tamoxifen and aromatase inhibitors⁸². ER bound to estradiol and tamoxifen induces different conformations in the receptor accounting for variation in functional activity. The drug tamoxifen, thus acts as an antagonist in breast cells and hence ideal for breast cancer treatment⁷⁰. As mentioned already, DNA methylation may influence the expression of ER⁶⁶⁻⁶⁹.

4. Demographic factors:

Demographic factors like age, race/ethnicity, body mass index (BMI), reproductive factors like age at menarche, age at menopause, parity, etc., often used as surrogates for exposures to endogenous hormones, and exogenous hormones like OC and HRT use have been widely recognized as factors that influence survival following diagnosis and treatment of breast cancer⁸³⁻⁸⁵.

Although effect modification of the association between breast cancer risk and

MTHFR by menopausal status has been investigated, the findings of the existing literature have been non-significant and inconsistent^{11 86}. Studies have not yet investigated the influence of menopausal status on breast cancer survival among women carrying various other polymorphisms in folate metabolizing pathway and genes responsible for folate intake. These covariates are, therefore, potential confounders in the investigation of the association between genes in the folate metabolizing pathway and breast cancer-specific survival.

5. Other nutritional and life style factors:

Biochemical studies have shown that B-complex vitamins are involved in the pathogenesis of breast cancer in several pathways. Deficiencies in vitamin B₆ (pyridoxine), B₁₂ (cyanocobalamin), niacin causes DNA breakage and oxidative lesion⁸⁷⁻⁸⁹. Both vitamin B₆ and B₁₂ are directly involved in the folate metabolizing pathway (Figure 1). For instance, SHMT1 enzyme is dependent on vitamin B₆ for purine synthesis necessary for conversion of homocysteine to methionine and also catalyzes the conversion from 5-methyltetrahydrofolate to 5-10-methyltetrahydrofolate. Vitamin B₁₂ is directly involved in the methylation process via SAM synthesis.

Studies have also shown the interaction of alcohol consumption and survival following diagnosis of breast cancer⁹⁰⁻⁹². Although the risk for early mortality due to breast cancer among women with 40 g/day alcohol consumption is elevated (HR= 2.00; 95% CI: 1.14-3.49) with low folate intake (200µd/day), high levels (400µd/day) of folate intake seems to have a protective effect against mortality (HR= 0.77; 95% CI: 0.33-1.80) due to breast cancer⁹⁰.

Dietary and lifestyle factors along with prognostic factors serve as potential confounders of the association between folate intake and survival following breast cancer diagnosis and treatment. Polymorphisms in the folate metabolizing pathway alter the concentration and cellular pathway of folate metabolism disrupting the sensitivity of chemotherapeutic drugs used in breast cancer treatment.

6. Comorbidities:

Studies have investigated the associations of the genes in folate metabolizing pathway on the etiology of colon cancer^{21 22 76 93}. It was therefore, intriguing to explore the percentage of the deaths due to cancer other than breast cancer in the HEAL sample can be attributable to colon cancer and if they serve as confounds. It was therefore, important to consider deaths due to other cancers that might confound the association between the genes in the folate metabolizing pathway and breast cancer survival.

Studies have also shown that higher concentrations of homocysteine, an amino acid involved in SAM synthesis (see figure 1), elevate the risk of cardiovascular disease (CVD)^{94 95}. In presence of vitamin B₁₂, *MTR*, *BHMT*, *MTRR*, and *MTHFR* catalyzes the conversion of homocysteine to methionine by remethylation. Under normal physiological conditions homocysteine is excreted by the kidney in moderate amounts⁹⁶ but polymorphisms of genes involved in homocysteine metabolisms could increase the plasma concentration of homocysteine^{97 98}, which is thought to damage the vascular epithelium and thereby increase the risk for CVD^{99 100}. Studies have also shown that vitamin B₁₂

and folate are protective against CVD development¹⁰¹. In the literature it is suggested that patients with vascular diseases and atherosclerosis have lower levels of folate and vitamin B12 concentrations¹⁰² and vitamin B₁₂ deficiency may elevate the homocysteine concentration and contribute plaque formation in the carotid artery¹⁰³. In other words, vitamin B₁₂ deficiency is an important risk factor for both hyperhomocysteinemia and CVD⁹⁵.

CHAPTER III

METHODS AND STATISTICAL PLAN

INTRODUCTION

This chapter presents the methods used for this dissertation. The chapter begins with a brief overview of the study population and the samples used in the study. It is followed by a list of study hypotheses, a description of the Cox Proportional Hazard method, primary dependent and independent variables and censorings used in the survival analysis. The section is followed by a list of the operational hypotheses and a description of the main effects and the interactions investigated in this dissertation project. There is a section on power analysis section and concludes with brief description of the genotyping process.

STUDY POPULATION

The Health, Eating, Activity, and Lifestyle (HEAL) Study is a population based, multicenter, multiethnic prospective cohort study in which 1,183 women with incident primary BC were enrolled to investigate the associations of weight, physical activity, diet, sex hormones, mammographic density, and other established risk factors with BC survival and quality of life. Women were recruited through three Surveillance, Epidemiology, and End Results (SEER) registries: at the Fred Hutchinson Cancer Research Center in Seattle, WA, the University of New Mexico and the University of Southern California. Details of the study aims

design, and recruitment procedures have been published previously¹⁰⁴⁻¹⁰⁶. Briefly, in the New Mexico site a total of 615 women, age 18 years or older, diagnosed with *in situ* to stage IIIA BC between July 1996 and March 1999 and living in Bernalillo, Sante Fe, Sandoval, Valencia, or Taos Counties were recruited. In the Seattle site, 202 women, between the age of 40 and 64 years, diagnosed with *in situ* to stage IIIA BC between September 1997 and September 1998 and living in King, Pierce, or Snohomish Counties were recruited. In Los Angeles (LA) site, 366 African American women with stage 0 to IIIA primary BC who either participated in the LA portion of the Women's Contraceptive and Reproductive Experiences Study, which is a case-control study of invasive BC, or in a parallel case control study of *in situ* BC. Other eligibility criteria for the LA site included were born in the US, capable of speaking English, between the ages of 35 to 64 years and diagnosed with BC between May 1995 and May 1998. Participants completed an in-person interview at baseline which was within their first year following diagnosis (an average of 7.5 months after diagnosis) and at 24 months after the baseline visit which was within their third year of diagnosis (an average of 31 months after diagnosis). During both time points biological specimens were collected. Data were collected again at 5 and 10 years. Person years of follow-up are calculated from the date of diagnosis to 12/31/2007 as the end point. For investigation of the SNPs associated with *MTHFR* gene samples from all three sites were used. A diagrammatic representation of the cohort is presented in Chapter 4.

To investigate the association of the SNPs corresponding to *BHMT*, *MTR*, *MTRR*, *SHMT1*, and *FOLR1* genes and breast cancer mortality only the samples from New Mexico site was available. Of the 615 women enrolled at New Mexico, DNA was collected for 406 women at baseline and 409 at 24 months follow-up. Because DNA was collected twice, some women who did not provide samples at baseline provided them at follow-up. Thus, the total number of women with DNA available for this study was 462. Three hundred and sixty- eight had provided DNA during baseline as well as at 24 months follow-up interview, 36 at baseline only and 58 at follow-up only. During the preparation phase, all 416 samples were inventoried and sample selection was based on availability with first preference given to baseline DNA and, if a sample did not have enough DNA from baseline, the sample from the 24 months follow-up was used. For 12 samples, there were insufficient amounts of DNA from both baseline and 24 months follow-up to perform genotyping and one sample was compromised for an unknown reason and could not be genotyped for all five SNPs. A total of 446 biological samples were used for the current investigation of which 137 (31%) were collected during baseline and 309 (69%) were from 24 months follow-up interview. Figure 8 provides a diagrammatic representation of the cohort.

Figure 8 Cohort definition for the New Mexico site of the Health, Eating, Activity, and Lifestyle (HEAL) Study and the number of events during 11.46 years of follow-up

HYPOTHESIS

Below are the study hypotheses:

1. Main effect:

Genes in one carbon metabolizing pathway (*MTHFR*, *BHMT*, *SHMT1*, *MTR*, *MTRR*, and *FOLR1*) are responsible for modulating key enzymes in folate metabolisms. Several case control studies¹¹⁻¹⁷ have investigated the association of *MTHFR* polymorphisms and breast cancer risk and breast cancer survival^{12 18 19} but only one cohort study has so far investigated the association of the other genes in the folate metabolizing pathway and breast cancer survival. In this study, therefore, we test the following hypothesis:

Women who carry at least one minor allele (dominant model) of the candidate/ TAG SNP polymorphisms experience an increase in mortality; specifically breast cancer mortality within the first 11.46 years of follow-up compared to women who carry two major alleles adjusting for relevant covariates.

For example, carriers of the variant allele of C677T, a polymorphism of *MTHFR* gene, are reported reduced of breast cancer specific mortality rates¹⁹.

2. Gene-Gene interaction:

Previous studies have not investigated the association of genes within the folate metabolizing pathway and breast cancer-specific survival. In this study, therefore, we test the following hypothesis:

Presence of other genes in the same pathway increases the all-cause, and breast cancer mortality rates among women who carried at least one minor allele of the candidate/TAG (*BHMT*, *SHMT1*, *MTR*, *MTRR*, and *FOLR1*) polymorphisms.

3. Gene-Environment interaction:

- a) The current literature suggests possible biological associations between chemotherapy and breast cancer survival^{60 61}. Genes in the folate metabolizing pathway may alter the levels of 5-10 methyleneTHF which may increase the levels of THF leading to increase in the cytotoxic levels of folate depleting chemotherapeutic agents, 5FU and MTX^{60 61}. In this study, therefore, the following hypothesis was tested:

Women who received chemotherapy and carried at least one minor allele (dominant model) of the candidate/TAG (*BHMT*, *SHMT1*, *MTR*, *MTRR*, and *FOLR1*) polymorphisms have increased rates of all-cause, and breast cancer mortality compared to women who did not receive chemotherapy and carried two major alleles.

- b) The assessment of ER status influences the type of treatment a women receives which may influence her survival^{28 82 107}. DNA methylation has been suggested to be associated with silencing of ER expression⁶⁶⁻⁶⁹. Results from the Long Island Breast Cancer Study¹⁹ suggested a significant interaction between *MTHFR* (C677T) and ER status for breast cancer survival. In this study, therefore, we test the following hypothesis:

Women diagnosed with ER negative tumors and carrying at least one minor allele (dominant model) of the candidate/TAG (*BHMT*, *SHMT1*, *MTR*, *MTRR*, and *FOLR1*) polymorphisms have increased rates of all-cause, and breast cancer mortality compared to women with ER positive tumor who carried two major alleles.

STATISTICAL PLAN

To test the study hypotheses, Cox proportional-hazards (PH) analysis (SAS 9.2, PROC PHREG) was used to estimate all cause and breast cancer-specific mortality for main effect and interaction with chemotherapy and ER status.

The formula for the Cox PH model:

$$h(t, X) = h_0(t) e^{\sum_{i=1}^p \beta_i X_i}$$

Here $h_0(t)$ is the baseline hazard at time $t = 0$ and $X = (X_1, X_2, \dots, X_p)$ are predictor variables that are independent of time. Here $h_0(t)$ is unspecified. Since the hazard function involves t and an exponential expression involving the X without t , it makes fitting the model non-negative. It is, therefore, robust non-parametric, non-linear and non-negative model¹⁰⁸. Parameters of the Cox model were obtained by maximizing the likelihood function which is the joint probability of obtaining the data that is observed on the study subjects as a function of the unknown parameter's (β) of the model under consideration¹⁰⁸.

Variables:

Multivariate Cox proportional-hazards models were created for each of the six SNPs and potentially important nutritional, behavioral and/or demographic covariates to investigate gene-environment interaction. Following are the primary dependent and independent variables.

Primary dependent variables or outcomes of interest:

1. Overall survival/all-cause mortality
2. Breast cancer-specific mortality

Primary independent variables:

1. Genotypes of interest: Genes (MTHFR (polymorphisms: C677T and A1298C), BHMT, MTR, MTRR, SHMT1, FOLR1 and RFC1) are coded as homozygous dominant (AA) = 0, heterozygous (AB) = 1 and homozygous recessive (BB) = 2.
2. Chemotherapy use: Although various chemotherapeutic agents were received either alone or in various combinations owing to sample size considerations (see chapter 4), chemotherapy was coded as a binary variable where no chemotherapy= 0 and chemotherapy= 1.
3. Estrogen receptor status: Estrogen receptor (ER) status of the tumor was re-coded into ER-positive= 0 and ER- negative = 1.

Covariates:

Covariates of interest were adjusted for all models based on biological plausibility, and frequency distribution. Relevant covariates were subjected to

correlation and leverage analysis prior to being incorporated into the models.

Covariates of interest based include:

Potential confounders were adjusted in all models based on biological plausibility and frequency distribution. Relevant covariates were subjected to correlation and leverage analysis prior to being incorporated into the models.

Covariates of interest included:

1. Age at diagnosis (continuous): Based on the eligibility criteria, all subjects in the study were between ages 29 to 87 years at the time of breast cancer diagnosis.
2. Ethnicity: Ethnicity was coded as Non-Hispanic White = 0 (reference), and Hispanic= 1.
3. Body Mass Index: The continuous variable BMI was created based on body weight and height. For the purpose of analysis it was re-coded into four categories based on WHO recommendations: BMI <18.5 (underweight), BMI between 18.5 and 24.9 (normal), BMI between 25.0 and 29.9 (overweight) and BMI ≥ 30.0 (obese). The variable underweight was considered as the reference category.
4. Menopausal status: Menopausal status was coded as a binary variable with pre-menopausal= 0 and post-menopausal= 1.
5. Stage at diagnosis: Based on eligibility criteria only cases with *in situ* to stage IIIA of breast cancer were included in the study. The stage of disease at diagnosis, was based on SEER defined,

Summary Stage 1977, composed of three sub-groups: *in situ*, localized, and regional.

6. Treatment: Treatment was categorized as: no surgery= 0, surgery only= 1, surgery and radiation= 2, surgery and chemotherapy = 3 and surgery, radiation and chemotherapy= 4.
7. Tamoxifen use: Tamoxifen use was coded as a binary variable; previous or present treated was coded as 1 and no treatment as 0.
8. Radiation therapy: Radiation therapy was coded as a binary variable; previous radiation was coded as 1 and no treatment as 0.
9. Oral contraceptive (OC) use: OC use was coded as a binary variable. If a subject reported receiving OC she was coded as 1 else 0.
10. Hormonal therapy (HRT): HRT was coded as a binary variable. If a subject reported receiving HRT she was coded as 1 else 0.
11. Age at menarche: Age at menarche was used as a continuous variable.
12. Age at menopause: Age at menopause was used as a continuous variable.
13. Parity: Parity was coded as 0 for nulliparous subjects, and 1 for subjects with one or more live births.
14. Family history of breast cancer: Family history of breast cancer was coded such that if a subject reported that she has no family history of breast cancer diagnosis she was coded as 0, she was coded as

1 if any 1st degree relative(s) were diagnosed with breast cancer and 2 if any 2nd degree relative(s) and no 1st degree relative were diagnosed with breast cancer.

15. Smoking status: Smoking is also coded as a binary variable. If a subject reported smoking the past or during the time of the interview (ever/current smoker) she was coded as 1. If she reported never smoking in her life she is coded as 0.
16. Alcohol intake: Like total folate intake variable, amounts of alcohol intake reported during 24 months interview was considered in the analysis. It was reported in mg/day of intake. Total alcohol intake was considered as continuous variable. For the purpose of analysis it was also re-coded in tertiles. All outliers were excluded from the analysis.
17. Folate intake: Since Los Angeles site did not collect food intake data at baseline, data collected during 24 months interview was used in the analysis for investigating the two *MTHFR* polymorphisms. Given that there was strong correlation between baseline and 24-months total folate intake for the New Mexico and Seattle data justified the used of the 24 months food frequency data. But for investigating the other five genes, where only New Mexico samples were used baseline food frequency data were used. It was reported in µg/day of intake. Total folate intake was considered as a continuous variable. For the purpose of analysis it

was also re-coded into tertiles where the lowest tertile was considered the reference category. All outliers ($> 2000 \mu\text{g/day}$) were excluded from the analysis.

18. B complex vitamin intake (B_1 , B_2 , B_3 , B_6 and B_{12}): Like folate, the data for the rest of the B-complex vitamins, namely: niacin (B_1), riboflavin (B_2), thiamin (B_3), vitamin- B_6 , and vitamin- B_{12} , were also used from the 24 months interview. It was reported in $\mu\text{g /day}$ of intake. All vitamin B variables were considered as continuous variables. For the purpose of analysis it was also recoded into tertiles.

19. Total energy intake: Information on total energy intake was also used from 24 months interview data. It was reported in Kcal/day. It was used as a continuous variable. It was also recoded into tertiles for the purpose of analysis. All outliers ($> 13000 \mu\text{g/day}$) were excluded from the analysis.

Censoring:

A censored observation is said to occur when there is incomplete information for survival time. Following are the reasons for censoring to occur in the study.

Primary outcomes:

For all-cause mortality:

1. End of follow-up: the subject does not experience death due to any cause before the study ends

2. Lost to follow-up: the subject withdraws from the study because of reasons not related to the study outcome (person moving to another location and not leaving any forwarding address, busy, not-interested in participating in the study, etc.)

For breast cancer specific mortality:

1. End of follow-up: the subject does not experience death due to breast cancer before the study ends
2. Lost to follow-up: the subject withdraws from the study because of reasons not related to the study (person moving to another location and not leaving any forwarding address, busy, not-interested in participating in the study, etc.)

Although four different kinds of censoring are possible: right truncation, left truncation, right censoring and left censoring, this study focuses only on right censoring because the central idea behind survival analysis is to follow subjects over time and observe at which point in time the event of interest occurs, which in this study is all-cause death or breast cancer death. Thus, when an observation is right censored it means that the information is incomplete because an event was not experienced during the study time period or the individual was lost to follow-up or withdrew from the study¹⁰⁸

OPERATIONAL HYPOTHESIS

Main effect:

Equation for the multivariable model:

$$h(t, X) = h_0(t) \exp [\beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \dots + \beta_p X_p]$$

Where X_1 is the gene under investigation (Example: *MTHFR* (C677T); 0= CC (wild type) and 1 = CT/TT(variant)) and X_2 through X_p are covariates of interest that are adjusted for in the model. The coefficients β_1 through β_p describes the main effects of the primary independent variables and covariates of interest.

Interaction:

Multivariable interaction model:

$$h(t, X) = h_0(t) \exp [\beta_1 X_1 + \beta_2 X_2 + \beta_3 (X_1 * X_2) + \beta_4 X_4 + \dots + \beta_p X_p]$$

Where X_1 is the gene under investigation (Example: *MTHFR* (C677T); 0= CC (wild type) and 1 = CT / TT (variant)) and X_2 is a primary independent variable (Example: chemotherapy, 0 = no and 1 = yes). X_4 through X_p are covariates of interest that are adjusted for in the model. The coefficients β_1 and β_2 describes the main effects of the gene and the primary independent variables respectively, β_3 describes the increase or decrease in hazard ratio due to interactions between the gene and the primary independent variable.

Multivariate interaction model testing dose or types of a treatment:

$$h(t, X) = h_0(t) \exp [bX + c_1 Z_1 + \dots + c_{k-1} Z_{k-1} + d_1 (X * Z_1) + \dots + d_{k-1} (X * Z_{k-1})]$$

Where X_1 is the gene under investigation (Example: *MTHFR*(C677T); 0= CC (wild type) and 1 = CT / TT (variant)), $Z_1, \dots, Z_{k-1}$ are types or levels of the primary

independent variable (Example: types of chemotherapy). The coefficients b and $c_1 \dots c_{k-1}$ describe the main effects of the gene and types or levels of the primary independent variable respectively, and $d_1 \dots d_{k-1}$ describe increase or decrease in hazard ratio due to the interactions of the gene and types or levels of the primary independent variable.

Gene-gene interaction was investigated based on allele and haplotype frequencies (PROC ALLELE and PROC HAPLOTYPE) of the genotype subgroups. Haplotype analysis was focused only on the two polymorphs of *MTHFR* gene. Covariates of interest were adjusted for all models based on biological plausibility, and frequency distribution. Relevant covariates were subjected to correlation and leverage analysis prior to being incorporated into the models.

POWER CALCULATIONS

The estimated minor allele frequencies of one carbon metabolizing genes in the general population are reported in table 3. The selection of the SNPs in each of the five genes is based on the following criteria:

- Estimated allele frequency of the SNP is greater than 10 % and less than 40 %
- The studies that helped estimate the allele frequencies had a sample size greater than 1000
- Allele frequencies used for power calculation (reported in table 3) were estimated based on studies which had samples that spread across the globe.

Power equations:

If f is the genotype frequency of interest and $g=1-f$, and D is the number of deaths after 10 years of follow-up (in this study), power to detect a hazard ratio Δ if we use a one-sided test is calculated using the following equations. Here it is assumed that there are no ties in survival times and the power of the log-rank test depends on the sample size which in turn depends the number of deaths¹⁰⁹. Let $Z_{1-\alpha}$ be the percentile corresponding to $\alpha\%$ probabilities, $P [Z > Z_{1-\alpha}] = \alpha$ and $P [Z < Z_{1-\alpha}] = 1- \alpha$, where $\alpha = 1-\alpha$ or $1-\beta$, then from Hsieh et al we have,

$$D = \frac{(Z_{1-\alpha} + Z_{1-\beta})^2}{fg[\log\Delta]^2} \text{-----} (1)$$

$$\Rightarrow D \times fg \times [\log\Delta]^2 = (Z_{1-\alpha} + Z_{1-\beta})^2$$

$$\Rightarrow \sqrt{D} \times \sqrt{fg} \times \log\Delta = Z_{1-\alpha} + Z_{1-\beta}$$

$$\Rightarrow Z_{1-\beta} = \sqrt{Dfg} \times \log\Delta - Z_{1-\alpha} \text{-----}(2)$$

For a two-sided test, where $\alpha = 5\%$ and $1- \beta = 80\%$, $Z_{1-\alpha} = 1.96$ then

$$P [Z < Z_{1-\alpha}] = P [Z < 1.96] = 0.975$$

and

$$P [Z > Z_{1-\alpha}] = P [Z > 1.96] = 0.025$$

For a one sided test, $Z_{1-\alpha} = 1.64$ then

$$P [Z < Z_{1-\alpha}] = P [Z < 1.64] = 0.$$

and

$$P [Z > Z_{1-\alpha}] = P [Z > 1.64] = 0.20$$

Genotype frequencies for the dominant and recessive models:

In genetics a trait is considered dominant, when it expresses a phenotypic effect with only one copy of the gene and it is considered recessive if a trait is expressed only if two copies of the gene are inherited. To illustrate, if we consider the gene *BHMT* (A742G), the population allele frequency for the major allele (A) and minor alleles (G) are 0.28 and 0.72 (1- 0.28= 0.72) respectively. If Hardy Weinberg equilibrium ($p^2 + 2pq + q^2 = 1$) is applied:

$$p^2 = GG = (0.72)^2 = 0.52$$

$$2pq = GA = 2 \times 0.72 \times 0.28 = 0.40$$

$$q^2 = AA = (0.28)^2 = 0.08$$

For the recessive model,

$$GG + GA = 0.52 + 0.40 = 0.92$$

$$AA = 0.08$$

For the dominant model,

$$GG = 0.52$$

$$GA + AA = 0.40 + 0.08 = 0.48$$

The following table shows the values of f and g corresponding to dominant and recessive model for all five SNPs in this study

Table 2: Values of f and g for dominant and recessive models

Gene	Allele freq	RECESSIVE MODEL				DOMINANT MODEL			
		f	g	f	g	f	g	f	g
BHMT (A742G)	0.28	GG/ GA	0.92	AA	0.08	GG	0.52	GA/ AA	0.48
MTR (A2756G)	0.19	AA/ AG	0.96	GG	0.04	AA	0.65	AG/ GG	0.35
MTRR (G66A)	0.35	AA/ AG	0.88	GG	0.12	AA	0.42	AG/ GG	0.58
SHMT1 (G1420A)	0.26	AA / AG	0.93	GG	0.07	AA	0.55	AG/ GG	0.45
FOLR1 (G606A)	0.32	GG/ GA	0.90	AA	0.10	GG	0.46	GA/ AA	0.54

Power for overall and stratified (chemotherapy Vs no chemotherapy) models:

For a one tailed test, by inserting the values corresponding to *BHMT* gene in equation 2 ($Z_{1-\alpha} = 1.64$), to detect a hazard ratio of 2.00 for a total of 107 deaths out of the 772 subjects enrolled in the study, we have:

$$\begin{aligned}
 Z_{1-\beta} &= \sqrt{Dfg} \times \log \Delta - Z_{1-\alpha} \\
 &= \sqrt{107 \times 0.48 \times 0.52} \times \log(2) - 1.64 \\
 &= 1.94
 \end{aligned}$$

So $1-\beta = 0.974$ which is 97.4% power

For a two-sided test:

$$\begin{aligned}
 &= \sqrt{107 \times 0.48 \times 0.52} \times \log(2) - 1.96 \\
 &= 1.62
 \end{aligned}$$

So $1-\beta = 0.948$ which is 94.8% power.

Applying the same equation the results for all five SNPs are reported in table 3. Microsoft Excel 2010 was used to analyze powers to detect a hazard ratio of 2.00, 1.75 and 1.50 for both two and one tailed tests. Power for stratified models (chemotherapy v. no chemotherapy) was also calculated for both two and one tailed tests and is shown in table 4.

Table 3: Power (two and one-tailed) to test association between genotype and breast cancer mortality for the main effects of the dominant models using all three HEAL sites (N= 772)

		RECESSIVE MODEL			DOMINANT MODEL		
Gene	Allele freq	HR 2.00	HR 1.75	HR 1.50	HR 2.00	HR 1.75	HR 1.50
Two- tailed test							
BHMT (A742G)	0.28	1.00	0.99	0.95	0.95	0.82	0.55
MTR (A2756G)	0.19	0.9	0.21	0.13	0.93	0.79	0.52
MTRR (G66A)	0.35	0.64	0.47	0.28	0.94	0.82	0.54
SHMT1 (G1420A)	0.26	0.45	0.32	0.19	0.95	0.82	0.55
FOLR1 (G606A)	0.32	0.58	0.41	0.24	0.95	0.82	0.55
One-tailed test							
BHMT (A742G)	0.28	1.00	1.00	0.98	0.97	0.89	0.68
MTR (A2756G)	0.19	0.41	0.30	0.21	0.96	0.87	0.64
MTRR (G66A)	0.35	0.76	0.59	0.39	0.97	0.89	0.67
SHMT1 (G1420A)	0.26	0.58	0.44	0.28	0.97	0.89	0.67
FOLR1 (G606A)	0.32	0.69	0.54	0.35	0.97	0.89	0.67

Table 4: Power (two and one-tailed) to test association between genotype and breast cancer mortality for the dominant models stratified by use of chemotherapy using all three HEAL sites

Gene	Allele freq	RECESSIVE MODEL						DOMINANT MODEL					
		Chemotherapy (N= 315)			No Chemotherapy (N= 457)			Chemotherapy (N= 315)			No Chemotherapy (N= 457)		
		HR 2.00	HR 1.75	HR 1.50	HR 2.00	HR 1.75	HR 1.50	HR 2.00	HR 1.75	HR 1.50	HR 2.00	HR 1.75	HR 1.50
Two-tailed test													
BHMT (A742G)	28	0.99	0.99	0.85	0.93	0.79	0.85	0.85	0.67	0.41	0.51	0.36	0.41
MTR (A2756G)	19	0.21	0.15	0.10	0.12	0.09	0.10	0.81	0.63	0.38	0.48	0.34	0.38
MTRR (G66A)	35	0.49	0.35	0.20	0.25	0.18	0.20	0.84	0.66	0.41	0.50	0.35	0.41
SHMT1 (G1420A)	26	0.33	0.23	0.14	0.17	0.13	0.14	0.84	0.67	0.41	0.51	0.36	0.41
FOLR1 (G606A)	32	0.43	0.30	0.18	0.22	0.16	0.18	0.84	0.67	0.41	0.51	0.36	0.41
One-tailed test													
BHMT (A742G)	28	1.00	0.99	0.91	0.96	0.87	0.64	0.91	0.78	0.54	0.64	0.49	0.32
MTR (A2756G)	19	0.32	0.24	0.17	0.19	0.15	0.12	0.89	0.74	0.51	0.60	0.46	0.29
MTRR (G66A)	35	0.62	0.47	0.31	0.37	0.28	0.19	0.90	0.77	0.53	0.63	0.48	0.31
SHMT1 (G1420A)	26	0.45	0.34	0.23	0.27	0.21	0.15	0.91	0.78	0.54	0.63	0.48	0.32
FOLR1 (G606A)	32	0.56	0.42	0.28	0.33	0.25	0.17	0.91	0.78	0.54	0.64	0.49	0.32

If the calculations are restricted to samples from the New Mexico site only, power for the main effect and stratified models (chemotherapy v. no chemotherapy) for both two and one-tailed tests are shown in Tables 5 and 6 respectively.

Table 5: Power (two and one-tailed) to test association between genotype and breast cancer mortality for the main effects of the dominant models using samples from New Mexico site (N= 446)

Gene	Allele freq	RECESSIVE MODEL			DOMINANT MODEL		
		HR 2.00	HR 1.75	HR 1.50	HR 2.00	HR 1.75	HR 1.50
Two- tailed test							
BHMT (A742G)	0.28	0.97	0.87	0.61	0.60	0.43	0.25
MTR (A2756G)	0.19	0.14	0.10	0.07	0.56	0.40	0.24
MTRR (G66A)	0.35	0.30	0.21	0.13	0.59	0.42	0.25
SHMT1 (G1420A)	0.26	0.20	0.15	0.09	0.59	0.43	0.25
FOLR1 (G606A)	0.32	0.26	0.19	0.12	0.59	0.43	0.25
One-tailed test							
BHMT (A742G)	0.28	0.99	0.92	0.72	0.72	0.56	0.37
MTR (A2756G)	0.19	0.22	0.17	0.13	0.68	0.53	0.34
MTRR (G66A)	0.35	0.42	0.32	0.21	0.71	0.55	0.36
SHMT1 (G1420A)	0.26	0.30	0.23	0.16	0.72	0.56	0.36
FOLR1 (G606A)	0.32	0.38	0.29	0.19	0.72	0.56	0.37

Table 6: Power (two and one-tailed) to test association between genotype and breast cancer mortality for the dominant models stratified by use of chemotherapy using samples from New Mexico site (N= 446)

Gene	Allele freq	DOMINANT MODEL						RECESSIVE MODEL					
		Chemotherapy (N= 118)			No Chem otherapy (N= 288)			Chemotherapy (N= 118)			No Chemotherapy (N= 288)		
		HR= 2.00	HR= 1.75	HR= 1.50	HR= 2.00	HR= 1.75	HR= 1.50	HR= 2.00	HR= 1.75	HR= 1.50	HR= 2.00	HR= 1.75	HR= 1.50
Two-tailed test													
BHMT (A742G)	0.28	0.83	0.65	0.39	0.69	0.51	0.30	0.39	0.28	0.17	0.29	0.21	0.13
MTR (A2756G)	0.19	0.09	0.08	0.06	0.80	0.06	0.05	0.37	0.26	0.16	0.28	0.19	0.12
MTRR (G66A)	0.35	0.19	0.14	0.09	0.15	0.11	0.08	0.38	0.27	0.16	0.29	0.21	0.13
SHMT1 (G1420A)	0.26	0.14	0.10	0.07	0.11	0.09	0.06	0.39	0.28	0.16	0.29	0.21	0.13
FOLR1 (G606A)	0.32	0.17	0.13	0.09	0.14	0.10	0.07	0.39	0.28	0.16	0.29	0.21	0.13
One-tailed test													
BHMT (A742G)	0.28	0.89	0.76	0.53	0.79	0.63	0.42	0.52	0.39	0.26	0.42	0.31	0.21
MTR (A2756G)	0.19	0.17	0.14	0.11	0.14	0.12	0.09	0.49	0.37	0.24	0.39	0.29	0.20
MTRR (G66A)	0.35	0.29	0.23	0.16	0.24	0.19	0.14	0.51	0.39	0.26	0.40	0.31	0.21
SHMT1 (G1420A)	0.26	0.22	0.17	0.13	0.18	0.15	0.11	0.52	0.39	0.26	0.41	0.31	0.20
FOLR1 (G606A)	0.32	0.27	0.21	0.15	0.22	0.17	0.13	0.52	0.39	0.25	0.42	0.31	0.21

Limitations of the power analysis:

For the purpose of this study, however, a one tailed test is used for the following reasons:

- ❖ Due to limited current literature on the association between the genes in the folate metabolizing pathway and breast cancer survival, the direction of the associations could not be determined *a priori*.
- ❖ A one sided test has greater power over a two-sided non-directional test

Power analysis was based on the entire HEAL cohort based on data from the preliminary investigation of the two polymorphs corresponding to the *MTHFR* gene (Chapter IV). The preliminary study included biological specimens from all three sites collected during baseline interview. Samples diagnosed with *in situ* tumors were excluded from the study (refer to Chapter IV for exclusion criteria). But for studying the association between the SNPs corresponding to *BHMT*, *MTR*, *MTRR*, *SHMT1*, and *FOLR1* and breast cancer mortality only the samples from New Mexico site were available. Blood specimens collected during both baseline and 24 months follow-up interviews were used for genotyping of these SNPs.

GENOTYPING

For the New Mexico site, 30mL of blood was collected from women who consented to provide biological specimens at baseline and 24 month follow-up visits. The blood samples were processed and stored at – 70 to – 80 °C within 3 hours of collection. DNA was isolated from stored buffy coat using Purogene (Gentra Systems, Inc.) extraction kits.. A StepOnePlus real-time polymerase

chain reaction (RT-PCR) system (Applied Biosystems by Life Technologies Corporations) was used to genotype the five SNPs under investigation. The RT-PCR was factory calibrated to generate accurate outputs with 99.7% confidence¹¹⁰. TaqMan SNP genotyping assays were used consisting of two PCR primers specific to the locus under investigation and two oligonucleotide probes specific to the allele of interest. Each probe has fluorescent dye, FAM and VIC at the 5' end and a non-fluorescent quencher with a minor groove binder (MGB) at the 3' end¹¹¹. The major allele of a SNP was identified by the FAM dye while the minor allele was identified by the VIC dye. A reaction cocktail of 10 µL of 2X master mix, 0.5 µL 40X assay, and 4.5 µL of de-ionized water was used with required amounts of DNA-TE mixture to initiate each RT-PCR reaction. During each RT-PCR cycle, the probe, which was hydrolyzed to the allele of interest, emitted a fluorescent signal when it cleaved to the 5' exonuclease action of the AmpliTaq Gold DNA Polymerase. The primers amplified the locus of interest in the DNA template and the dye specific to the alleles reported the presence or absence of the allele in the DNA sample^{112 113}. The presence of two probes, each specific to an allele of the SNP under investigation and labeled with two dyes, enabled two alleles to be detected in a single tube¹¹³. Appendix III provides the details of the genotyping procedure.

After genotyping was completed, results were tested for the Hardy Weinberg Equilibrium ($p^2 + 2pq + q^2 = 1$). Table 7 shows the calculations for the Hardy Weinberg Equilibrium in this study. There was significant difference

between the observed and the expected frequencies as is reflected in the chi square p values.

Table 7: Calculations for Hardy Weinberg Equilibrium for the SNPs investigated in this study

Genes	Obs AA	Obs AB	Obs BB	Exp AA	Exp AB	Exp BB	p	q	Chi-sq P value
BHMT	204	198	44	205.8	194.3	45.8	0.679	0.321	0.92
MTR	280	138	25	274.9	148.1	19.9	0.788	0.212	0.35
MTRR	104	216	121	101.9	220.2	118.9	0.481	0.519	0.92
SHMT	211	194	38	214.1	187.7	41.1	0.695	0.305	0.78
FOLR	210	187	48	206.9	193.0	44.9	0.682	0.318	0.81

Obs AA: observed homozygous wild-type; Obs AB: observed heterozygous; Obs BB: observed homozygous variant; Exp AA: expected homozygous wild-type; Exp AB: expected heterozygous; Exp BB: expected homozygous variant.

CHAPTER IV

PRELIMINARY STUDY: MTHFR POLYMORPHISMS AND BREAST CANCER SURVIVAL

INTRODUCTION

The preliminary investigation reports results from the analyses of the associations between the C677T and A1298C *MTHFR* polymorphisms with breast cancer-specific survival and how chemotherapy modifies the effect of the polymorphisms on survival using data from the HEAL Study.

MATERIALS AND METHODS

Statistical methods

Cox Proportional Hazard analysis was applied to compare the effect of *MTHFR* polymorphisms on women who received chemotherapy and those who did not receive chemotherapy on three outcomes: all-cause mortality and breast cancer-specific mortality. Analyses adjusted for relevant covariates including stage at diagnosis, ER-status, menopausal status, site, race, BMI, tamoxifen use, and family history of BC. Because race and site were highly correlated, site was included in the final model. Menopausal status, BMI, tamoxifen use and family history of BC did not influence the effect estimates and were not included in the final model. Univariate models for the two *MTHFR* polymorphisms, chemotherapy use, stage at diagnosis, ER-status and site were constructed for

the three outcomes. Since stage at diagnosis, ER-status and chemotherapy are highly correlated, only chemotherapy was used in the final model. Multivariate models tested for interactions between the variant alleles and chemotherapy. Chemotherapy was stratified into two categories based on folate-dependence and used in multivariate models. Diploidy and compound homozygous and heterozygous models were created to test if the effect of combined genotype modifies chemotherapy use on the three outcomes.

RESULTS

Below is a diagrammatic representation of the cohort. Also shown in the figure is the number of all-cause and breast cancer specific deaths stratified by chemotherapy. Only subjects diagnosed with invasive tumors with biological specimens were used in the analysis.

Figure 9 Health, Eating, Activity, and Lifestyle (HEAL) cohort definition and number of events during 10 years of follow-up stratified by chemotherapy

Figure 9 shows the distribution of relevant demographic, genotype and clinical characteristics for the cohort stratified by treatment with chemotherapy (yes, no). Fifty-four percent of the participants were non-Hispanic white, 31% African American and 13% Hispanic. Sixty-two percent were post-menopausal, and 35% were overweight or obese. Forty-eight percent had the T allele (CT/TT) for C677T and 45% had the C allele (AC/CC) for A1298C. Those who received

chemotherapy tended to be African American, younger, premenopausal women with more advanced disease and ER negative tumors at diagnosis.

Table 8: Demographic and medical characteristics of breast cancer survivors (N= 772) in the Health, Eating, Activity, and Lifestyle study ¹

Descriptive Characteristics	No Chemotherapy N=457		Chemotherapy N=315		χ^2 P-value
Genotype					
<u>C677T</u>					0.137
CC	229	50	175	56	
CT/TT	228	50	140	44	
<u>A1298C</u>					0.291
AA	245	54	181	57	
AC/CC	212	46	134	53	
Age at Diagnosis²	58.3	11.41	49.2	8.22	
Body Mass Index (Kg/m²)					0.333
Normal (< 25.0)	283	62	213	68	
Over weight (25.0-29.9)	99	22	57	18	
Obese (>=30)	70	15	45	14	
Study site					<0.001
New Mexico	288	63	118	37	
Seattle	64	14	63	20	
Los Angeles	105	23	134	43	
Race					<0.001
Hispanic	61	13	37	12	
Non-Hispanic white	281	61	135	43	
African American	105	23	134	43	
Other	10	3	9	2	
Menopausal Status					<0.001
Pre-menopause	113	26	158	54	
Post-menopause	317	74	133	46	
Breast cancer stage					<0.001
Localized	401	88	134	43	
Regional	54	12	181	57	
ER Status					<0.001
Positive	345	87	168	57	
Negative	53	13	125	43	
PR Status					<0.001
Positive	267	73	142	53	
Negative	97	27	128	47	

¹ Cell size may vary because of missing values. Only participants in the HEAL study diagnosed with invasive tumor and with available DNA from the 24-months interview were used in the analysis; ² mean $\pm$ SD

Table 9 shows that carriers of the variant alleles of both C667T and A1298C polymorphisms, had lower breast cancer mortality compared to the homozygous wild-type (C677T: HR= 0.76, 95 % CI: 0.51, 1.11; A1298C: HR= 0.71, 95 % CI: 0.48, 1.05).

Table 9: Crude Hazard Ratios for any cause mortality and breast cancer-specific mortality for study variables (11.46 years)

Variables	ALL CAUSE MORTALITY				BREAST CANCER-SPECIFIC MORTALITY			
	Deaths N	%	Hazard Ratio 95% CI	P- value	Deaths N	%	Hazard Ratio 95% CI	P- value
<u>C677T</u>								
CC	96	55	1.00		63	59	1.00	
CT/TT	79	45	0.89 0.66-1.20	0.45	44	41	0.76 0.5-1.10	0.15
<u>A1298C</u>								
AA	105	60	1.00		67	63	1.00	
AC/CC	70	40	0.79 0.59-1.07	0.13	40	37	0.71 0.48-1.05	0.08
<u>Chemo therapy</u>								
No	85	49	1.00		33	31	1.00	
Yes	90	51	1.66 1.23-2.23	<0.01	74	69	3.48 2.31-5.25	<0.01

*Information on ER-status and stage at diagnosis was not available for all subjects

Table 10 Prognosis: stratified by chemotherapy and genotype showing breast cancer mortality

Chemo therapy	Genotype	Deaths N	%	Hazard Ratio 95% CI	P- value	Deaths N	%	Hazard Ratio 95% CI	P- value	Deaths N	%	Hazard Ratio 95% CI	P- value
	C677T	FIVE YEARS				SEVEN YEARS				TEN YEARS			
No	CC	11	12	Reference	0.06	11	11	Reference	0.05	16	15	Reference	0.23
	CT/TT	16	18	2.24		16	16	2.26		17	16	1.58	
				0.97-5.17				0.98-5.22				0.75-3.34	
Yes	CC	40	45	Reference	0.32	46	46	Reference	0.19	47	44	Reference	0.34
	CT/TT	22	25	0.75		24	25	0.70		27	25	0.78	
				0.43-1.31				0.42-1.19				0.47-1.29	
	A1298C	FIVE YEARS				SEVEN YEARS				TEN YEARS			
No	AA	18	20	Reference	0.31	18	19	Reference	0.29	21	22	Reference	0.49
	AC/CC	9	10	0.65		9	9	0.64		12	12	0.76	
				0.28-1.49				0.28-1.47				0.37-1.63	
Yes	AA	39	44	Reference	0.46	44	45	Reference	0.43	46	47	Reference	0.52
	AC/CC	23	26	0.82		26	27	0.82		28	29	0.86	
				0.49-1.39				0.50-1.34				0.53-1.38	

*Adjusted for age at diagnosis, and race. Stage at diagnosis and ER status are not adjusted for in the model due to strong correlation with chemotherapy

Table 11: Interaction of genotype with chemotherapy on breast cancer mortality

Treatment	C677T						A1298C					
	CC			CT/TT			AA			AC/CC		
FIVE YEARS	Deaths		Hazard Ratio	P-value	Deaths		Hazard Ratio	P-value	Deaths		Hazard Ratio	P-value
	N	%	95% CI		N	%	95% CI		N	%	95% CI	
No Chemotherapy	11	12	Reference		16	18	1.73	0.17	18	20	Reference	
Chemotherapy	40	45	4.76		22	25	3.99		39	44	3.27	
			2.39-9.46	<0.01			1.89-8.44	<0.01			1.82-5.89	<0.01
P for the interaction	0.13						0.72					
SEVEN YEARS	Deaths		Hazard Ratio	P-value	Deaths		Hazard Ratio	P-value	Deaths		Hazard Ratio	P-value
	N	%	95% CI		N	%	95% CI		N	%	95% CI	
No Chemotherapy	11	11	Reference		16	16	1.73		18	19	Reference	
Chemotherapy	46	47	5.39		24	25	4.18		44	45	3.57	
			2.74-10.59	<0.01			2.00-8.72	<0.01			2.01-6.34	<0.01
P for the interaction	0.08						0.71					
TEN YEARS	Deaths		Hazard Ratio	P-value	Deaths		Hazard Ratio	P-value	Deaths		Hazard Ratio	P-value
	N	%	95% CI		N	%	95% CI		N	%	95% CI	
No Chemotherapy	16	15	Reference		17	16	1.29		21	22	Reference	
Chemotherapy	47	44	3.77		27	25	3.25		46	47	3.13	
			2.09-6.76	<0.01			1.71-6.17	<0.01			1.82-5.37	<0.01
P for the interaction	0.37						0.95					

*Adjusted for age at diagnosis and race.

Table 10 shows results for analyses of the interaction of the variant alleles in C677T and A1298T with chemotherapy by duration of follow-up, adjusting for age, study site, race, stage of disease and ER status. In a multiplicative model, in the absence of interaction the expected HR for those with the minor allele (CT/TT or AC/CC) who received chemotherapy should be approximately equal to the HR for those with minor allele who did not received chemotherapy multiplied by the HR for the homozygous wild-type (CC or AA) group who received chemotherapy. The results, however, suggest antagonistic, multiplicative interaction between the T allele of C677T and chemotherapy because the observed HR for their joint presence is substantially less than expected. For example, for 7 years of follow-up, the expected result should be 1.18 (HR for CT/TT not receiving chemotherapy) x 3.33 (HR for CC receiving chemotherapy) = 3.93 (HR_{exp} for CT/TT receiving chemotherapy). The observed HR, however, is 2.51, or 30% less than the expected result, and the formal test of interaction also approaches statistical significance (p = 0.09). The results in Table 11 also suggest that the interaction between the C677T polymorphism and chemotherapy is stronger during the first 5 to 7 years and attenuates somewhat thereafter. This is reflected in the p-values for interaction as well as in comparison of the expected to observed HRs for the joint effect group; for example the expected HR for CT/TT and chemotherapy at 10 years is 4.86 compared to the observed value of 3.25. We also computed an interaction model for C677T with chemotherapy without adjustment for stage of disease and ER status. While the absolute magnitudes of the HRs were larger across all

strata, the relative size of the interaction was similar (the observed HR was approximately 45% less than the expected value). In contrast to C677T, there was no evidence whatsoever for interaction between A1298C and chemotherapy at 5, 7 or 10 years of follow-up.

Because stage of disease and ER status are criteria used by clinical oncologists to assign breast cancer patients to receive chemotherapy rather than hormonal therapy, the possibility exists for residual confounding of the interaction between C677T and chemotherapy by these prognostic factors. As a result, we also explored the interactions of the MTHFR polymorphisms with stage of disease (local versus regional) and ER (negative versus positive) independent of chemotherapy. None of the interactions were statistically significant or approached significance (p 's > 0.40; Hazard Ratios not shown) for at 5, 7 or 10 years of follow-up. Once again, there was no evidence for interaction between the A1298C polymorphism and either stage of disease or ER status on breast cancer mortality.

The Kaplan Meier graphs shown in Figures 10 and 11 provide additional evidence for an interaction between C677T and chemotherapy. The difference between chemotherapy and no chemotherapy groups is visibly greater in the women with the CC genotype than in those with CT/TT genotypes, or T allele.

To further investigate the effect of chemotherapy on breast cancer-specific, chemotherapy was stratified based on folate dependence (folate depleting chemotherapy (170 (54%)) versus non-folate depleting chemotherapy (145(46%)) and adjusted for relevant covariates. No significant change in hazard

ratios were observed (C677T: HR= 1.75, 95% CI: 0.79, 3.84; A1298C: HR=0.63, 95% CI: 0.28, 1.42).

Figure 10: Kaplan-Meier curve for ten years breast cancer-specific mortality by chemotherapy for carriers of CC genotype (C677T polymorphism) showing number of months from diagnosis to breast cancer death using data from Health, Eating, Activity, and Life-style study (N= 772)

Figure 11: Kaplan-Meier curve for ten years breast cancer-specific mortality by chemotherapy for carriers of CT/TT genotype (C677T polymorphism) showing number of months from diagnosis to breast cancer death using data from Health, Eating, Activity, and Life-style study (N= 772)

Figure 12: Kaplan-Meier curve for ten years breast cancer-specific mortality for carriers of A1298C polymorphism showing number of months from diagnosis to breast cancer death using data from Health, Eating, Activity, and Life-style study (N= 772)

DISCUSSION

The results of this investigation suggest that carriers of the variant alleles of both the C677T and A1298C *MTHFR* polymorphisms may be protected against breast cancer mortality. Our results additionally suggest that the C677T polymorphism may modify breast cancer survival in women receiving chemotherapy. While formal tests of statistical significance for both main effects and interactions of these *MTHFR* polymorphisms with chemotherapy were not significant due to the small sample size ($n = 772$), the effect sizes observed are potentially, clinically meaningful and should be replicated in larger cohort studies or clinical trials. These polymorphisms have been established to modify folate metabolism, which is important for DNA methylation and repair, and have been previously reported to be associated with breast cancer survival as well as breast

cancer risk¹¹⁻¹⁷. Because common chemotherapeutic agents used in breast cancer, such as MTX and 5FU, also impact folate metabolism, we hypothesized that these polymorphisms might also modify the efficacy of these drugs.

Biochemical studies have shown that besides being an anti-metabolite of folate, two metabolites of 5-FU, 5-fluoro-2'-deoxyuridine-5'-triphosphate and 5'-flurouridine-5'-triphosphate when incorporated into DNA and RNA respectively could interfere with the level of DNA stability and RNA functioning⁶⁰. Again, 5'-formylTHF, the precursor of 5, 10-methyleneTHF potentiates the cytotoxic effects of 5-FU by stabilizing the inhibition of 5, 10-methyleneTHF-TS- 5FdUMP complex. Studies have shown that the *MTHFR* polymorphisms that increases the concentration of 5, 10 –methyleneTHF may result in elevation the cytotoxic levels of 5FU⁶⁰. Conversely, *MTHFR* polymorphisms that increases accumulation of 5,10 –metheleneTHF is shown to alter the cytotoxic effects of MTX⁶¹. MTX inhibits dihydrofolate reductase, decreases intracellular 5,10 –methyleneTHF levels for the synthesis of thymidylate and inhibits the synthesis of purine⁶¹. In short, these chemotherapeutic agents affect DNA synthesis and intimately down-regulate cell replication. In fact, experimental studies investigating the relationship between *MTHFR* polymorphisms and commonly used chemotherapeutic agents on breast cancer cell lines^{23 24}, mice models²⁴ and breast cancer patients^{20 25-27} have shown that *MTHFR* polymorphisms influences the concentration and cellular pathway of folate metabolism altering their chemosensitivity.

The results suggest that the C677T polymorphism may modify the effects of chemotherapeutic treatment. This interpretation is supported by the following arguments. The presence of the T allele for C677T was not associated stage at diagnosis or ER status, and the interaction with chemotherapy was not meaningfully diminished by adjusting for these covariates of chemotherapy. Moreover, it is difficult to see how the T allele might modify the associations of these prognostic variables with subsequent survival because they are fixed characteristics established at the time of diagnosis. Adjusting for these variables diminished the absolute values of the HRs, but did not affect the relative strength of interaction, indicating confounding between chemotherapy and these prognostic characteristics. Thus, the better than expected survival of women receiving chemotherapy who have the T allele may reflect a modification of the effectiveness of chemotherapeutic treatment. Once again, the plausibility of this interpretation is supported by the fact that the women receiving chemotherapy were predominantly taking methotrexate and/or 5FU, which are known to affect folate metabolism.

The data suggests that there is no correlation between the C677T and chemotherapy (Pearson correlation coefficient; $r = -0.003$), so it may support the notion that the variant allele of the C677T alter the metabolism of the chemotherapeutic drug, reducing the probability of adverse events or may reduce the side effects of the drug, thus contributing to decrease in breast cancer mortality among carriers of the T allele compared to those not carrying the T allele. The direct association of A1298C, on the other hand, suggests protection

against breast cancer mortality, even after adjusting for established prognostic markers of breast cancer. A1298C may influence breast cancer survival through a different pathway from C677T.

To test the association between the two polymorphisms and breast cancer-specific mortality we performed diplotype analysis. The results of the diplotype analysis or compound genotype analysis suggest although recombination between the variant alleles of the two polymorphisms was rare (CTAC; n= 130, TTAC; n= 3, TTCC; n=0) (figure 12). There was strong linkage disequilibrium between the two polymorphisms (Lewontin's $D' = 0.94$) suggesting that the two polymorphisms may be associated with breast cancer survival in two different ancestral pathways. The fact that there was no correlation (0.001) between them further supports the assumption.

Figure 12 Patterns of recombination showing change in C677T and A1298C polymorphisms

Although no correlation among the polymorphisms and ER-status, and stage at diagnosis was observed, there were strong correlations among both

covariates and chemotherapy because they are significant prognostic markers of breast cancer and are important deciding factors in chemotherapeutic regimen. Both ER-status and stage at diagnosis may, therefore, be mediators of the relationship between genotype and chemotherapy. The notion is further supported by the results of the univariate analyses which show approximately three times higher breast cancer-specific mortality rates for all three variables. Both covariates were, therefore, not adjusted in our multivariate models. The effects of ER-status and stage at diagnosis were investigated in this study. Unlike the results of Long Island Breast Cancer Study¹⁹ and Shanghai Breast Cancer Study²⁰ which suggested that ER-status modified the effect of C677T and breast cancer survival and C677T significantly increased the survival of women diagnosed with late stage (stage III-IV) tumor respectively, no such interactions were observed in this study.

CHAPTER V

RESULTS

INTRODUCTION

The current chapter describes the findings of the dissertation. In all the tables in this chapter the two polymorphisms of *MTHFR* are also listed along with the five genes investigated in this dissertation project. The purpose of having the two *MTHFR* polymorphisms was to compare the findings of this analysis based on samples from the New Mexico site with findings based on an earlier analysis using all available samples from the three HEAL study sites (see Chapter IV), and (b) to test for gene-gene interaction between the *MTHFR* polymorphisms and breast cancer-specific mortality.

DESCRIPTIVE STATISTICS

Out of the 446 women in the New Mexico site, a total of 67 (15%) were deceased as of 12/31/2007 at 11.46 years; 22 (5%) deaths were due to breast cancer for women diagnosed with invasive disease. More than three-fourths (78%) of the participants were non-Hispanic white, 22% were Hispanic and 3% belonged to the other category. The majority of the participants were normal (43%; BMI: 18.5-24.9) or over-weight (31%; BMI: 25.0-29.9), 8% were below normal (BMI: <18.5) and 18% were obese (BMI: >=30). Sixty-eight percent of the sample was post-menopausal and 32 % were pre-menopausal. More than half of the participants were diagnosed with a localized tumor (62%), 18% were

diagnosed with regional tumor and 20% with *in situ* breast disease. Table 12 describes the demographic and medical characteristics of the participants (N= 446) in the New Mexico site and table 13 provides the frequency distribution of the genotypes of the breast cancer survivors.

Table 12: Demographic and medical characteristics of breast cancer survivors for the New Mexico site in the Health, Eating, Activity, and Lifestyle Study (n= 446)¹

Descriptive Characteristics	Breast Cancer Deaths	Total N	%/SD
Age at Diagnosis (yrs)²	22	57.6	12.1
Race³	22		
Hispanic		97	22
Non-Hispanic white		346	78
Body Mass Index (Kg/m²)⁴	22		
Below normal (<18.5)		37	8
Normal (18.5-24.9)		190	43
Over weight (25.0-29.9)		137	31
Obese (>=30)		78	18
Menopausal Status	22		
Pre-menopause		136	32
Post-menopause		294	68
Stage at Diagnosis	22		
In situ		89	20
Localized		276	62
Regional		81	18
Estrogen Receptor Status⁵	17		
Positive		257	82
Negative		56	18
Progesterone Receptors Status⁶	18		
Positive		213	71
Negative		89	29
Chemotherapy	22		
No		334	75
Yes		112	25
Tamoxifen use	22		
No		242	54
Yes		204	46
Oral Contraceptive Use⁷	22		
No		160	36

Yes		286	64
Hormone replacement Therapy⁷	22		
No		186	42
Yes		259	58
Family History of Breast Cancer⁸	21		
No		197	48
1 st degree		121	29
2 nd degree		93	23
Current Smoker⁹	10		
No		201	81
Yes		48	19
Total Folate Intake (µg)¹⁰	20	316.2	126.7
Vitamins (mg/d)¹⁰	20		
Total B1(Thiamin)		1.1	0.5
Total B2 (Riboflavin)		1.6	0.7
Total B3 (Niacin)		14.9	6.9
Total B6 (pyridoxine, pyridoxal, & pyridoxamine)		1.4	0.6
Total B12 (Cobalamin)		4.5	2.9
Alcohol (gm/day)¹⁰	20	4.3	9.7
Energy Intake (kcal)¹⁰	20	1401.3	611

¹ Percentages calculation excludes missing values; ² mean ± SD; ³ other in race: 3; ⁴ missing values in BMI: 4; ⁵ missing values in ER-status: 133; ⁶ missing values in PR-status: 144; ⁷ missing values in OC and HRT use: 1; ⁸ missing values in family history of breast cancer: 35; ⁹ missing values among current smoker: 197; ¹⁰ missing values in energy intake, vitamins, folate and alcohol intake: 14.

Table 13: Genotypes of breast cancer survivors for the New Mexico site in the Health, Eating, Activity, and Lifestyle Study (n= 446)

Genotypes	Breast Cancer Deaths	Total N	%	P value HWE ¹
BHMT (N= 446; rs3733890)				
A742G	22			0.92
AA		204	46	
AG		198	44	
GG		44	10	
AG/GG		242	54	
MTR (N= 443; rs1805087)				
A2756G	21			0.36
AA		280	63	
AG		138	31	
GG		25	6	
AG/GG		163	37	

MTRR (N= 441; rs1801394)				
G66A	21			0.92
GG		104	24	
AG		216	50	
AA		121	27	
GA/AA		307	76	
SHMT1 (N= 443; rs1979277)				
C1420T/ G1420A	22			0.78
GG		211	48	
AG		194	44	
AA		38	9	
GA/AA		232	52	
FOLR1 (N= 445; rs3796517)				
G606A	22			0.80
GG		210	47	
AG		187	42	
AA		48	11	
GA/AA		235	53	
MTHFR(N= 397; rs1801133)				
C677T	20			0.96
CC		165	42	
CT		184	46	
TT		48	12	
CT/TT		232	58	
MTHFR(N= 397; rs1801131)				
A1298C	20			0.99
AA		197	50	
AC		165	42	
CC		35	8	
AC/CC		200	50	

[†]Abbreviation: HWE: Hardy Weinberg Equilibrium.

AIM 1: MAIN EFFECTS

The associations between the genes in the folate metabolizing pathway and all-cause and breast cancer-specific mortality are summarized in table 14. All models reported in table 14 were adjusted for age. Adjustments for the potential confounders, race/ethnicity, menopausal status, BMI, stage at diagnosis, treatment types, ER status, folate, B-vitamin intake, and alcohol intake did not change the point estimates by more than 10%.

Table 14 Age-adjusted hazard ratios¹ and 95% confidence intervals for the associations of polymorphisms in the folate metabolizing pathway and all-cause mortality and breast cancer-specific mortality using data from the New Mexico site of the Health, Eating, Activity, and Lifestyle study (N= 446).

Genotypes	ALL-CAUSE MORTALITY						BREAST CANCER-SPECIFIC MORTALITY					
	Deaths N	Censored N	%	HR	(95% CI)	P value	Deaths N	Censored N	%	HR	(95% CI)	P value
BHMT (N= 446)												
A742G												
AA	30	174	85		1.00		8	196	96		1.00	
AG	26	172	87	0.98	0.6-1.7	0.93	12	189	94	1.52	0.6-3.7	0.36
GG	11	33	75	1.61	0.8-3.2	0.18	2	42	95	1.17	0.3-5.5	0.84
AG/GG	37	205	85	1.10	0.7-1.8	0.89	14	228	94	1.45	0.6-3.5	0.39
MTR (N= 443)												
A2756G												
AA	42	238	85		1.00		14	266	95		1.00	
AG	21	117	85	1.19	0.7-2.0	0.52	6	132	96	0.82	0.3-2.1	0.82
GG	3	22	88	0.79	0.3-2.6	0.69	1	24	96	0.82	0.1-6.2	0.85
AG/GG	24	139	85	1.12	0.7-1.9	0.67	7	156	96	0.82	0.3-2.0	0.67
MTRR (N= 441)												
G66A												
GG	27	94	78		1.00		10	111	92		1.00	
GA	28	188	87	0.52	0.3-0.9	0.01	7	209	97	0.38	0.2-1.0	0.05
AA	11	93	89	0.47	0.2-0.9	0.03	4	100	96	0.42	0.1-1.4	0.15
GA/AA	39	281	88	0.51	0.3-0.8	<0.01	11	309	97	0.40	0.2-0.9	0.04
SHMT1(N= 443)												
G1420A												
GG	35	176	83		1.00		10	201	95		1.00	
AG	29	165	85	0.93	0.6-1.5	0.78	11	183	94	1.17	0.5-2.8	0.71
AA	3	35	92	0.57	0.2-1.9	0.35	1	37	97	0.51	0.1-3.9	0.52
GA/AA	32	200	86	0.87	0.5-1.4	0.57	12	220	95	1.06	0.5-2.5	0.89

¹ Adjusting for clinical covariates stage at diagnosis, chemotherapy use, ER-status did not affect point estimates by more than 10%.

Table 14 continued: Age-adjusted hazard ratios¹ and 95% confidence intervals for the associations of polymorphisms in folate metabolizing pathway and all-cause mortality and breast cancer-specific mortality using data from the New Mexico site of the Health, Eating, Activity, and Lifestyle study (N= 446).

Genotypes	ALL-CAUSE MORTALITY					BREAST CANCER-SPECIFIC MORTALITY				
	Deaths N	Censored N	%	HR	(95% CI) P value	Deaths N	Censored N	%	HR	(95% CI) P value
FOLR1(N= 446)										
G606A										
GG	27	183	87	1.00		9	201	96	1.00	
AG	32	155	83	1.26	0.8-2.1 0.38	8	179	96	1.02	0.4-2.7 0.96
AA	8	40	83	1.08	0.5-2.4 0.84	5	43	90	2.51	0.8-7.5 0.10
GA/AA	40	195	83	1.22	0.8-1.9 0.42	13	222	95	1.33	0.6-3.1 0.52
MTHFR(N= 397)²										
C677T										
CC	22	143	87	1.00		5	160	97	1.00	
CT	26	158	86	0.98	0.6-1.7 0.94	12	172	93	2.22	0.8-6.3 0.13
TT	9	39	81	1.56	0.7-3.4 0.27	3	45	94	2.14	0.5-8.9 0.29
CT/TT	35	197	85	1.08	0.6-1.8 0.78	15	217	94	2.17	0.8-5.9 0.13
MTHFR(N= 397)²										
A1298C										
AA	29	168	85	1.00		10	187	95	1.00	
AC	23	142	85	0.89	0.5-1.5 0.67	10	155	94	1.19	0.5-2.9 0.69
CC	5	30	86	0.89	0.3-2.3 0.81	0	35	100	N/A	
AC/CC	28	172	86	0.88	0.5-1.5 0.62	10	190	95	0.98	0.4-2.4 0.97

¹Adjusting for clinical covariates stage at diagnosis, chemotherapy use, ER-status did not affect point estimates by more than 10%;

²Only baseline DNA included in the analyses.

There was a statistically significant inverse association between the *MTRR* G66A polymorphism and breast cancer-specific mortality: carriers of the variant alleles (GA/AA) had reduced risk compared to carriers of the homozygous wild-type genotype (GG) (HR: 0.40; 95% CI: 0.2-0.9; P- value: 0.04). The association remained statistically significant when the heterozygous GA (HR: 0.38; 95% CI: 0.2-1.0; P-value: 0.05) and homozygous AA (HR: 0.42; 95% CI: 0.1-1.4; P-value: 0.15) were compared to the GG genotype. Similar significant inverse associations were also observed between carriers of the variant alleles of the *MTRR* G66A polymorphism and all-cause mortality compared to carriers of the homozygous dominant genotype.

A non-significant, positive association was observed between the *MTHFR* C677T polymorphism and breast cancer-specific mortality: carriers of the variant allele (CT/TT) had increased risk compared to the women who were homozygous for the major allele (CC) (HR: 2.17; 95% CI: 0.8-5.9; p- value: 0.13). This positive trend for association also was evident when the heterozygous CT (HR: 2.22; 95% CI: 0.8-6.3; p- value: 0.13) and homozygous TT (HR: 2.14; 95% CI: 0.5-8.9; p- value: 0.29) genotypes were separately compared to the CC referent group. Although there was no association between carriers of CT genotype and all-cause mortality, there was a positive association among carriers of homozygous TT genotype and all-causes mortality (HR: 1.56; 95% CI: 0.7-3.4; p- value: 0.27).

A non-significant, inverse association with breast cancer mortality also was observed among carriers of the variant allele (G) of the *MTR* A2756G

polymorphism. No consistent association was, observed for all-cause mortality and *MTR* A2756G genotype. The three other SNPs, *BHMT* A742G, *SHMT1* G1420A, and *FOLR1* G606A, were not statistically, significantly associated with either breast cancer-specific or all-cause mortality in this sample (table 14).

Test for confounding:

To explore if demographic and clinical covariates such as race/ethnicity, BMI, menopausal status, stage at diagnosis, chemotherapy, ER-status confounded the associations between the genes in the folate metabolizing pathway and breast cancer-specific mortality, multivariate Cox proportional hazard analysis models were tested. Models with covariates including dietary intakes of folate and B complex vitamins, smoking status and alcohol consumption were examined. Age-adjusted HRs reported in tables 14 did not change with the incorporation of these covariates of interest in the models. None of these covariates in multivariate Cox proportional hazard analysis models produced a change greater than 10% in the main effects of the SNPs.

AIM 2: GENE - GENE INTERACTION

Gene-gene interaction was significant for two interactions (*BHMT* A742G X *FOLR1*G60A and *MTRR* G66A X *MTHFR* C677T, (see table 15)).

Table 15: Age- adjusted breast cancer-specific mortality stratified by genotypes in the New Mexico samples of the Health, Eating, Activity, and Lifestyle Study (n= 446).

Genotype	Deaths N	Censored N %	Hazard Ratio ¹ 95% CI	P-value	Deaths N	Censored N %	Hazard Ratio ¹ 95% CI	P-value
BHMT A742G								
FOLR1 G606A (GG)					FOLR1 G606A (GA/AA)			
AA	6	95 94	1.00		2	101 98	0.33 0.1-1.6	0.17
AG/GG	3	107 97	0.44 0.1-1.8	0.25	11	121 92	1.42 0.5-3.8	0.49
P for interaction	0.03							
MTRR G65A								
MTHFR C677T (CC)					MTHFR C677T (CT/TT)			
GG	1	53 93	1.00		8	47 84	3.96 1.2-13.2	0.02
GA/AA	4	109 96	0.89 0.2-3.6	0.87	6	171 96	0.87 0.2-3.1	0.82
P for interaction	0.03							

¹Adjusting for clinical covariates stage at diagnosis, chemotherapy use, ER-status did not affect point estimates by more than 10%;

²Only baseline DNA included in the analyses.

For the model examining interaction between *BHMT* A742G and *FOLR1*G60A, women with AA genotype for *BHMT* A742G and the GG genotype for *FOLR1* G606A were considered the referent group. Women with at least one variant allele for *BHMT* A742G (GA/GG) and *FOLR1* G606A (GG) (HR: 0.44; 95% CI: 0.1-1.8; p-value: 0.25) and those with the AA genotype for *BHMT* A742G and GA/AA for *FOLR1* G606A (HR: 0.33; 95% CI: 0.1-1.6; p-value: 0.17) had an inverse association with breast cancer-specific mortality (Figures 13 and 14). Women with at least one variant allele for both *FOLR1* G606A polymorphism (GA/AA) and *BHMT* A742G (AG/GG) had a positive association with breast cancer-specific mortality rates (HR: 1.42; 95% CI: 0.5-3.8; p-value: 0.49). The P value for interaction was 0.03. There was synergistic effect of the variant alleles of the two polymorphisms on breast cancer-specific mortality.

Figure 13: Kaplan-Meier survival curves among carriers of the *BHMT* A742G, AA genotype stratified by *FOLR1* G606A polymorphism, HEAL study, New Mexico (N= 446)

Figure 14: Kaplan-Meier survival curves among carriers of the *BHMT* A742G, AG/GG genotype stratified by *FOLR1* G606A polymorphism, HEAL study, New Mexico (N= 446)

For the model investigating the interaction between *MTRR* G66A and *MTHFR* C677T, women carrying the genotypes GG for *MTRR* G66A and CT/TT for *MTHFR* C677T were almost four times more likely to die due to breast cancer

(HR: 3.96; 95% CI: 1.2-13.2; p- value: 0.02) compared to women who were homozygous carriers of both major alleles (GG for *MTRR* G66A and CC for *MTHFR* C677T) (figure 15). There was non-significant inverse association with breast cancer-specific mortality for women carrying the genotypes GA/AA for *MTRR* G66A and CC for *MTHFR* C677T (figure 16). Non-significant associations were also observed for those with at least one variant genotype for both polymorphisms (GA/AA for *MTRR* G66A and CT/TT for *MTHFR* C677T) compared to women with GG for *MTRR* G66A and CC for *MTHFR* C677T. In this multiplicative model P for interaction was 0.08. There was borderline antagonistic effect of the variant allele on breast cancer-specific mortality.

Figure15: Kaplan-Meier survival curves among carriers of the *MTRR* G66A, GG genotype stratified by *MTHFR* C677T polymorphism, HEAL study, New Mexico (N= 446)

Figure 16: Kaplan-Meier survival curves among carriers of the *MTRR* G66A, GA/AA genotype stratified by *MTHFR* C677T polymorphism, HEAL study, New Mexico (N= 446)

AIM 3: GENE - ENVIRONMENT INTERACTION

Interaction with chemotherapy:

To explore if chemotherapy influences the effect of the genes in the folate metabolizing pathway on breast cancer-specific mortality, the associations between the polymorphisms and breast cancer mortality stratified by chemotherapy was examined in table 16.

Table 16: Age- adjusted breast cancer-specific mortality stratified by chemotherapy and genotype in the New Mexico samples of the Health, Eating, Activity, and Lifestyle Study (n= 446).

Genotype	Deaths N	Censored N	%	Hazard Ratio [†] 95% CI	P- value	Deaths N	Censored N	%	Hazard Ratio [†] 95% CI	P- value		
No Chemotherapy						Chemotherapy						
BHMT A742G (N= 446)												
AA	4	146	97	1.00		4	50	93	3.26	0.8-13.8	0.11	
AG/GG	4	180	98	0.81	0.2-3.3	0.77	10	48	83	8.59	2.4-30.7	<0.01
P for interaction	0.20											
MTR A2756G (N= 443)												
AA	6	208	97	1.00		8	60	88	4.06	1.3-12.4	0.01	
AG/GG	2	118	98	0.52	0.1-2.5	0.41	5	38	88	3.99	1.2-13.6	0.03
P for interaction	0.64											
MTRR G66A (N= 441)												
GG	4	87	95	1.00		6	28	82	3.70	1.0-13.2	0.04	
GA/AA	4	240	98	0.29	0.1-1.1	0.07	7	69	91	1.87	0.5-6.4	0.32
P for interaction	0.69											
SHMT G1420A (N= 443)												
GG	5	157	97	1.00		5	47	90	3.73	1.0-13.9	0.05	
GA/AA	3	170	98	0.56	0.1-2.3	0.44	9	50	85	6.49	1.9-21.9	<0.01
P for interaction	0.21											

¹ Adjusting for clinical covariates stage at diagnosis, chemotherapy use, ER-status did not affect point estimates by more than 10%;

² Only baseline DNA included in the analyses.

Table 16 continued: Age- adjusted breast cancer-specific mortality stratified by chemotherapy and genotype in the New Mexico samples of the Health, Eating, Activity, and Lifestyle Study (n= 446).

Genotype	Deaths N	Censored N	%	Hazard Ratio ¹ 95% CI		P- value	Deaths N	Censored N	%	Hazard Ratio ¹ 95% CI		P- value
No Chemotherapy							Chemotherapy					
FOLR1 G606A (N= 445)												
GG	4	157	98	1.00			5	45	90	4.63	1.2-18.1	0.02
GA/AA	4	169	98	0.89	0.2-3.6	0.87	9	53	85	7.07	2.0-24.5	<0.01
P for interaction						0.54						
MTHFR C677T (N= 397) ²												
CC	1	175	98	1.00			4	32	89	7.23	1.5-33.8	0.01
CT/TT	6	166	97	2.00	0.5-8.0	0.33	9	51	85	10.56	2.7-41.4	<0.01
P for interaction						0.39						
MTHFR A1298C (N= 397) ²												
AA	4	192	97	1.00			6	42	88	4.88	1.5-16.0	<0.01
AC/CC	3	149	98	0.63	0.2-2.5	0.51	7	41	85	5.52	1.7-17.6	<0.01
P for interaction						0.59						

¹Adjusting for clinical covariates stage at diagnosis, chemotherapy use, ER-status did not affect point estimates by more than 10%;

²Only baseline DNA included in the analyses.

In these analyses, the referent group was women homozygous for the major allele and did not receive chemotherapy as part of their treatment following breast cancer diagnosis. For *BHMT* A742G, breast cancer-specific mortality was higher among those with AA genotype who received chemotherapy (HR: 3.26; 95% CI: 0.8-13.8; p- value: 0.11) compared to those with the same genotype who did not receive chemotherapy (table 17). Among women who did not receive chemotherapy, the AG/GG genotype had a non-significant inverse association with breast cancer-specific mortality (HR: 0.81; 95%CI: 0.2-3.3; p- value: 0.77) compared to carrying the AA genotypes (figure 18). Women with the same genotype who received chemotherapy had significantly increased breast cancer-specific mortality rates (HR: 8.59; 95%CI: 2.4-30.7; p- value: <0.01) compared to not carrying the variant allele who did not receive chemotherapy suggesting a possible synergistic effect of the polymorphism and chemotherapy on breast cancer-specific mortality (p for interaction: 0.21).

Figure 17: Kaplan-Meier survival curves among carriers of the *BHMT* A742G, AA genotype stratified by chemotherapy, HEAL study, New Mexico (N= 446)

Figure 18: Kaplan-Meier survival curves among carriers of the *BHMT* A742G, AG/GG genotype stratified by chemotherapy, HEAL study, New Mexico (N= 446)

For *SHMT* G1420A polymorphism, women with GG genotype, who received chemotherapy were at increased risk of breast cancer-specific mortality (HR: 3.73; 95% CI: 1.0-13.9; p-value: 0.05) compared to women without chemotherapy (figure 19). Women with the variant alleles of the polymorphisms (GA/AA) who did not receive chemotherapy had inverse association with breast cancer-specific mortality (HR: 0.56; 95% CI: 0.1-2.3; p-value: 0.44) (figure 20) while those with the same genotype who received chemotherapy were at increased breast cancer-specific mortality rates (HR: 6.49; 95% CI: 1.9-21.9; p-value: <0.01) compared to women with the homozygous dominant genotype (GG) without chemotherapy suggesting a borderline synergistic effect of *SHMT* G1420A polymorphism and chemotherapy on breast cancer-specific mortality (p for interaction: 0.21).

No significant interactions with chemotherapy were observed with the *MTR*, *MTR*, and *FOLR1* polymorphisms and breast cancer-specific mortality.

Figure 19: Kaplan-Meier survival curves among carriers of the *SHMT* G1420A, GG genotype stratified by chemotherapy, HEAL study, New Mexico (N= 446)

Figure 20: Kaplan-Meier survival curves among carriers of the *SHMT* G1420A, GA/AA genotype stratified by chemotherapy, HEAL study, New Mexico (N= 446)

Interaction with ER-status:

To investigate if ER-status modifies the effect of the genes in the folate metabolizing pathway on breast cancer-specific mortality, the associations between the polymorphisms and breast cancer-specific mortality stratified by ER status were examined in table 17.

Table 17: Age- adjusted breast cancer-specific mortality stratified by ER status and genotype in the New Mexico samples of the Health, Eating, Activity, and Lifestyle Study (n= 446).

Genotype	Deaths N	Censored N	%	Hazard Ratio ¹ 95% CI		P- value	Deaths N	Censored N	%	Hazard Ratio ¹ 95% CI		P- value
ER- Positive							ER- Negative					
BHMT A742G (N= 313)												
AA	3	120	98	1.00			3	26	90	4.33 0.9-21.5		0.07
AG/GG	7	127	95	2.18 0.6-8.4		0.26	4	23	85	6.21 1.3-28.7		0.01
P for interaction	0.69											
MTR A2756G (N= 313)												
AA	8	154	94	1.00			2	29	94	1.09 0.2-5.1		0.91
AG/GG	2	93	98	0.37 0.1-1.7		0.19	4	20	83	2.92 0.9-9.7		0.08
P for interaction	0.11											
MTRR G66A (N= 313)												
GG	5	62	91	1.00			2	18	90	1.06 0.2-5.3		0.95
GA/AA	4	185	98	0.23 0.1-0.8		0.02	5	31	86	1.51 0.5-5.1		0.50
P for interaction	0.11											
SHMT G1420A (N= 313)												
GG	4	125	97	1.00			4	25	86	4.41 1.1-17.9		0.03
GA/AA	6	122	95	1.51 0.4-5.4		0.52	3	24	89	3.68 0.8-16.6		0.09
P for interaction	0.57											

¹Adjusting for clinical covariates stage at diagnosis, chemotherapy use, ER-status did not affect point estimates by more than 10%;

²Only baseline DNA included in the analyses.

Table 17continued: Age- adjusted breast cancer-specific mortality stratified by ER status and genotype in the New Mexico samples of the Health, Eating, Activity, and Lifestyle Study (n= 446).

Genotype	Deaths N	Censored N	%	Hazard Ratio ¹ 95% CI	P- value	Deaths N	Censored N	%	Hazard Ratio ¹ 95% CI	P- value	
ER- Positive						ER- Negative					
FOLR1 G606A (N= 313)											
GG	3	119	98	1.00		3	25	89	4.32	0.9-21.5	
GA/AA	7	128	95	2.19	0.6-8.5	0.26	4	24	95	6.07	1.3-27.3
P for interaction	0.67										
MTHFR C677T (N= 397) ²											
CC	2	131	97	1.00		2	17	89	3.60	0.7-19.8	
CT/TT	6	122	95	1.60	0.5-5.7	0.46	5	26	84	5.59	1.5-21.1
P for interaction	0.72										
MTHFR A1298C (N= 397) ²											
AA	3	139	97	1.00		4	23	85	4.54	1.2-17.1	
AC/CC	5	114	96	1.20	0.3-4.1	0.77	3	20	87	3.59	0.8-15.2
P for interaction	0.54										

¹Adjusting for clinical covariates stage at diagnosis, chemotherapy use, ER-status did not affect point estimates by more than 10%;

²Only baseline DNA included in the analyses.

In these survival analysis models, in the referent group was women with the homozygous dominant genotype with ER-positive tumor. For *MTR* A2756G, for women with AA genotype, there was no association between women with ER-negative tumors and breast cancer-specific mortality compared to women with ER-positive tumor (figure 21). Figure 21 shows violation of the proportional hazard assumption. Among women who carried at least one variant allele of the polymorphism (AG/GG), those with ER-positive tumor had an inverse association with breast cancer-specific mortality (HR: 0.37; 95% CI: 0.1-1.7; p-value: 0.19) (figure 22), while women with ER-negative tumor had significantly increased risk for breast cancer-specific mortality (HR: 2.92; 95% CI: 0.9-9.7; p-value: 0.08) suggesting multiplicative interaction (p for interaction: 0.11).

Figure 21: Kaplan-Meier survival curves among carriers of the *MTRR* G66A, GG genotype stratified by ER-status, HEAL study, New Mexico (N= 446)

Figure 22: Kaplan-Meier survival curves among carriers of the *MTRR* G66A, GA/AA genotype stratified by ER-status, HEAL study, New Mexico (N= 446)

For *MTRR* G66A polymorphism, there was no association between women with ER-negative tumor and GG genotype and breast cancer-specific mortality compared to women with the same genotype and ER-positive tumor (figure 23). The Kaplan-Meier curve in figure 23 shows the violation of the proportional hazard assumption. In this sample, among women who carried at least one

variant allele of the polymorphism (GA/AA), there was significant inverse association among women with ER-positive tumor and breast cancer-specific mortality (HR: 0.23; 95% CI: 0.06-0.79; p-value: 0.02) compared to women with GG genotype and ER-positive tumor (figure 24). There was non-significant association between women with ER-negative tumor and GA/AA genotype and breast cancer-specific mortality (HR: 1.51; 95% CI: 0.5-5.1; p-value: 0.50) compared to women with GG genotype and ER-positive tumor suggesting multiplicative interaction (p for interaction: 0.11).

Figure 23: Kaplan-Meier survival curves among carriers of the *MTR* A2756G, AA genotype stratified by ER-status, HEAL study, New Mexico (N= 446)

Figure 24: Kaplan-Meier survival curves among carriers of the *MTR* A2756G, AG/GG genotype stratified by ER-status, HEAL study, New Mexico (N= 446)

CHAPTER VI

DISCUSSION

INTRODUCTION

This chapter summarizes the findings of the dissertation project and provides potential interpretations that establish integration between biochemical mechanisms of the folate metabolizing pathway reported in the literature and the statistical findings of the current study. Strengths and limitations of this dissertation are evaluated also.

In this population-based breast cancer cohort with 446 participants from New Mexico, 5% of the study participants died due to breast cancer related complications within the 11.46 years of follow-up. Since 1990 there has been a steady decline in the breast cancer-specific mortality rates mostly due to early detection, advancement in treatment options and decreased incidence¹. Although the literature on the genes in the folate metabolizing pathway and their influence on breast cancer survival are limited, studies have investigated the associations between *MTHFR* polymorphisms and breast cancer risk¹¹⁻¹⁷. The genes in the folate metabolizing pathway are responsible for enzymes that maintain DNA methylation, synthesis and repair^{2 7 8}. Since abnormal DNA synthesis and repair may lead to carcinogenesis and irregularity in DNA methylation may modify response to chemotherapy^{5 6}, genes in the folate metabolizing pathway may influence breast cancer survival.

AIM1: MAIN EFFECTS

In this exploratory study, *MTRR* G66A had a statistically significant inverse association with breast cancer-specific mortality and all-causes mortality. Similar inverse associations were also observed in the Long Island Breast Cancer study (Breast cancer-specific mortality: HR: 0.77; 95% CI: 0.49-1.23; all-causes mortality: HR: 0.75; 95% CI: 0.52-1.08)¹⁹. Non-significant associations were also observed between four SNPs, *MTR* G66A, *BHMT* A742G, *SHMT1* G1420A, and *FOLR1* G606A and breast cancer-specific mortality. In the New Mexico sample, unlike the larger HEAL sample, there was positive association between *MTHFR* C677T and breast cancer-specific mortality.

AIM 2: GENE - GENE INTERACTION

Significant and borderline gene-gene interactions were observed between *FOLR1* G606A and *BHMT* A742G polymorphisms and between *MTRR* G66A and *MTHFR* C677T polymorphisms respectively.

Potential interpretations for gene-gene interactions:

Gene-gene interaction between *FOLR1* G606A and *BHMT* A742G (p for interaction: 0.03) in predicting breast cancer-specific mortality was observed in this sample. One of the reasons for interaction may be related to their respective functions in the folate metabolizing pathway. The role of *FOLR1* is to bind with folic acid and its derivatives and facilitate transportation of 5-methylTHF. The role of *BHMT* enzyme is the re-methylation of homocysteine to methionine⁴⁷, a process which uses 5-methylTHF transported by *FOLR1*. Variations in *FOLR1* may influence the levels of 5-methylTHF, while variation in *BHMT* polymorphisms

may compromise the conversion of homocysteine to methionine. The plausibility of this argument is supported by the synergistic effect of the two polymorphisms of these genes in predicting breast cancer survival. Hence, each of these polymorphisms may modify the effect of the other in predicting breast cancer-specific mortality.

In this exploratory study, a statistically significant interaction between *MTRR* G66A and *MTHFR* C677T (p for interaction: 0.08) in predicting breast cancer-specific mortality was observed also. Biochemical studies suggest that *MTHFR* acts as a catalyst in the conversion of 5, 10-methylHF to 5-methylTHF, which serves as a donor for the re-methylation of homocysteine to methionine⁹. Methionine synthase reductase activates methionine synthase by acting as a carbon donor for homocysteine re-methylation and substitutes isoleucine with methionine⁴³. Variations in *MTHFR* and *MTRR* polymorphisms may, therefore, influence homocysteine levels, although in different directions. Since studies are inconsistent about the direction of the association between *MTRR* polymorphism and homocysteine levels⁴³ and it is also unknown if homocysteine levels are associated with breast cancer-specific mortality in this population, the antagonistic effect observed in this exploratory study should be interpreted with caution. In spite of low to moderate power of this study (tables 5 and 6, chapter III) women carrying the variant alleles for both polymorphism had statistically significant reduced breast cancer-specific mortality rates compared to women carrying the homozygous dominant genotypes for both polymorphism while significantly increased breast cancer-specific mortality was observed among

women carrying variant allele for *MTHFR* C677T and homozygous GG genotype for *MTRR* G66A.

AIM 3: GENE - ENVIRONMENT INTERACTION

Although it did not reach statistical significance, chemotherapy modified the effects of *BHMT* A742G and *SHMT1* G1420A. In this sample, there were also borderline significant interactions between ER-status and *MTRR* G66A polymorphism and *MTR* A2756G polymorphism.

Potential interpretations for gene-environment interactions:

In this sample, women carrying the AG/GG genotype of *BHMT* A742G polymorphism who received chemotherapy may have elevated rates of breast cancer related deaths. The apparent synergistic effect of chemotherapy with *BHMT* A742G polymorphism may be supported by the following arguments. Two common chemotherapeutic agents 5 FU and MTX affect DNA synthesis and down-regulate cell replication. 5'-formylTHF, the precursor of 5, 10-methyleneTHF potentiates the cytotoxic effects of 5-FU by stabilizing the inhibition of 5, 10-methyleneTHF-TS- 5FdUMP⁶⁰. MTX inhibits dihydrofolate reductase, decreases intracellular 5,10 –methyleneTHF levels for the synthesis of thymidylate and inhibits the synthesis of purine⁶¹. Although not directly involved in folate metabolism, BHMT helps in the conversion of homocysteine to methionine by donating carbon when the re-methylation process is compromised due to genetic and/or dietary factors⁴⁷. It is also suggested to be associated with homocysteine levels⁴⁷. Although future research should confirm the findings of this exploratory study, it is plausible that since chemotherapy may influence DNA

synthesis and repair and the *BHMT* A742G polymorphism may affect the methylation process, the two simultaneous processes may result in the elevation of the breast cancer-specific mortality.

In this study, there was also borderline interaction between chemotherapy and *SHMT* G1420A polymorphism. It has been suggested that carriers of the minor allele of *SHMT* G1420A polymorphism may decrease the production of the 5-10-methylTHF for pyrimidine synthesis which may reduce the levels of DNA methylation through negative feedback resulting in decreased 5-methylTHF production and accumulation of THF⁵³. The common chemotherapeutic agents 5FU and MTX interferes with the levels of 5-10-methylTHF and increases their cytotoxic effects resulting in abnormal DNA synthesis⁶⁰. Owing to the synergistic effect of the variant allele and the effect of chemotherapy women who received chemotherapy and carried at least one variant allele of *SHMT* G1420A polymorphism may have increased breast cancer-specific mortality rates compared to carriers of the homozygous dominant genotype GG who did not receive chemotherapy.

Studies have suggested that DNA methylation may be associated with inhibition of ER expression⁶⁶⁻⁶⁹. Binding of estrogen to the ER is thought to stimulate mammary cell proliferation, which may lead to decrease in cell division, DNA replication and mutation¹¹⁴. Although studies have reported the presence of association between *MTR* A2756G and plasma homocysteine in humans, the results are inconsistent for the levels of homocysteine across genotypes of A2756G³⁴⁻³⁶. Like the *MTR* polymorphism, although studies are consistent about

the association between homocysteine levels and *MTRR* G66A there is inconsistency in the literature regarding the direction of the association⁴⁴⁻⁴⁶. Thus, variations in either of these polymorphisms may compromise DNA methylation which could be associated with ER-induced carcinogenesis. Future studies in this area may explain the synergistic of these polymorphisms and ER status.

STRENGTHS

One of the strengths of this study is that although the sample for this population based case cohort is relatively small it has a maximum follow-up time of 11.5 years. Secondly, no other study has so far investigated the interactions among the genes in the folate metabolizing pathway and breast cancer-specific mortality. Interactions of the genes in the folate metabolizing pathway with chemotherapy and ER-status were also investigated. Since the enzymes associated with these genes in the folate metabolizing pathway influence each other and are known in the biochemical literature to be influenced by prognostic markers of breast cancer, it is important to investigate the effects of these interactions in predicting breast cancer-specific mortality. Hence, the study is an important contribution to the literature on folate metabolizing pathway and breast cancer-specific mortality.

LIMITATIONS

One of the potential limitations of this dissertation project is its small sample size. However, the study participants were followed for a maximum of 11.5 years which helped enhance the power of the study to a great extent.

Secondly, in this study only the samples from New Mexico site of the HEAL study were used. The sample consisted of 22% Hispanic and 78% non-Hispanic white ethnicity. There was, however, limited power to investigate ethnic-specific associations. Third, the study also had limited power to investigate the effects of folate and B- complex vitamins on the association of these genes in the folate metabolizing pathway and breast cancer mortality. Although interactions with chemotherapy and genes in the folate metabolizing pathway in predicting breast cancer-specific mortality rates were investigated, effect modifications with individual chemotherapeutic regimens and breast cancer-specific mortality could not be studied due to lack of power. Potential three way interactions may exist between genes in the folate metabolizing pathway in predicting breast cancer-specific mortality rates, but was unable to investigate due to limited sample size in this study.

Fourth, all analyses in this dissertation were based on dominant models. It would be of interest to know the strengths of the associations between genes in the folate metabolizing pathway and breast cancer-specific mortality and all-causes mortality when other models are used but owing to limited power in this cohort, results of other model types would be unstable.

Fifth, the current study did not investigate three other genes that influence folate metabolism: dihydrofolate reductase (*DHFR*) and thymidylate synthetase (*TYMS* or *TS*) and replication factor C1 (*RFC1*), which were studied in the Long Island Breast Cancer Study¹⁹ and have been investigated by researchers who have studied the pathway in relation to etiology of other cancers. *TYMS* and

DHFR serve important roles in folate metabolizing pathway. *TYMS* catalyzes the conversion of dUMP to dTMP, which is important for DNA synthesis and repair¹¹⁵, while 5,10-methylene-THF, a substrate of *TYMS*, is obtained from thymidine synthesis, purine synthesis, and DNA methylation²¹. Commonly used chemotherapeutic agents, 5-FU and MTX, affect *TYMS* and *DHFR* and thereby block DNA synthesis and cell replication¹⁹. However, unlike the SNPs corresponding to *MTHFR*, *BHMT*, *MTR*, *MTRR* and *SHMT* genes, where there was only one nucleotide change among the two variants of the polymorphs, *TYMS*, with repeats at 5'-untranslated region (5'-UTR) and insertion-deletion at 3'-UTR and *DHFR*, which is a 19 bp deletion, requires more advanced laboratory capabilities than were available for this research and therefore were not included. Like *FOLR1*, *RFC1* is responsible for transportation of 5-methylTHF from plasma to cytosol¹¹⁶. Although a commonly investigated SNP, A80G (rs1051266) polymorph of *RFC1*, was not investigated in this dissertation because its population allele frequency (Minor allele frequency: 0.45), was beyond the range that was set *a priori* for a SNP to be eligible for investigation in this study (refer to chapter 3 for eligibility criteria for SNP selection).

Sixth, in the preliminary study the association between *MTHFR* polymorphisms and breast cancer survival (see Chapter IV for details) was investigated using data from all three HEAL Study sites (N= 772). Unlike the dissertation project where both baseline and follow-up data were used, only baseline DNA was used while genotyping the *MTHFR* polymorphisms. In the preliminary study sample, 41 out of the 107 women who died during the follow-up

were from the New Mexico site. For the current project, there was insufficient archival DNA left for genotyping 22 out of the 41 women leading to a systematic selection bias in the sample used in this dissertation. The effect of this bias is reflected in the point estimates of the *MTHFR* polymorphisms in the two studies (compare table 9, chapter IV and table 14, chapter V). In the larger sample, carriers of the variant alleles of *MTHFR* C677T (CT/TT: HR: 0.76; 95% CI: 0.5-1.10) and *MTHFR* A1298C (AC/CC: HR: 0.71; 95% CI: 0.48-1.05) polymorphisms had an inverse association with breast cancer-specific mortality. The direction of the association between *MTHFR* C677T and breast cancer-specific mortality reverses when the analysis is restricted to the New Mexico sample (CT/TT: HR: 2.17; 95% CI: 0.8-5.9).

Lastly, the current research also did not take into consideration competing risks which may have influenced mortality. Previous work has suggested that the genes in folate metabolizing pathway influence the etiology of colon cancer^{21 22 76 93}. It is, therefore, intriguing to test what percentage of the deaths due to cancer other than breast cancer in this sample could be attributable to colon cancer and if they serve as confounding factors in the association between the genes in the folate metabolizing pathway and breast cancer survival. Studies have shown that higher concentration of homocysteine, an amino acid involved in SAM synthesis, elevates the risks of CVD^{94 95} and *MTR*, *BHMT*, *MTRR*, and *MTHFR* catalyzes the conversion of homocysteine to methionine by remethylation (chapter II, figure 1). Homocysteine is excreted by the kidney in moderate amounts⁹⁶ but polymorphisms of genes involved in

homocysteine metabolisms could increase the plasma concentration of homocysteine^{97 98} which is suggestive of increasing the risk for CVD^{99 100}. Since the genes in the folate metabolizing pathway may be involved in altering CVD mortality, it may be considered an important competing risk against the association between genes in folate metabolizing pathway and breast cancer mortality. In spite, of its importance in the literature on the folate metabolizing pathway, it could not be investigated in the current dissertation project owing to limited power.

CONCLUSION

To summarize, genes in the folate metabolizing pathway may play an important role in breast cancer-specific mortality. Gene-gene interactions and interactions between prognostic markers like chemotherapy and ER-status and the genes in the folate metabolizing pathway may influence breast cancer-specific mortality. Because folate metabolism is a complex interconnected mechanism, future research should further investigate the findings of this study and help integrate the biochemical mechanisms of the folate metabolizing pathway with statistical findings of Genetic Epidemiological studies. It will also be valuable to the literature on the folate metabolizing pathway, if future studies investigate the effects of these genes on disease-free survival following breast cancer diagnosis and the role of competing risks on all-causes mortality. Larger cohort and randomized control trails should investigate the finding of the current study in relation to breast cancer survival.

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APPENDIX I

LIST OF ABBREVIATIONS

BHMT: betaine-homocysteine-methyltransferase

BMI: Body Mass Index

CVD: Cardiovascular Disease

DHF: dihydrofolate

DHFR: dihydrofolate reductase

DNA: Deoxyribonucleic acid

dTMP: deoxythymidine monophosphate

dUMP: deoxyuridine monophosphate

ER: estrogen receptor

FOLR1: Folate receptor 1

HR: Hazard Ratio

HEAL: Health, Eating, Activity and Lifestyle study

MTHFR: methylenetetrahydrofolate reductase

MTRR: 5-methyltetrahydrofolate-homocysteine methyltransferase reductase

MTR: 5-methyltetrahydrofolate-homocysteine

MTX: Methotrexate

PR: progesterone receptor

RFC: reduced folate carrier

RT-PCR: real-time polymerase chain reaction

SAH: S-adenosylhomocysteine

SAM: S-adenosylmethionine

SHMT1: serine hydroxymethyltransferase 1

SNP: single nucleotide polymorphisms

THF: tetrahydropfolate

TYMS: thymidylate synthetase

5-FU: fluoropyrimidine

APPENDIX II

INSTITUTIONAL REVIEW BOARD- APPROVAL NOTICE

**UNIVERSITY OF
LOUISVILLE**
It's Happening Here.

Human Subjects Protection Program Office
MedCenter One – Suite 200
501 E. Broadway
Louisville, KY 40202-1798
Office: 502.652.5188 Fax: 502.652.2154

August 17, 2011

Richard Baumgartner, Ph.D.
Department of Epidemiology & Population Health
485 East Gray Street, Room 227A
Louisville, KY 40202

RE: IRB# 319.05/ Health, Eating, Activity and Lifestyle (HEAL) Study

Dear Dr. Baumgartner:

The following items for the aforementioned study have been received by the Human Subjects Protection Program Office and approved by the chair of the Institutional Review Board (IRB) through the expedited review procedure according to 45 CFR 46.110(B):

- Amendment #11, dated 08/01/2011

The modifications include:

- The addition of Ratan Das to the study as key personnel.
- Within an established cohort study, specimens from approximately 800 eligible women will be used (includes all 3 sites). This research includes carrying out DNA extraction and re-aliquoting, as well as, conduct of assays focused on approximately 6 specific polymorphisms conducted on a PCR machine. The purpose of the assay work is to meet aims included in the doctoral dissertation proposal of Nandita Das.

Training for this individual has been verified.

This action will be reported promptly to the IRB at a scheduled full Board meeting. Please send all inquiries and electronic revised/requested items to our office email address at hsppo@louisville.edu.

Sincerely,

Julie L. Goldman, MD
Vice Chair, Biomedical Institutional Review Board
JLG/tkp

APPENDIX III

TAQMAN SNP GENOTYPING

RT-PCR uses TaqMan SNP genotyping assay which includes two PCR primers specific to the locus under investigation that flanks the SNP, and two oligonucleotide TaqMan probes specific to the allele of interest. Each probe has fluorescent dye at the 5' end and a non-fluorescent quencher with a minor groove binder (MGB) at the 3' end¹¹¹. The two fluorescence dyes used here are FAM and VIC. Conventionally the major allele of a SNP is identified by the FAM while the minor allele is identified by the VIC dye. TaqMan genotyping uses a universal master mix to detect the variants of the six SNPs. In a nut-shell, during each PCR cycle, the probe which is hydrolyzed to the allele of interest, emits a fluorescent signal when it cleaves to the 5' exonuclease action of the AmpliTaq Gold DNA Polymerase. The primers amplify the locus of interest in the DNA template and the dye specific to the alleles reports the presence or absence of the allele in the DNA sample^{112 113}. Taq polymerase, which is isolated from thermophilic bacteria, is capable of withstanding very high temperatures, hence used for the purpose. The presence of two probes, each specific to an allele of the SNP under investigation and labeled with two dyes, enables two alleles to be detected in a single tube¹¹³. The following is an animated version of the technique shown in three chronological steps.

Figure 26: Animated representation of the 5' nuclease TaqMan assay process. Adopted from TaqMan SNP genotyping assay protocol^{113 117}

Step 2: Annealing the components of TaqMan assay

Step 3: Polymerization

Below is the protocol developed for genotyping the six SNPs:

Steps for Genotyping:

Instructions to make working and reaction plates for genotyping using RT-PCR

Prepare for bench work:

1. wipe the work bench with 0.01X bleach solution
2. lay out a long sterile sheet
3. Wear gloves

Working plate:

1. Dilute 1X TE buffer to 0.1X by adding de-ionized or double distilled water.
 2. Label each working plate. Be as complete as possible <WP_Experiment name_SNP ID_plate #>
 3. Four designated wells are considered as controls and are filled with de-ionized water. Make sure they are spread out randomly across the plate. (Consult the template for their exact location). Keep their positions constant for all plates for convenience.
 4. Get the DNA box out of the freezer
 5. Thaw each vial by rubbing it with hand in circular motion
 6. Allocate required amount of DNA into the labeled working plate well and dispose the pipette tip (consult excel sheet for DNA volume and concentration)
 7. Using another pipette tip, allocate required amount of TE into each well.
- To determine the amount of DNA and 0.1X TE required to make working plates is derived from the equation:

$$C_1V_1 = C_2V_2$$

$$\Rightarrow V_1 = [C_2V_2] / C_1$$

Here, C_1 = Initial DNA concentration

V_1 = Total final volume of DNA after addition of 0.1X TE

C_2 = Final DNA concentration

V_2 = Final volume of DNA and 0.1X TE mixture in each well of the working plate

For example, for DNA with initial concentration of 47ng/ μ L (C_1), we

allocate 75 μ L of 0.1X TE to 2 μ L of DNA (V_1),

$$\text{Volume of DNA needed} = [2 \times 75] / 47$$

$$\sim 3$$

$$\text{Volume of 0.1X TE needed} = 75 - 3 = 72$$

In other words, we allocated 3 μ L of DNA (of 47ng/ μ L concentration) in each plate and added 72 μ L of 0.1X TE to make our working stock. Here, enough DNA-TE mixture is made as working stock to allow room for evaporation.

8. Mix the DNA and the TE by using the pipette (counting 1-15 help)
9. Repeat step 2-6, 92 times to fill 92 wells for the working plate
10. Seal the plate with adhesive covers (less expensive non-optical works fine) making sure the edges are smoothed tightly.

11. Centrifuge for 1 minute at 1200 rpm if you are ready to prepare reaction plates. Otherwise, cover with aluminum foil and store in refrigerator for 6-8 months. If stored for long check the amounts in each well for evaporation.

Reaction plate:

1. To prepare the reaction mixture for a particular SNP we need to start with mixing required amounts of assay, de-ionized water and master-mix in a reservoir used for multi-channel pipettor. Consult corresponding charts to ensure the exact amount required for one plate times the total number of plates that will be run in the RT-PCR on a particular lab day (table 18). As the name suggest, in a 96 well plate, there are 96 wells. To make reaction mixture for one well, we need

2X master mix:	10 μ L
40X assay:	0.5 μ L
De-ionized water:	4.5 μ L
Total:	10 μ L

However, additional amount of 10-15% made for each plate to account for evaporation. Therefore, after accounting for the excess, for one plate, we made a reaction mixture of the following:

2X master mix:	1200 μ L
40X assay:	60 μ L

De-ionized water: 540 μ L

Total: 1800 μ L

Below is a quick look at the amounts corresponding to each of the three ingredients required to prepare reaction mixtures

Table 18: Amounts of ingredients required to make reaction mixtures:

# of plates	# of wells	additional Amts*	2X Master Mix	40X assay	Water	Total
	1		10	0.5	4.5	15
1	96	120	1200	60	540	1800
2	192	220	2200	110	990	3300
3	288	330	3300	165	1485	4950
4	384	450	4500	225	2025	6750
5	480	540	5400	270	2430	8100
6	576	660	6600	330	2970	9900

The “total” column can be double checked by multiplying column “additional amts” by 15; for example, 330 X 15 = 4950. Stocks can be stored in the refrigerator for no more than 2-3 days.

2. Label each reaction plate. Be as complete as possible <RP_Experiment name_SNP ID_plate #>
3. Using an 8 tip pipettor, allocate required amount of standardized DNA-TE into all 96 wells (Consult the template for each experiment for the exact location of each ID). Dispose the pipette tips after every row.
4. Using an 8 tip pipettor, pipette the master-mix cocktail into the wells, one row at a time. Dispose the tips after every row.
5. Once all the 96 wells are filled, seal the plate with optical adhesive cover
6. Centrifuge the plate for 1 min at 1200 rpm.

7. Open the relevant template in the computer/RT-PCR corresponding to the experiment.
8. In the "Advance Set-up" tab type in the experiment name and number, and select the following tabs:
 - Step-one Plus instrument (96 well plates)
 - Genotype
 - TaqMan reagent
 - Standard (speed)

The rest of the conditions are pre-set.

9. In the "Plate Set-up" tab, double check the " View plate layout" and "View well table" to make sure the right templates are open for the experiment
10. Stick the plate into the RT-PCR and let the reaction begin. The reaction last for about 1hr 30 mins to complete.
11. Once the reaction is over check if the controls are correctly detected.
12. Save the output in the computer and the USB drive.

Thermal cycler:

13. While one plate is running in the RT-PCR machine, a second one can be inserted in the thermal cycler. Select "SNP assay PCR" and click "run".

The reaction last for about 1hr 30 minutes.

14. Once it is over, if the RT-PCR is running a plate or you are running out of time for the day at the lab, it is okay for the plate to be stored in the refrigerator for a day. Just remember to (a) cover it with aluminum foil prior

to storage and (b) centrifuge the plate for 1 min at 1200 rpm prior to running it on the RT-PCR machine.

15. Make sure you have opened the right template for the reaction.

16. In the “Run Methods” tab, open the Graphical view tab, select and delete the following sections:

- Pre-PCR Reading (Holding stage)
- Holding Stage
- Cycling Stage

Only the final step, called the “Post-PCR Read (Holding Stage)” where the end point of the reaction is processed in the RT_PCR machine.

17. Make sure options “Pre-PCR Reading” and “Amplification” are checked.

18. Insert the plate in the RT-PCR machine and click “run”.

19. Reaction will last for approximately 2 minutes.

20. Once the reaction is over check if the controls are correctly detected.

21. Save the output in the computer and the USB drive.

If there is not enough time on the day reaction plates are prepared, the reaction plates can be stored in the refrigerator (for about 2-3 days) after step 6 is completed. Start by centrifuging (step 6 onwards) the following lab day.

CURRICULUM VITAE

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Education

Ph.D. (2012): Epidemiology and Population Health, University of Louisville
Mentor: Dr. Richard N. Baumgartner, PhD
Area of Specialization: **Cancer and Nutrition**; Current GPA: **3.9 out of 4.0**

M.A. (2006): Sociology, University of Louisville.
Mentor: Dr. Susan E. Kelly, PhD
Area of Specialization: **Medicine and Health**; GPA: **4.0 out of 4.0**

M.A. (2001): English, Department of English and Foreign Languages, Tezpur University, Naapam, Tezpur, Assam, India
Area of Specialization: English Literature, British Drama; Award: **1st Class 2nd Position**

B.A. (1999): English Major, Darrang College, Tezpur, Assam, India

Employment History

01/2012-05/2012: Dissertation Completion Award

01/2010- 12/2011: Graduate Assistant, Susan G. Komen trainee
Long-Term Quality of Life study, a follow-up of the New Mexico Women's Health Study

- ❖ Data cleaning: LTQOL study
- ❖ Data analysis: LTQOL study, NMWHS
- ❖ Report writing and presentation
- ❖ Subject recruitment using NMTR info
- ❖ Data collection (telephone interview): LTQOL study

- ❖ Data management (subject tracking using Oracle software and Microsoft Access, data filing system, data entry, and quality control)
- ❖ Genotyping using PCR technology: HEAL sample

08/2008-12/2009: Graduate Assistant

Department of Epidemiology and Population Health, University of Louisville

Teaching assistant: Tutoring (Fall 2009), Epidemiological Methods (Fall 2008, Spring 2009)

Research assistant: for Cancer research projects:

- ❖ Data analysis: HEAL study and KCR data
- ❖ Data collection (telephone interview): LTQOL study
- ❖ Data management: LTQOL study

06/2008-07/2008: Program Assistant

Cancer Prevention and Control Program, James Graham Brown Cancer Center, University of Louisville

- ❖ Data analyze: SEER Cancer Registries
- ❖ Write proposals for research on subsequent cancers following first primary cancer of the breast, cervix uteri, prostate, lung, or colorectal

01/2008-05/2008: Research Assistant, (Part-time)

Diabetes Self-Management program, Department of Psychology, University of Louisville

- ❖ Subject recruitment at UofL Hospital
- ❖ Data collection (Mail-in-interview)
- ❖ Data entry

09/2006- 12/2006: Research Coordinator, (Volunteer) Urogynecology

Specialists of Kentuckiana, Louisville

- ❖ Subject recruitment at Norton Hospital
- ❖ Data collection
- ❖ Preliminary data analyses

10/2001- 02/2002 and 09/2002-12/2002: Lecturer (Part-Time)

Darrang College, Tezpur, Assam, India

- ❖ Taught English language and phonetics
- ❖ Taught critical thinking and writing skills

Skills

- ❖ Excellent leadership and inter-personal skills
- ❖ Excellent organizational capacity
- ❖ Expertise in multi-tasking and trouble shooting
- ❖ Experience in working individually and with multi-disciplinary teams
- ❖ Expertise in research and analysis of robust data

- ❖ Expertise in comprehensive documentation of methodologies and findings of analysis and writing reports
- ❖ Expertise in presentation of analysis findings

Memberships

Since 2008	American Association for Cancer Research Associate member
Since 2008	Society for Epidemiological Research
Since 2006	North Central Sociological Association
2005-2006	Graduate Student Council University of Louisville, Louisville, KY
Since 2005	Alpha Kappa Delta International Sociological Honor Society

Honors and Awards

Spring, 2012	Dissertation Completion Award
2010	Society of Epidemiological Research (SER) Student Caucus Travel Award to present poster at 2010 SER Annual Meeting, Seattle, WA.
2009	Graduate Teaching Academy, University of Louisville
2008	1st Prize at Research! Louisville for the poster: Home-based Tele-monitoring with or without Disease Management: Impact on Quality of Life and Self Efficacy in Patients with Heart Failure.
2006	Future Faculty Certificate awarded from North Central Sociological Association
2005	Alpha Kappa Delta, International Sociological Honor Society, for the study: <i>Psychosocial Factors of Pregnancy</i>
2001	1 st class second position in M.A. (English)
1994- 1996	Special Merit Scholarship, State Level Advisory Committee, Students and Youth Welfare, Government of Assam, India

1994-1995 Junior Combined Merit Scholarship,
Government of Assam, India

Community Service

2006- 2010 **Adult Tutor**, Adult Literacy Program of
The Life-Long Learning Center,
Down Syndrome of Louisville, Inc.

2006 **Note Taker**, Disability Resource Center,
University of Louisville

Studies in process for presentation and publication

Submitted Presentation:

Das, N., Baumgartner, K.B., Baumgartner, R.N. (March 4-5, 2010) MTHFR polymorphisms modifies the association of chemotherapy with breast cancer survival, Annual Healthy Eating Activity Lifestyle (HEAL) Meeting, National Institute of Health (NIH), National Cancer Institute (NCI), Rockville, Maryland.

Das, N., Tate, S., Kelly, S.E. (March 23-26, 2006) Success of Laparoscopic Sacrocolopexy Using Synthetic Mesh, North Central Sociological Association, Indianapolis, Indiana.

Paper/Poster Published:

Das, N., Baumgartner, K.B., Ulrich, C.M., Rai, S.N., Kerber, R.A., Baumgartner, R.N. (September 8-11, 2012) Genetic polymorphisms in the folate metabolizing pathway and breast cancer-specific survival
Abstract Submitted: Annual meeting of the American College of Epidemiology, Chicago, Illinois.

Das, N., Baumgartner, R.N., Pinkston, C.M., Baumgartner, K.B. (Sep 18-21, 2011)
Antioxidant intake and breast cancer survival among Hispanic and non-Hispanic white women: the New Mexico Women's Health Study.
Poster presentation: 4th AACR conference on Science of Cancer Health Disparities in Race/Ethnicity and the medically underserved and Susan G. Komen Annual PI and Trainee meeting.

Das, N., Baumgartner, R.N., Baumgartner, K.B. (June 18- 24, 2011) Consistency among participants in a breast cancer follow-up study.
Poster presentation: North American Association for Central Cancer Registries (AACCR), Louisville, Kentucky.

Das, N., Baumgartner, K.B., Kerber, R.A., Rai, S.N., Ulrich, C., Neuhouser, M.L., Bernstein, L., McTiernan, A., Ballard-Barbash, R., Baumgartner, R.N. (April 2-6, 2011) Associations between genetic variability in folate metabolism and breast cancer survival.

Poster presented: 102nd Annual Meeting, American Association for Cancer Research (AACR), Orlando, Florida.

Das, N., Baumgartner, K.B., Kerber, R.A., Baumgartner, R.N. (Nov 5, 2010) MTHFR polymorphisms modifies the effect of chemotherapy on breast cancer mortality and disease free survival: the Healthy, Eating, Activity, and Lifestyle (HEAL) study.

(with updated results from poster presented at Society of Epidemiological Research)

Poster presented: James Graham Brown Cancer, 9th Annual Retreat, Louisville, Kentucky.

Das, N., Baumgartner, K.B., Kerber, R.A., Baumgartner, R.N. (Oct 11-15, 2010) MTHFR Polymorphisms modify the association of chemotherapy with breast cancer survival. (with updated results from poster presented at Society of Epidemiological Research)

Poster presented: Research! Louisville, Louisville, Kentucky.

Das, N., Baumgartner, R.N., Baumgartner, K.B., Ulrich, C., Bernstein, L., McTiernan, A., Ballard-Barbash, R. (June 23-26, 2010) MTHFR polymorphisms modifies the association of chemotherapy with survival in breast cancer.

American Journal of Epidemiology, 171 (11)

Poster presented: 43rd Annual Meeting of the Epidemiological Research, Seattle, Washington. Society of Epidemiological Research (SER) Student Caucus Travel Award

Das, N., Groves, F., Gupta, R.C. (June 23-26, 2009) Racial disparities in recurrence-free cervical cancer survival in Kentucky. American Journal of Epidemiology, 169 (11)

Poster presented: 42nd Annual Meeting of the Society of Epidemiological Research, Anaheim, California.

Das, N., Groves, F., Gupta, R.C. (2008) Risk of recurrence among cervical cancer survivors in Kentucky, 1995-2005

Poster presented: James Graham Brown Cancer, 7th Annual Retreat, Louisville, Kentucky.

Das, N., Hornung, C.A. for the "Home Heart Failure Care Comparing Patient Driven Technology Models" Investigators. (2008) Home-based Tele-monitoring with or without Disease Management: Impact on Quality of Life and Self Efficacy in Patients with Heart Failure.

Poster presented: Research!Louisville, Louisville, Kentucky. 1st place award for poster presentation.

Rogers, W., Meyer, J., Rothschild, C., Bonner, J., **Das, N.**, Richardson, K., Mokshagundam, S.P., Krishnasamy, S., Kong, M., Stetson, B. (2009). Social isolation is associated with geographic socioeconomic status and quality of life in at-risk, underserved adults with type 2 diabetes. *Annals of Behavioral Medicine*, 37, s199

Poster presented: 30th Annual Meeting of the Society of Behavioral Medicine. Montreal, Canada.

Richardson, K., Rogers, W., Bonner, J., Rothschild, C., Meyer, J. **Das, N.**, Mokshagundam S.P., Krishnasamy, S. Kong, M., Stetson, B. (2009). Geographic socioeconomic status, health literacy and perceived barriers to self-care in at-risk, underserved adults with type 2 diabetes. *Annals of Behavioral Medicine*, 37, s197

Poster presented: 30th Annual Meeting of the Society of Behavioral Medicine. Montreal, Canada.

Furr A, **Das N**: The Effects of Social Autonomy on BMI Scores: A study of women in Nepal. *Contributions to Nepalese Studies*: January 1, 2006; 33(1), 17

Manuscript in the process of publication:

Das, N., Baumgartner, K.B., Kerber, R.A., Ulrich, C., Neuhaus, M.L., Bernstein, L., McTiernan, A., Ballard-Barbash, R., Baumgartner, R.N. Association between MTHFR polymorphisms and the effect of chemotherapy on breast cancer mortality: the Healthy, Eating, Activity, and Lifestyle (HEAL) study

Das, N., Baumgartner, K.B., Kerber, R.A., Ulrich, C., Rai, S.N., Neuhaus, M.L., Bernstein, L., McTiernan, A., Ballard-Barbash, R., Baumgartner, R.N. Competing risk factors of breast cancer mortality and genes in one-carbon metabolizing pathway: the Healthy, Eating, Activity, and Lifestyle (HEAL) study

Das, N., Baumgartner, R.N., Pinkston, C.M., Baumgartner, K.B. Vitamins and minerals intake and breast cancer survival among Hispanic and non-Hispanic white women: the New Mexico Women's Health Study.

Das, N., Baumgartner, R.N., Pinkston, C.M., Baumgartner, K.B. Treatment related risk factors of arm lymphedema among Hispanic and non-Hispanic white breast cancer survivors

Das, N., Baumgartner, R.N., Pinkston, C.M., Baumgartner, K.B. Association between antioxidant intake and risk for arm lymphedema among Hispanic and non-Hispanic white breast cancer survivors

Past Research Support:

- ❖ **Role: Graduate Research Assistant**
Susan G. Komen Foundation. Grant No: KG090926
Long Term Quality of Life, follow-up of New Mexico Women's Health Study
University of Louisville, Louisville, Kentucky
- ❖ **Role: Graduate Research Assistant**
National Institute of Health Grant, "Ethnicity, Breast Cancer Recurrence and Long-Term QOL" Grant No: 5R01CA105266
- ❖ **Role: Graduate Assistant**
Department of Epidemiology and Population Health, School of Public Health and Information Sciences
- ❖ **Role: Program Assistant**
Gupta R C: Agnes Brown Duggan Endowment, Cancer Prevention and Control Program, James Graham Brown Cancer Center.
- ❖ **Role: Research Assistant**
Stetson B A (PI) Validation of Diabetes Self-Management Survey with Minority and Underserved Adults. University of Louisville Intramural Research Grant 2007-2008

Current research projects:

Baumgartner RN and Baumgartner KB (PI): Folate metabolizing pathway and breast cancer survival (Healthy Eating Activity and Lifestyle (HEAL)). Data collection funded by National Cancer Institute, National Institutes of Health, SEER Grant No: N01-PC-05031-20. Additional funding assistance through the Susan G. Komen Foundation, Grant No: KG090926.

Baumgartner KB (PI): Long Term Quality of Life, New Mexico Women's Health Study. Grant No: 5R01CA105266.