GENETIC VARIATION OF THE FOLATE METABOLIC NETWORK AND CARDIOVASCULAR DISEASE

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Farbod Raiszadeh

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Farbod Raiszadeh, Ph.D.

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Common chronic diseases including cardiovascular disease (CVD) are the leading cause of morbidity and mortality in developed countries. These diseases are multifactorial in origin with both genetic and environmental components that act through a complex network of gene-gene and gene-environment interactions to generate the health or disease phenotype. The folate metabolic network plays an important role in a variety of fundamental intracellular functions including remethylation of homocysteine to methionine, DNA synthesis and repair, DNA methylation, protein synthesis and cell-signaling. The overall objective of this work is to understand the role of genetic variation in the human folate metabolic network in cardiovascular risk. We have focused on five potentially-functional candidate SNPs in four genes involved in sequential reactions, namely *methylene-tetrahydrofolate reductase (MTHFR), methylene-tetrahydrofolate dehydrogenase (MTHFD), methionine synthase (MTR),* and *cystoplasmic serine hydroxymethyl transferase (cSHMT)*. We use data from nested case control studies of cardiovascular disease conducted in the framework of two large epidemiological cohort studies, the Nurses' Health Study (NHS) and the Normative Aging Study (NAS). Our main findings are the presence of gene-nutrient interaction between folate and the *MTHFR* 677 polymorphism in predicting serum homocysteine levels, the presence of gene-gene

interaction between the *MTHFR* 677 and *MTHFD* 1958 polymorphisms in predicting CVD risk, the presence of gene-gene interaction between the *MTHFR* 1298 and *MTR* 2756 polymorphisms, strengthening of the above two interactions with inclusion of serum homocysteine levels in the models, and the partial replication in a nested case-control study of women of a gene-gene interaction between *MTHFR* 677 and *cSHMT* 1420 polymorphism previously detected in a study on men. In summary, we have found evidence for the presence of gene-gene interaction between variants in genes encoding sequential reactions in the folate metabolic network. Lack of mediation by homocysteine suggests that other folate-related markers need to be studied to understand the pathophysiologic route from genotype to disease phenotype. Our findings suggest the importance of evaluating gene-gene interactions, especially among genes with functional connections, in epidemiologic studies of complex disease in general, and cardiovascular disease in particular.

BIOGRAPHICAL SKETCH

Farbod Raiszadeh was born to Pourandokht Zahedi and Hossein Raiszadeh in 1973 in Tehran, Iran. He attended medical school at Tehran University of Medical Sciences, graduating with an MD degree in 1999. For two years, he studied the epidemiology of cardiovascular disease and vitamin D deficiency at the Endocrine Research Center in Tehran. In 2001, he started his PhD study in the field of Human Nutrition at the Division of Nutritional Sciences at Cornell University. He has minored in the fields of Genetics and Development and Statistical Genomics. After graduation from Cornell University, he is pursuing clinical training in Internal Medicine in New York City.

Dedicated to the living memory of Farzad

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CHAPTER 1

FOLATE METABOLISM AND CARDIOVASCULAR DISEASE: BACKGROUND AND SIGNIFICANCE

Cardiovascular Disease: Genetic and Environmental Web

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in industrialized, developed nations [1]. By the year 2020, it is estimated that CVD will surpass infectious diseases as the world's leading cause of death and disability [1]. CVD is multifactorial in origin with both genetic and environmental components. Typically, CVD shows some degree of familial aggregation, but it does not segregate in families as single gene disorders do. In fact the distribution of disease among individuals, families, and populations is a direct consequence of the distribution of interactions between the effects of many susceptibility genes and many environmental exposures [2]. This complex web of interactions works through dynamic, epigenetic, and regulatory mechanisms to ultimately become integrated and generate the health or disease phenotype [3-8].

Traditionally, the epidemiologic study of human disease has focused on selected possible risk factors, and determining the degree of contribution of that factor to the risk of disease in a population. The field of epidemiology has succeeded in determining a considerable number of constitutional as well as modifiable lifestyle factors that contribute to a person's risk of developing cardiovascular disease. These so-called "traditional" risk factors include male gender, age, hypertension, diabetes mellitus, family history of premature CVD, elevated levels of plasma low-density lipoprotein cholesterol, decreased levels of high-density lipoprotein cholesterol, and elevated levels of plasma total homocysteine. The study of genetic factors that

contribute to human disease has been mostly driven by the "nature vs. nurture" paradigm, in which the focus of the investigation is on determination of the competing contribution of genetic and environmental factors. The observation that genetic variation and environmental exposure variation are elements that work together to produce an outcome is well understood in the theory of human genetics, and is increasingly being incorporated into the design of epidemiologic studies of disease.

The completion of the human genome project has provided the full inventory of genes that specify all of the components that constitute human cells and has therefore provided an unprecedented opportunity to add multiple levels of complexity to the study of human disease risk. Equally important, ongoing initiatives to identify and map single nucleotide polymorphisms (SNP) [9, 10] and human haplotypes [10] within the human genome offer the possibility of understanding the molecular basis for gene-environment interactions that determine human phenotypic variation [11]. Given the new tools and opportunities of the genomic age, investigators have shown that genetic factors comprise a considerable amount of interaction among genes, manifested as a high level of inter-dependence among genes and an extreme sensitivity to subtle background genetic variation [12]. The presence of this highly interconnected, highly interactive system has been shown in different model systems [13, 14].

The implication of considering networks as functional systems of action in biology is that network elements are highly dependent on the background state of the system, and are highly versatile and can assume new roles in the system if conditions call for it. In addition, the relationship between a factor and an outcome is not necessarily linear. Most of these network-dependent aspects of biological systems have been ignored in the study of human disease, and have therefore limited the potential for greater understanding of the true interactive mechanisms that underlie the development of disease [2].

The path from a genotypic variant to an observable clinical outcome is not always a straightforward linear relationship. Often, there are intermediate risk factors and intermediate phenotypes that are affected by the genotypic variant. Changes in homocysteine level due to genotypic variants in the folate metabolism genes and the ensuing alteration in endothelial function, blood pressure regulation, and plaque build-up are examples of these intermediary outcomes (Figure 1.1). Consideration of these factors in the design of epidemiologic studies of complex disease can provide valuable insights into the pathophysiology.

Genetic variation in folate metabolism and cardiovascular risk

The consequences of altered folate homeostasis in neural tube defects are well-known, and recent studies implicate disrupted folate metabolism in the pathogenesis of both cardiovascular disease and cancer. Cytoplasmic folate is needed for the biosynthesis of purines, thymidylate, and methionine. These products play a fundamental role in remethylation of homocysteine to methionine, DNA synthesis and repair, DNA methylation, protein synthesis and cell-signaling. Folate metabolism may be disrupted by vitamin deficiency, genetic predisposition, and by medical therapies

Such metabolic disruptions lead to various biochemical consequences including elevated levels of plasma homocysteine (a proposed causal pathway for cardiotoxic effects), increased uracil misincorporation into DNA due to disrupted thymidylate synthesis, and hypomethylation of DNA due to reduced methylation potential by S-adenosyl methionine (SAM) (the latter two are proposed causal pathways for carcinogenic effects). Thus, genetic polymorphisms in enzymes that regulate the distribution of intracellular forms of folate and control serum homocysteine levels have the potential to produce diverse clinical consequences including heart disease and cancer.

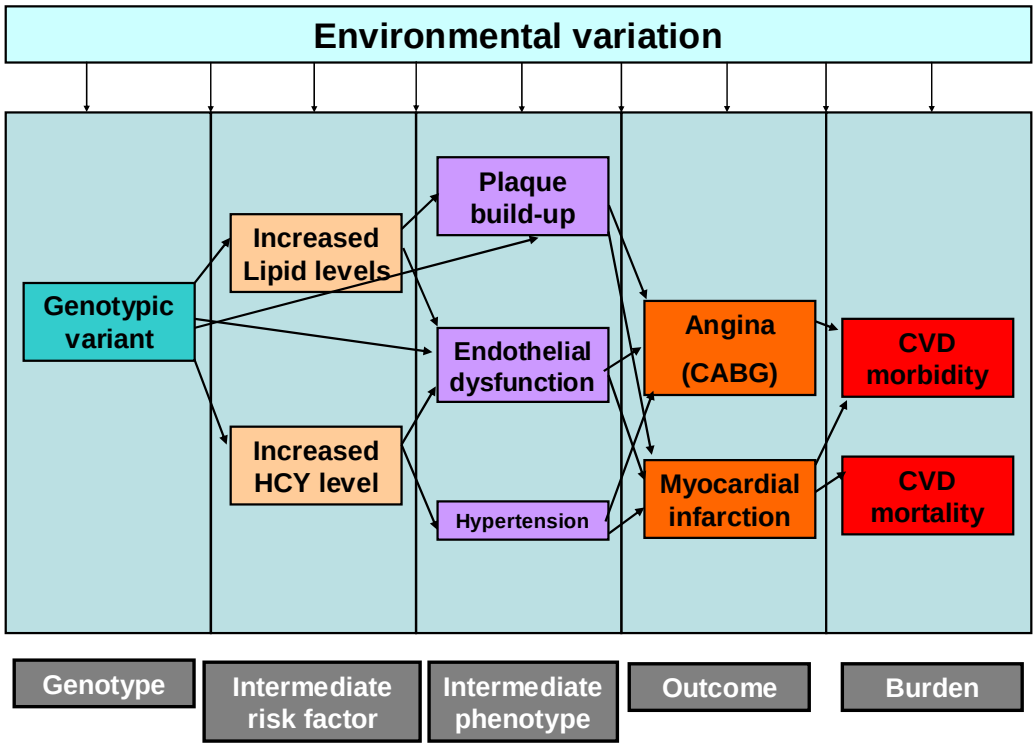

Figure 1.1. The pathway postulated to connect genotype to phenotype in atherosclerosis. (abbreviations: HCY, homocysteine; CABG, coronary artery bypass graft; CVD, cardiovascular disease)

Homocysteine is hypothesized to be a key player in the pathogenesis of cardiovascular disease [15]. It is a sulphur amino acid that is formed by the demethylation of the essential amino acid methionine via S-adenosylmethionine (SAM or AdoMet) and S-adenosylhomocysteine (SAH or AdoHcy) [16]. SAM is the methyl donor in numerous methylation reactions including DNA and RNA methylation, and is synthesized from methionine in a reaction catalyzed by the enzyme, methionine adenosyltransferase (MA). Homocysteine is in turn metabolized through two separate pathways, transsulfuration and remethylation (Figure 1.2). The first step in the transsulfuration pathway is the condensation of homocysteine and serine to produce cystathionine. This step is catalyzed by cystathionine β-synthase (CBS). Cystathionine is subsequently hydrolyzed to cysteine by γ-cystathionase. Homocysteine can be remethylated to methionine by two different enzymatic reactions: methionine synthase (MTR), with 5-methyltetrahydrofolate (5-methyl-THF) as methyl donor; or betaine homocysteine methyltransferase (BHMT), with betaine (trimethylglycine) as methyl donor. 5-methyl-THF is in turn formed by the reduction of 5,10-methylene-tetrahydrofolate, a reaction that is catalyzed by the enzyme 5,10-methylene tetrahydrofolate reductase (MTHFR). The reversible conversion of serine and tetrahydrofolate (THF) to glycine and 5,10-methylene-THF is catalyzed by cytosolic serine hydroxymethyltransferase (cSHMT). Methylenetetrahydrofolate dehydrogenase (MTHFD) catalyzes three sequential reactions in the conversion of derivatives of THF. Given the complexity of the folate and homocysteine metabolism network, the genes coding for the various enzymes detailed above are plausible candidates for consideration in studies of cardiovascular disease risk.

MTHFR is one of the first genes whose alleles have been linked to alterations in serum homocysteine concentration and cardiovascular risk. In 1988, a variant of the MTHFR enzyme was found to be associated with decreased enzyme activity and

reduced stability after heating[17]. This thermolabile variant of MTHFR was later found to be due to a single base nonsynonymous substitution of C to T at nucleotide 677 (causing an alanine to valine substitution at amino-acid position 222), and to be more prevalent in patients with cardiovascular disease[18, 19]. The association of this polymorphism with coronary artery disease was recently established in a large meta-analysis [20] involving 11,162 cases and 12,758 controls. This meta-analysis estimated that the odds ratio (OR) for 677 TT genotype vs. CC genotype was 1.16 (95% confidence interval (CI) 1.05, 1.28) [21]. The relation between *MTHFR* 677C→T and cardiovascular disease clearly shows evidence of a gene-nutrient interaction, as the associations among the polymorphism, cardiovascular disease, and elevated homocysteine are stronger in persons and in populations with low folate status [21-23]. *MTHFR* variation due to the 677C→T polymorphism is a strong genetic determinant of homocysteine concentration that has been consistently observed in numerous studies, but it accounts for only 25% of the mild hyperhomocysteinemia observed in subjects with vascular disease [24]. This indicates that additional mutations in the *MTHFR* gene (for example *MTHFR* 1298A→C, studied herein) or in other genes within the folate metabolic network may contribute to variation in homocysteine concentrations [16].

The association of common polymorphisms in other genes of the folate metabolic network with arteriosclerosis has been analyzed in only a limited number of studies and remains to be determined [16]. Another gene encoding a critical enzyme in the folate metabolic network is *methionine synthase (MTR).* MTR, a vitamin B12-dependent enzyme, catalyzes the remethylation of homocysteine to methionine using a methyl group donated by 5-methyl-THF, which is the major circulating form of folate in the human body (Figure 1.2). It is therefore biologically plausible that functional genetic variants of the *MTR* gene would alter the homocysteine and folate levels and

in turn affect the pathogenesis of vascular disease. The *MTR* gene was simultaneously cloned by three groups[25-27] and analyzed for the presence of common polymorphisms. The only common polymorphism in the *MTR* gene reported to date is an A→G transition at nucleotide 2756 (*MTR* 2756 A→G) in the open reading frame of the gene, which results in replacing an aspartic acid (D) residue with a glycine (G) at codon 919 (D919G), a potentially functional site of the protein [28]. Studies on the biological effect of this polymorphism have been mostly case-control or cross-sectional in design and also limited by sample size. These studies yielded inconsistent results [29-33], and most have focused on the single effect of the *MTR* polymorphism on cardiovascular risk without consideration of the plausible interaction with the *MTHFR* polymorphisms. In this study, we plan to consider this interaction.

Investigating interactions between genetic polymorphisms is one of the primary objectives of this work. Many of the biologically-relevant SNPs are part of a functional biochemical network, and therefore interact with other elements of the network[2, 12]. While evaluating multiple interactions is severely limited by the power issues resulting from small sample size in many cross-sectional epidemiologic studies, using the data obtained in the large nested case-control study within the Normative Aging Study, we have previously shown that such interactions exist and can be detected between different SNPs of the folate metabolic network [34] (Figure 1.2).

Previous findings from the Normative Aging Study (NAS) relate to a polymorphism in the human cytoplasmic serine hydroxymethyltransferase (*cSHMT* 1420C→T) gene that affects the regulation of intracellular folate [35]. Factors affecting the activity of the cSHMT enzyme, including the polymorphism in question, are expected to relate to the flux of one-carbon folate forms within the cell [36], and ultimately may be associated with health outcomes including neural tube defects, cardiovascular disease and cancer. Intracellular folate cofactors exist in limited

concentrations and numerous folate- dependent reactions compete for available folate.

The competition for 5,10-methylenetetrahydrofolate (5,10-methyleneTHF) is keen given the role of this substrate in three known reactions, and the cSHMT enzyme is likely to be key given its direct effects on the supply or depletion of 5,10-methyleneTHF. Prior research has indicated that complete absence of the functional domain where the *cSHMT* polymorphism resides causes disruptions in one-carbon homeostasis [37]. Under these circumstances (absence of functional domain in cSHMT) reaction pressure may favor serine synthesis, affecting the thymidylate cycle with resultant increased uracil misincorporation into DNA [38]. At the same time, the *cSHMT* polymorphism may lead to the depletion of 5,10-methyleneTHF and subsequently, to the depletion of 5-methylTHF with two results. First, as a required cofactor for homocysteine remethylation, depleted 5-methylTHF leads to homocysteine accumulation, and second, reduced homocysteine remethylation leads to reduced production of SAM and therefore to reduced SAM-mediated methylation (thus, hypomethylation of DNA). Recent evidence suggests the *cSHMT* 1420C→T polymorphism's putative action might be related to sumoylation of the cSHMT protein, affecting transport from the cytoplasm to the nucleus (Stover P, personal communication). In summary, the metabolic disruptions brought about by the *cSHMT* polymorphism have the potential to play a role in the pathogenesis of cardiovascular disease and cancer.

Although the functional consequences of the *cSHMT* 1420C→T polymorphism are not proved, studies demonstrate effects on biochemical phenotype [39], leukemia risk[40], and cardiovascular risk[41]. In a study of mothers of neural tube defect cases, Heil and colleagues reported lower plasma homocysteine in women with the *cSHMT* 1420C→T *CT* or *TT* genotypes compared to women with the *CC* genotype [39]. In a

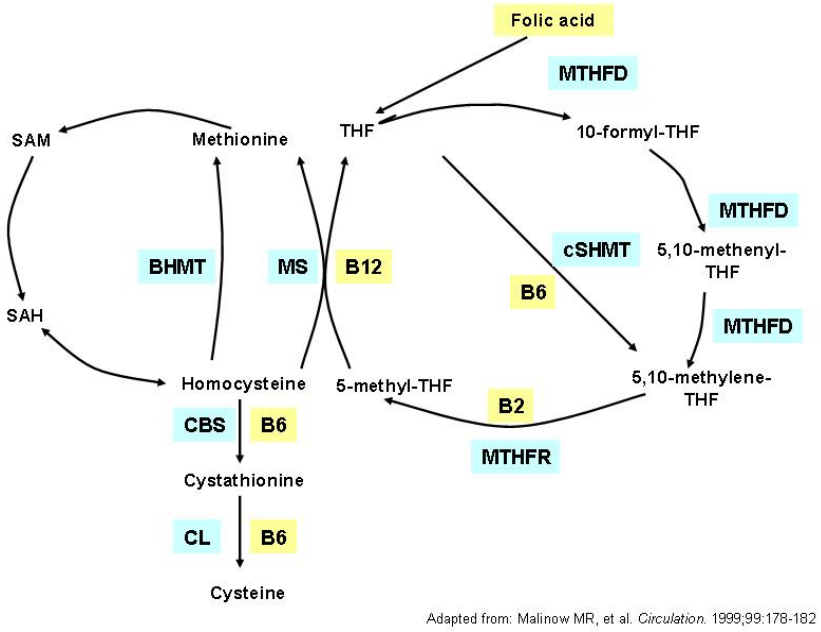

Figure 1.2. Folate metabolic network and homocysteine metabolism. : (BHMT: betaine-homocysteine methyltransferase, MTHFR: Methylenetetrahydrofolate reductase, cSHMT: Cytoplasmic serine hydroxymethyltransferase, B6: Vitamin B6, B12: vitamin B12; CL: cysthationine lyase, ; CBS: cysthationine β-synthase, MTHFD: Methylenetetrahydrofolate dehydrogenase, ; MS: Methionine synthase)

study of 71 individuals diagnosed with acute lymphocytic leukemia (ALL), both the *cSHMT* 1420C→T *CT* genotype and the *TT* genotype were associated with a reduced risk of ALL (odds ratios [OR] 0.48; 0.31, respectively) [40]. In a nested case control study of 535 CVD cases in the Normative Aging Study[41] our group found a strong inverse association of the *cSHMT* 1420C→T *TT* genotype (compared to *CT* and *CC*) with cardiovascular disease risk (OR = 0.45; 95% CI: 0.25, 0.81) among men with the *MTHFR* C→T *CC* genotype. However, the *cSHMT* 1420C→T *TT* genotype was associated with an increased risk of CVD among men heterozygous (OR = 1.59; 95% CI: 1.40, 1.80) or homozygous (OR = 3.11; 95% CI: 1.52, 6.36) for the variant of *MTHFR* 677C→T (*CT*, *TT*, respectively). Thus, the *cSHMT* polymorphism affects cardiovascular disease risk, with strong evidence for a gene-gene interaction ($p<0.05$), making a compelling case for further studies to confirm this finding, and to explore the biochemical phenotype to reveal the metabolic consequences of this genetic variation.

Research Questions

The overall objective of the research presented herein is to understand the role of genetic variation in specific genes in the folate metabolic network in relation to the pathogenesis of cardiovascular disease. The studies describe the extent of genetic variation in selected genes encoding proteins involved in folate metabolism, the effect of that variation on cardiovascular risk, and the role of changes in folate and homocysteine as the intermediary between the genotype and the disease phenotype. Our analysis is guided by the conceptual framework summarized in Figure 1.3, which has been divided into three sections to reflect the three chapters of the dissertation.

The work is focused on four genes that are directly involved in homocysteine remethylation or transsulfuration or that indirectly provide the substrates needed for these reactions. These genes are important in maintaining adequate intracellular folate

pools, and therefore contribute to the *de novo* biosynthesis of purines and thymidylate as well as DNA methylation. This dissertation investigates five potentially-functional candidate single nucleotide polymorphisms in four genes related to folate metabolism, including *MTHFR* 677C→T and 1298A→C, *cSHMT* 1420C→T, *MTR* 2756A→G, and *MTHFD* 1958G→A.

The three chapters address the following specific research questions:

1. To investigate the single, joint, and interactive effects of three single-nucleotide polymorphisms in the *MTHFR* and *cSHMT* genes on plasma homocysteine levels and cardiovascular disease in a case control study of cardiovascular disease in women (NHS), and to explore whether these effects are modified by plasma levels of folate, vitamin B6 and vitamin B12 (Figure 1.3, section B).

2. and **3.** To examine the association of *MTR* 2756A→G, and *MTHFD* 1958G→A with plasma homocysteine levels and with cardiovascular disease in the Normative Aging Study, while considering the effects of gene-gene interaction, mediation by homocysteine levels, and effect modification by folate levels (Figure. 1.3: sections A and C).

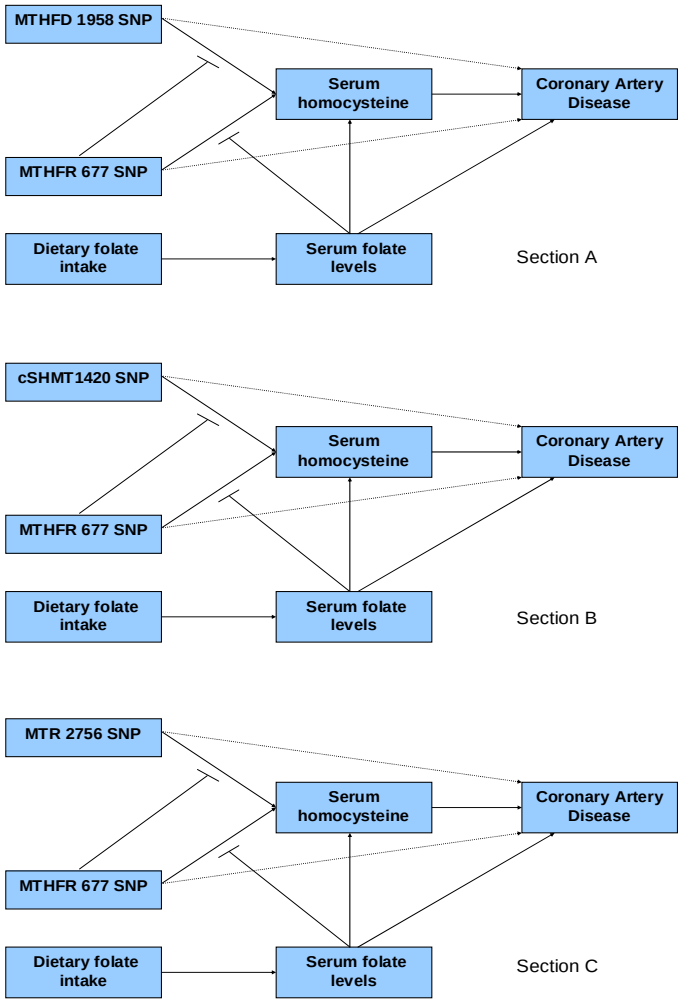

Figure 1.3. Pathway diagram demonstrating the relation between *MTHFR*, *cSHMT*, *MTHFD*, and *MTR* genotypes, serum homocysteine level, and cardiovascular disease risk, including the role of dietary and serum folate as potential effect modifiers (gene-nutrient interaction). (*MTHFR*: *Methylenetetrahydrofolate reductase*, *cSHMT*: *Cytoplasmic serine hydroxymethyltransferase*, *MTHFD*: *Methylenetetrahydrofolate dehydrogenase*, *MTR*: *Methionine synthase*)

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CHAPTER 2

MTHFD1 1958G→A AND MTHFR 677C→T IN RELATION TO SERUM HOMOCYSTEINE CONCENTRATION AND CARDIOVASCULAR DISEASE RISK: THE NORMATIVE AGING STUDY

Abstract

Genetic variants in enzymes of the folate metabolic network are associated with a variety of outcomes. A well-known genetic polymorphism in methylenetetrahydrofolate reductase (*MTHFR* 677C→T) is associated with higher homocysteine level and cardiovascular disease (CVD) risk. Methylenetetrahydrofolate dehydrogenase (*MTHFD1*) catalyzes the interconversion of tetrahydrofolate derivatives needed for purine, thymidylate and methionine synthesis. The *MTHFD1* 1958G→A polymorphism is associated with an increased risk of neural-tube defects. In a matched case-control study nested within the Normative Aging Study, we studied the association of these two polymorphisms with homocysteine and CVD risk, considering gene-nutrient and gene-gene interactions. In regression models with homocysteine as an outcome, folate deficiency and *MTHFR* 677 variant T allele were significantly associated with homocysteine levels. There was also a significant *MTHFR*-folate interaction in models predicting homocysteine concentration. No such interaction was observed for *MTHFD*1 polymorphism. In adjusted conditional logistic models, *MTHFD1 GA/AA* (vs. *GG*) had a statistically non-significant protective effect on CVD risk (OR 0.8; 95% CI 0.6, 1.1), and this effect was similar in folate subgroups. A gene-gene interaction between the two polymorphisms was observed

(overall p=0.24, individual coefficient p values .09 and .35). The increased risk of CVD association with the *MTHFR* 677C→T *TT* genotype was only observed among men with *MTHFD1 GA/AA* genotype (OR 1.6, 95% CI 1.1, 2.4), and was not evident among those with *MTHFD1 GG* genotype (OR 1.1, 95% CI 0.6, 2.2). The *MTHFR* 677C→T *CT* genotype increased CVD risk among men with *MTHFD1 GA/AA* genotype (OR 1.2; 95% CI 0.9, 1.7), but had little or no effect in men with the *MTHFD1 GG* genotype (OR 0.8; 95% CI 0.5, 1.3). The findings support a gene-gene interaction between two important genes of the folate metabolic network. This interaction does not seem to be mediated by alterations in plasma homocysteine levels.

Introduction

Genetic variants in enzymes of the folate metabolic network are associated with a variety of disease outcomes, including cardiovascular disease (CVD)[1]. The most widely-studied variant is the *MTHFR* 677 C→T polymorphism. This common polymorphism results in an alanine to valine substitution in position 222 of the encoded protein, is relatively common in populations of Caucasian descent, and is associated with lower catalytic activity of the MTHFR enzyme[2]. Epidemiologic studies have demonstrated that the *MTHFR* 677 C→T polymorphism is associated with increased serum homocysteine concentration and CVD risk, especially in folate-deficient populations, indicating a gene-nutrient interaction[3]. This *MTHFR* genotype explains some of the variation in disease risk observed in different populations, but since folate metabolism and homocysteine remethylation are mediated by a complex network of enzymes, attention has shifted to include other genes.

Another important enzyme in the folate metabolic network is methylenetetrahydrofolate dehydrogenase (*MTHFD1*), a trifunctional enzyme that catalyzes the sequential interconversion of tetrahydrofolate derivatives required for

purine, thymidylate, and methionine synthesis. The trifunctional enzyme comprises the following functions: N10-formyltetrahydrofolate synthetase; N5,N10-methenyltetrahydrofolate cyclohydrolase; and N5,N10-methylenetetrahydrofolate dehydrogenase[4]. The final step produces a key substrate in folate metabolism, namely 5,10-methylenetetrahydrofolate. A common polymorphism in the *MTHFD1* gene, 653R→Q, resulting from an A to G transition at nucleotide position 1958, has been detected in the synthetase domain of the protein[5]. The effect of this polymorphism on the catalytic activity of the enzyme is unknown. Several recent reports investigated this variant in relation to developmental outcomes and chronic diseases, and reported that *AA* variants were at increased risk of neural tube defects and placental abruption compared to the *GA/GG* genotypes[4-10]. The variant had little or no association with the risk of colorectal cancer [11]. In the three studies that investigated the association of the *MTHFD1* 1958G→A genotype with homocysteine levels, no statistically significant relation was observed between the variant allele and serum homocysteine levels[11-13].

A key intersection in folate metabolism is 5,10-methylenetetrahydrofolate (5,10-methyleneTHF), which is the substrate for three enzymes: methyleneTHF reductase (MTHFR), cytosolic serine hydroxymethyl transferase (cSHMT), and thymidylate synthase (TS). TS uses 5,10- methyleneTHF as a substrate to synthesize the thymidine residues needed for DNA replication and repair[11]. cSHMT uses 5,10-methyleneTHF as a substrate in the interconversion of glycine to serine[14]. MTHFR uses 5,10- methyleneTHF for provision of 5-methylTHF to the homocysteine remethylation reaction. Genetic variants of *MTHFR* or *MTHFD1* that result in reduced enzyme activity or changes in gene expression are hypothesized to result in higher serum homocysteine concentration through decreased availability of 5-methylTHF for folate-dependent remethylation of homocysteine. In addition, since these two enzymes catalyze sequential steps at a critical point in the folate metabolic network, we

hypothesized a biological interaction between the enzymes.

The objective of this study was to evaluate the relation of *MTHFR* 677C→T and *MTHFD1* 1958G→A genotypes with serum homocysteine concentration and with cardiovascular disease risk: prior research on *MTHFR* supports increased risk associated with the *T* allele, and prior research on *MTHFD1* suggests increased risk associated with the *A* allele. We also sought to investigate the *MTHFD1* by *MTHFR* interaction. Effects on CVD risk are hypothesized to be mediated in part by homocysteine elevation (a biological marker of folate-dependent methylation), thus the role of homocysteine as a mediator was assessed. Finally, folate status is a possible effect modifier of these associations, and thus gene-nutrient interactions were considered.

Methods

The study population and data collection have been described previously[15]. In brief, a nested case—control study was conducted within the prospective Normative Aging Study (NAS) cohort. Initially 2,280 men aged 21-81 (mean age 42 years at study entry) were included in the study: intake into the cohort occurred between 1961 and ’63. Exclusion criteria included past or current chronic conditions, including coronary heart disease, hypertension, diabetes, cancer, peptic ulcer, gout, asthma, chronic bronchitis, and chronic sinusitis[16]. Participants have undergone comprehensive clinical examinations at 3- to 5-year intervals, with a response rate of greater than 90% for mailed questionnaires. The overall annual attrition rate from all causes was less than 1%.

From 1961 to 1998, 749 incident cases of cardiovascular disease (CVD) occurred (including coronary heart disease (CHD) and stroke). Possible occurrences of CHD, including angina pectoris and nonfatal myocardial infarction (MI), were

evaluated based on medical records and physician examination[17]. Possible occurrences of stroke were confirmed by neurologists who reviewed individual medical records[18]. CHD or stroke mortality was confirmed by death certificates, which were coded according to the eighth revision of the International Classification of Diseases[18]. About six percent of cases had only stroke. DNA was available only for more recent cases, thus 535 incident CVD cases were studied. The cases without DNA were older at study entry, had slightly higher systolic blood pressure and greater cumulative smoking exposure.

A total of 1,048 matched controls were selected from the cohort by risk set sampling, and two controls were matched to each case by age at onset and birth period (in five-year intervals) of the case. The final list of distinct individuals selected included 535 cases and 547 non-cases, with a maximum of 2 controls matched to each case. The study was approved by the Brigham and Women's Hospital Human Subjects committee, the Veterans' Administration R & D committee, and the Cornell University Committee on Human Subjects.

At each follow-up examination, extensive information on lifestyle variables and physical examination results were collected and a blood sample was drawn and stored. Beginning in 1987, men completed the Willett semi-quantitative food frequency questionnaire (FFQ) on dietary intake in the past year. Estimations of dietary intake, including B vitamins, were derived from the FFQ using software developed by the Nurses Health Study[19]. At least one FFQ measurement was available prior to the date of diagnosis for about half of the CVD cases (246 out of 535) and half of the non-cases (307 out of 547). Lipids were assayed over the course of the follow-up as follows: serum cholesterol was assayed enzymatically (SCALVO Diagnostics, Wayne, New Jersey); HDL cholesterol was measured in the supernatant after

precipitation of the LDL cholesterol and very low density lipoprotein fractions with dextran sulfate and magnesium, using the Abbott Biochromatic Analyzer 100 (Abbott Laboratories, South Pasadena, California); triglyceride was measured with a Dupont ACA discrete clinical analyzer (Biomedical Products Department, Dupont Company, Wilmington, Delaware). Total plasma homocysteine was assayed in an unselected subset of stored blood samples. Plasma samples were stored at -80° C, and transferred to the Jean Mayer USDA Human Nutrition Research Center on Aging where they were analyzed. Total homocysteine in plasma was determined by an adaptation of the method described by Araki and Sako[20]. The coefficient of variation for this assay was 4.0%. Homocysteine data were available for about 54% of cases and 72% of controls. Most (93%) of the plasma homocysteine data were obtained from blood samples collected at a regular study visit after the CVD event had occurred.

In 1999, DNA was extracted from stored frozen buffy coat of 7 cc whole blood, using the Qiamp DNA blood kits (Qiagen, Valencia CA): DNA was successfully extracted for 1,584 cohort participants. Genotypes of the two polymorphisms (*MTHFR* 677C→T, and *MTHFD1* 1958G→A) were determined by the TaqMan procedure using the allelic discrimination technique (ABI Prism 7900 Sequence Detection System, Applied Biosystems, Foster City, CA). The primers and probes for both polymorphisms were created according to standard methods for the TaqMan procedure.

Student's t-test and chi-square were used to compare means of continuous and categorical variables, respectively, and to assess Hardy-Weinberg equilibrium. Conditional logistic regression, which accounts for the matched design, was used to assess the relation of genotype with disease risk. Two-way interaction terms were included in the adjusted conditional logistic regression model to evaluate the presence

of gene-gene interaction. To assess the inclusion of interaction terms in the models, a likelihood ratio test was used to calculate the global p-value comparing the model with interaction terms to that without. The coefficients provided by this analysis were used to calculate the effect size for each genotype in subgroups of the other genotype. To examine the influence of genotypes on serum levels of homocysteine, ANOVA was used to calculate the mean level of homocysteine in each genotype subgroup after adjusting for age at homocysteine measurement. All p-values presented are from two-tailed tests and all analyses were performed using SAS v. 9.0 (SAS Institute, Cary, NC).

Results

The pattern of cardiovascular risk factors was consistent with greater CVD risk in cases compared to controls (Table 2.1). The pattern of vitamin intake, as assessed by total dietary intake of folate, vitamin B6, and vitamin B12 by FFQ, was similar between cases and controls, and mean plasma homocysteine concentration was 0.5 μmol/L higher in cases. Genotype had little or no association with the traditional cardiovascular risk factors (data not shown).

Among controls, the frequency of the variant allele was 34.7% for the *MTHFR T* allele and 47.1% for the *MTHFD1 A* allele (Table 2.2), similar to reports from other studies[3, 6, 11, 12]. Both *MTHFR* 677C→T and *MTHFD1* 1958G→A were in Hardy-Weinberg equilibrium (P value = 0.97 and 0.15, respectively).

Homocysteine was measured in a subset of all cases and controls due to the date of the blood draw for this assay (early 1990s), thus serum variables are available on 620 (60%) of the full case-control study population. In regression models with homocysteine as the dependent variable and ignoring case-control status, the *MTHFR*

677C→T polymorphism and folate status (coded as tertiles of serum folate based on the distribution in controls: tertile cutpoints were 7.40 and 12.50 ng/ml) were both statistically significantly associated with serum homocysteine levels (P= 0.0005 and P < 0.0001, respectively), and there was a statistically significant two-way interaction between the *MTHFR* 677C→T genotype and serum folate (P= 0.01). The least-squares mean of serum homocysteine was greatest in men with the *MTHFR* 677C→T *TT* genotype compared to men with the *CT* or *CC* genotypes (11.6 nmol/l [95% CI 11.2, 12.7], 10.3 nmol/l [95% CI 9.9, 10.7], and 10.2 nmol/l [95% CI 9.7, 10.7], respectively). Serum folate was inversely associated with serum homocysteine such that men in the lowest serum folate tertile had the highest homocysteine levels. The mean serum homocysteine concentration by serum folate tertile (low to high) was: 12.7 nmol/l (95% CI 12.2, 13.2), 10.6 nmol/l (95% CI 9.96, 11.2), and 9.20 nmol/l (95% CI 8.6, 9.8). Finally, among men with the lowest serum folate, men with the *MTHFR* 677C→T *TT* genotype had significantly higher serum homocysteine levels in comparison to all other groups (mean in this subgroup was 14.9 nmol/L [95% CI 13.8, 16.0]; P<0.01 for all comparisons) (Table 2.3).

The *MTHFD1* polymorphism had little or no association with homocysteine (P= 0.89), but there was statistical evidence for an interaction between the *MTHFR* 677C→T and *MTHFD1* 1958G→A genotypes (P= 0.02). Mean homocysteine in *MTHFR* genotype groups varied by the *MTHFD1* genotype: in the *MTHFD1 GG* genotype group, serum homocysteine concentrations of 9.7, 10.3 and 12.8 nmol/L were observed for *MTHFR* 677 *CC*, *CT*, and *TT* genotypes, respectively. In the *MTHFD1 GA/AA* genotype group, serum homocysteine concentrations of 10.6, 10.4, and 11.1 nmol/L were observed for *MTHFR* 677 *CC*, *CT*, and *TT* genotypes, respectively. Men with *MTHFD1 GG*/*MTHFR TT* genotype (mean = 12.8 nmol/L) had statistically significantly higher homocysteine compared to four of the other genotypes

(P < 0.05 for all comparisons). There was no statistical evidence for a three-way interaction between the two polymorphisms and folate tertiles in relation to homocysteine (P=0.2) (data not shown).

In unadjusted conditional logistic regression models assessing the genotype—CVD association, both *MTHFD1* 1958G→A *AA* vs. *GG* and *GA* vs. *GG* had similar associations: the odds ratios (OR) were 0.84 (95% CI 0.6, 1.2) and 0.88 (95% CI 0.7, 1.1), respectively. Further models therefore considered the combined *MTHFD1* 1958G→A *AA/GA* genotype in comparison to the *GG* genotype. The *MTHFR* 677C→T polymorphism has a well-documented graded effect on phenotype[2], hence *CT* and *TT* were considered separately in comparison to the *CC* genotype.

MTHFR 677C→T *CT* vs. *CC* increased risk by 13% (OR 1.13; 95% CI 0.9, 1.4) and *TT* vs. *CC* increased risk by 54% (OR 1.54; 95% CI 1.1, 2.2). Adjustment for covariates had little or no effect on these estimates. In the subgroup with measured serum folate (~60% of total group) serum folate deficiency (defined as serum folate level < 7.5 nmol/L) was not an effect modifier of the *MTHFR*-CVD association (P values for the genotype*folate interaction terms were 0.5 and 0.9).

There was little or no association of *MTHFD1* genotype with CVD risk in unadjusted models (*AA/GA* vs. *GG* OR 0.87; 95% CI 0.7, 1.1), and adjusting for covariates had little or no effect on model coefficients. There was no evidence for an interaction between folate deficiency and the *MTHFD1* polymorphism.

The addition of the *MTHFR* genotype to the *MTHFD1*-CVD model did not affect the coefficients for the *MTHFD1* polymorphism, but in models evaluating the interaction of the two genotypes (Table 2.4), there was weak statistical evidence for an interaction (P value = 0.24 for the set of interaction coefficients, individual coefficient P values of 0.36, 0.095). The effect sizes for the *MTHFR* polymorphism within strata of the *MTHFD1* variant were calculated from model coefficients. The deleterious

effect of homozygosity for the *MTHFR T* allele was observed in men with *MTHFD1 GA/AA* genotype (OR 1.6, 95% CI 1.1, 2.4), but not in men with *MTHFD1 GG* genotype (OR 1.1, 95% CI 0.6, 2.2). The *MTHFR* 677C→T *CT* genotype had a deleterious effect in men with *MTHFD1 GA/AA* genotype (OR 1.2; 95% CI 0.9, 1.7) and a statistically non-significant inverse effect in men with *MTHFD1 GG* genotype (OR 0.8; 95% CI 0.5, 1.3). Further adjustment for the *cSHMT* 1420C→T genotype had little or no effect on these findings. There was no evidence for a three-way interaction between serum folate concentration, the *MTHFR* 677 polymorphism, and the *MTHFD1* 1958 polymorphism (P value = 0.6 and 0.5 for the three-way interaction terms).

To investigate the mediating role of homocysteine levels on the genotype-CVD relationship, serum homocysteine concentration was added to the conditional logistic regression models. Paradoxically, the addition of homocysteine to the model strengthened the interaction between the *MTHFD1* and *MTHFR* polymorphisms; the overall P value for the interaction model dropped to 0.14, and the individual regression coefficients P values decreased to 0.058 and 0.21 (Table 2.5). Adjusting for serum homocysteine slightly attenuated the effect of homozygosity for *MTHFR T* allele in men with *MTHFD1 GA/AA* genotype (OR 1.4; 95% CI 0.8, 2.5) (Table 2.5).

Discussion

We studied the association of two genetic polymorphisms in the folate metabolic network (*MTHFR* 677 C→T and *MTHFD1* 1958G→A) with cardiovascular disease in this nested case-control study of the Normative Aging Study cohort. The main clinical endpoint is cardiovascular disease, with serum homocysteine level studied as a secondary outcome and as a mediating risk factor. We investigated the main and interactive effects of two single-nucleotide polymorphisms in the *MTHFR*

Table 2.1. Characteristics at study entry of cases and controls, Normative Aging Study, 1961-1998[1].

Variable	Cases	Controls
Questionnaire and Physical Exam[2]		
BMI	26.3 ± 2.8	25.7 ± 2.9
Alcohol intake, usual ≥ 2/day (%)	13.0	11.4
Current smokers (%)	33.3	29.1
Duration of smoking (years)	14.8 ± 12.8	13.0 ± 11.8
Cumulative smoking (pack-years)	19.8 ± 21.4	15.9 ± 18.9
Systolic blood pressure (mmHg)	125.0 ± 13.1	121.4 ± 12.2
Diastolic blood pressure (mmHg)	77.7 ± 8.4	76.4 ± 8.4
Food Frequency Questionnaire[3]		
Folate (µg/d)	423 ± 255	427 ± 221
Vitamin B6 (mg/d)	3.4 ± 5.6	3.4 ± 6.3
Vitamin B12 (µg/d)	9.0 ± 6.4	9.8 ± 7.8
Serum Biomarkers[4]		
Total cholesterol (mg/dL)[5]	211 ± 45	198.8 ± 43.0
HDL cholesterol (mg/dL)[5]	45.0 ± 12.2	48.3 ± 13.3
Triglycerides (mg/dL)	146 ± 69	133 ± 68
Folate (ng/ml)	10.2 ± 5.4	10.5 ± 5.0
Total homocysteine (nmol/L)	10.9 ± 3.2	10.4 ± 3.8
Vitamin B6 (nmol/L)	80 ± 74	88 ± 90
Vitamin B12 (pg/ml)	455 ±182	471 ±272

[1] Values are means ± SD or %, total n=1034.
[2] n=979 minimum, due to missing data
[3] n=546 minimum, due to missing data
[4] n=630 minimum, due to missing data
[5] To convert mg/dL to SI units (mmol/L), multiply by 0.0259.

Table 2.2. Genotype frequency of the *MTHFR* and *MTHFD1* polymorphisms in cases and controls, Normative Aging Study, 1961-1998.

Polymorphism		*Cases (n=505)*	*Matched controls*[1] *(n=676)*
MTHFR	*CC*	196 (38.8%)	290 (42.9%)
	CT	226 (44.8%)	300 (44.4%)
	TT	83 (16.4%)	86 (12.7%)
MTHFD1	*GG*	141 (27.9%)	175 (25.9%)
	GA	267 (52.9%)	365 (54.0%)
	AA	97 (19.2%)	136 (20.1%)

[1] Includes disease-free controls and cases who were used as matching controls prior to the disease onset.

Table 2.3. Mean serum levels of total homocysteine and 95% confidence interval stratified by *MTHFR* 677 genotype status and folate tertiles in men in the nested case control study, Normative Aging Study, 1961-1998.

		*Folate Tertile**			
Genotype		Low	Medium	High	Total
MTHFR	*CC*	11.5 (10.7-12.2)	9.8 (9.0-10.6)	9.2 (8.5-9.9)	10.2 (9.7-10.7)
	CT	11.7 (11.1-12.3)	9.8 (9.0-10.6)	9.3 (8.6-10.0)	10.4 (10.0-10.8)
	TT	14.9 (13.7-16.0)	11.6 (10.3-12.8)	9.3 (7.9-10.7)	11.9 (11.1-12.7)
	Total	12.7 (12.2-13.2)	10.5 (9.9-11.0)	9.3 (8.7-9.9)	

* Low folate tertile is defined as serum folate <7.5 nmol/L, medium as ≥7.5 and ≤ 12.7 nmol/L, and high as >12.7 nmol/L)

Table 2.4. Multivariate conditional logistic models evaluating *MTHFD1* 1958G→A, *MTHFR* 677C→T genotypes, and their interaction in relation to CVD risk, Normative Aging Study, 1961-1998.

***Model*[1]:**	***Crude***			***Covariate-adjusted***			***Covariate and homocysteine adjusted***		
Model variables:	**β[2]**	**SE**	**P**	**β**	**SE**	**P**	**β**	**SE**	**P**
MTHFD1 1958G→A (*GA/AA* vs. *GG*)	-0.33	0.20	0.10	-0.48	0.22	0.03	-0.70	0.32	0.028
MTHFR 677C→T (*CT* vs. *CC*)	-0.16	0.23	0.49	-0.27	0.25	0.28	-0.58	0.35	0.10
MTHFR 677C→T (*TT* vs. *CC)*	0.25	0.33	0.44	0.096	0.36	0.79	-0.36	0.49	0.46
Interaction: *MTHFD1* 1958 (*GA/AA* vs. *GG*) and *MTHFR* 677 (*CT* vs. *CC*) [3]	0.35	0.27	0.19	0.48	0.29	0.095	0.78	0.41	0.058
MTHFD1 1958 (*GA/AA* vs. *GG*) and *MTHFR* 677 (*TT* vs. *CC)* [3]	0.21	0.38	0.59	0.37	0.41	0.36	0.70	0.56	0.21

[1] Models are crude (unadjusted for covariates), covariate-adjusted (adjusted for body mass index, total cholesterol level, triglycerides level, drinking alcohol, smoking status, and pack-years of smoking), and covariate and homocysteine adjusted (adjusted for covariate list and homocysteine).

[2]Values are β=regression coefficient, SE=standard error of regression coefficient, and P=P value.

[3] Likelihood-ratio test of the statistical significance of the set of two interaction terms:

Unadjusted model: $\chi^2 = 1.691$; P=0.4293; Covariate adjusted model: $\chi^2 = 2.827$; P=0.2432; Covariate and homocysteine-adjusted model: $\chi^2 = 3.855$; P=0.1455

Table 2.5. *MTHFD1* 1958G→A and *MTHFR* 677C→T genotype effect sizes, stratified by the levels of the other genotype, based on the estimates provided in Table 2.3, Normative Aging Study, 1961-1998.

	***MTHFR* effect:**	***MTHFD1* subgroup**	
		MTHFD1 GG	***MTHFD1 GA/AA***
Covariate-adjusted model*			
	MTHFR CT vs. *CC*	0.76 (0.46- 1.25)	1.24 (0.92- 1.66)
	MTHFR TT vs. *CC*	1.10 (0.55- 2.21)	1.60 (1.06-2.42)
Covariate + serum homocysteine adjusted model			
	MTHFR CT vs. *CC*	0.56 (0.28-1.12)	1.22 (0.78- 1.90)
	MTHFR TT vs. *CC*	0.70 (0.27- 1.81)	1.41 (0.79- 2.49)

gene (*MTHFR* 677 C→T) and the *MTHFD1* gene (*MTHFD1* 1958 G→A) on the risk of cardiovascular disease and on serum homocysteine levels.

We observed significant independent effects of the *MTHFR* 677C→T genotype and folate deficiency on serum homocysteine levels, as well as a statistically significant gene-nutrient interaction. The effect sizes for the interaction of *MTHFR* and folate may be underestimated due to randomly missing data: the strongest effect of homozygosity for *MTHFR* variant *T* allele on CVD risk was seen in men with missing serum folate data (data not shown). Overall, these findings are consistent with previous findings on the effect of *MTHFR* polymorphism on homocysteine levels in populations with suboptimal folate nutritional status, indicating the suitability of this population for the study of further gene-nutrient and gene-gene interactions in the folate metabolic pathway. Folate-replete populations (such as North American populations after the institution of folate fortification) show an attenuated relation between *MTHFR* and homocysteine levels[1-3]. Thus, these data provide an optimal population for studying folate metabolism and disease risk.

MTHFD1 1958 G→A had little or no independent association with CVD, but an interaction with the *MTHFR* 677 C→T genotype was found. The effect of the *MTHFR* genotype was limited to the subgroup of men with the *MTHFD1 GA/AA* genotype: there was a 60% increase in CVD risk in this subgroup, compared to little or no increased risk of the *MTHFR TT* or *CT* genotype in the subgroup of men with the *MTHFD1 GG* genotype. The interaction was independent of another polymorphism, namely *cSHMT* 1420 C→T, which has been shown to be important in one-carbon metabolism in general and in cardiovascular disease pathology in this population[15] .

The role of the *MTHFD1* 1958 polymorphism was originally identified in a study of neural tube defects[5]: the allele frequency was not significantly different between cases and control in that study. However, the *A* allele was associated with an

elevated risk of developmental and obstetric outcomes in other studies of neural tube defects (NTDs), pregnancy loss, and placental abruption[4, 6, 7, 9, 10]. The *MTHFD1 A* allele was associated with increased risk of NTD (OR 1.5, 95% CI 1.2, 2.0) in a family-based study, but no difference was observed in serum folate and homocysteine levels between genotype groups[4]. Similarly, the *A* allele was associated with an increased risk of NTDs in an Italian population (OR 1.1 for *AA* vs. *GG*, and 1.7 for *AG* vs. *GG*)[6]. These two studies did not investigate gene-gene interactions. In two studies on second-trimester pregnancy loss and placental abruption in Ireland, the variant *A* allele increased the risk of the outcome, but the gene-biomarker association was not reported[9, 10]. The effect of the *MTHFD1* genotype in both studies was independent of *MTHFR* genotype, but gene-gene interactions were not assessed. A small study of omphalocele found no effect of *MTHFD1* genotype[21]. Three studies investigated the association of *MTHFD1* genotype with chronic disease outcomes, including colorectal cancer[11], migraine[22], and spontaneous cervical artery dissection[12]. None of these studies reported a relation of the *MTHFD1* polymorphism to the clinical outcome, but, a statistically significant interactive effect with *MTHFR* was reported in migraine study. Overall, the *MTHFD1 A* allele, increased risk of some outcomes, particularly in homozygotes.

The MTHFD1 and MTHFR enzymes catalyze sequential steps in the folate metabolic network, leading to the hypothesis of interaction between them in relation to the risk of CVD. The three reaction steps catalyzed by MTHFD1 provide the substrate for MTHFR, which is responsible for the provision of 5-methylTHF for the remethylation of homocysteine to methionine. *MTHFD1* or *MTHFR* variants with decreased functional capacity are therefore expected to result in increased homocysteine levels and increased cardiovascular disease risk. We have observed that the effect of the *MTHFR* polymorphism is modified by *MTHFD1* genotype. Other

studies have investigated the relation of *MTHFD1* to a variety of outcomes, but most have not considered an interaction with *MTHFR* 677 genotype. Only one prior study of migraine reported a differential effect of *MTHFR T* allele across *MTHFD1* subgroups: the *MTHFR* T allele was associated with a protective effect in the *MTHFD1 GG* subgroup and with an increased risk in the *MTHFD1 GA/AA* subgroup: subgroup-specific effects were not reported[22].

Folate nutritional status has been shown to be an effect-modifier of the *MTHFR*—homocysteine and *MTHFR*—CVD relations[3]. Serum folate concentration as a proxy for folate nutritional status was available in a subset of the study population, and a significant gene-nutrient interaction was detected in models predicting serum homocysteine. The gene-nutrient interaction was not as strong in models with CVD as an outcome: the *MTHFR—C*VD association was strongest in men with missing data on serum folate, suggesting that this lack of interaction was mainly due to missing data.

Serum homocysteine is hypothesized to partially or wholly mediate the association of the *MTHFR* and *MTHFD1* polymorphisms with CVD risk. If the effect of genotype were entirely mediated by homocysteine, then the addition of homocysteine to the multiple conditional logistic regression models of CVD would be expected to attenuate and/or eliminate the effect of genotype. In these data, the addition of serum homocysteine to regression models did not decrease the magnitude of the gene—gene interaction coefficients. Indeed, the statistical significance of the gene—gene interaction was strengthened in the homocysteine- and covariate-adjusted models vs. the covariate-adjusted models (P values of 0.058 and 0.21 vs. 0.095 and 0.36, respectively: Table 2.4). A limitation is that serum biomarker data were available only on a subset of the study population; therefore evaluating the effect of mediation by the biomarkers does not include the entire set of cases and matched controls. The

strengthening of interaction in homocysteine-adjusted models suggests that the observed gene-gene interaction is mediated through homocysteine, but not in the expected way.

While mild hyperhomocysteinemia has been proposed to be a causal factor in atherogenesis based on a large body of observational epidemiologic evidence[23, 24], this view has been challenged by two recent homocysteine-lowering randomized controlled trials showing no effect of lowering homocysteine levels through vitamin administration on cardiovascular disease risk or recurrence[25, 26]. Since homocysteine is part of a larger metabolic network that provides substrates for many biological functions (methylation reactions, nucleotide biosynthesis, provision of folate cofactor forms), functional genetic polymorphisms in the genes of the network might exert their effect through pathways other than methionine biosynthesis.

The prospective nature of the NAS cohort is important because it minimizes the biases associated with case-control studies of cardiovascular disease. Another strength of the study is the availability of serum biomarker data on folate and homocysteine. This allowed us to examine the role of effect modification by folate and effect mediation by homocysteine. However, a potential weakness was the limited availability of biomarker data in a subset of the original case-control population. Another feature of our study was the consideration of genetic variants on two genes that encode sequential enzymes in the folate metabolic network. It is biologically plausible to observe gene-gene interaction between two variants that affect functional capacity of sequential steps in a biochemical pathway.

The *MTHFD1* 1958 G→A is a common polymorphism, minor allele frequency is 64%, therefore the observed effect may have important implications at the population level. The NAS nested case-control study has a relatively large sample size of about 500 cases and 1000 matched controls. This provides adequate power to

evaluate independent main effects, with odds ratios between 1.4 (81% power) and 1.5 (93%). However, the power to evaluate interactions of similar magnitude is restricted in the stratified analysis given the cross-tabulation of genotypes (power for OR=1.4 equals 52% when sample size is reduced in half). Inadequate sample size can explain the marginally significant P values for interaction in our study. A study twice as large would have adequate power (81%) to evaluate an odds ratio of 1.4 in an interaction model.

In summary, we investigated the role of the *MTHFD1* 1958G→A and *MTHFR* 677C→T polymorphisms in relation to serum homocysteine level and cardiovascular disease risk. There is some evidence for a gene-gene interaction such that the effect of the *MTHFR* 677C→T genotype is limited to men with the *MHTFD1* variant genotype. The observed effects are independent of homocysteine. This is the first report of the relation of the *MTHFD1* polymorphism to cardiovascular disease in a cohort study. Evaluation of this interaction in future studies on genetic aspects of cardiovascular disease is recommended.

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CHAPTER 3

CYTOPLASMIC SERINE HYDROXYMETHYLTRANSFERASE (CSHMT) AND METHYLENE-TETRAHYDROFOLATE REDUCTASE (MTHFR) GENE POLYMORPHISMS AND CORONARY HEART DISEASE RISK IN US WOMEN

Abstract

Genetic variation in folate-regulating enzymes contributes to metabolic and nutritional influences on cardiovascular disease. Prior findings in a case-control study of men show that genetic variation in the cytoplasmic serine hydroxymethyltransferase gene (*cSHMT* 1420C→T) modifies the effect of *MTHFR* 677 genotype on CVD risk. This study investigates the same question in a cohort of women (the Nurses' Health Study, NHS). The NHS,a cohort study of 121,700 female nurses established in 1976, followed participants biennially through mailed questionnaires. 250 cases of cardiovascular disease (CVD) occurred among individuals who provided a blood sample. Controls were matched to cases on age and smoking status. The variant allele frequency of the *cSHMT* and *MTHFR* polymorphisms was 29.8% and 30.9%, respectively. There was little or no main effect of *cSHMT* 1420C→T *TT* on the risk of CVD (OR= 0.94; 95% CI: 0.5, 1.7) compared to *cSHMT* 1420C→T *CC/CT*. There was a complete absence of cases with the *MTHFR TT*/*cSHMT TT* genotype, therefore no estimate could be made for the risk of CVD associated with the *MTHFR* 677C→T polymorphism among women with the *cSHMT* 1420C→T *TT* genotype. An interaction between the effect of the *MTHFR* 677C→T *CT* genotype (vs. *CC*) and the *cSHMT* 1420C→T genotype (P=0.09) was evident. The effect of *MTHFR* 677C→T

CT (vs. *CC*) on CVD risk differed markedly by the *cSHMT* genotype. There was little or no association of the *MTHFR* 677C→T *CT* genotype with CVD in the *cSHMT CC/CT* subgroup (OR 1.1, 95% CI 0.8, 1.5), but there was about a 3-fold increased risk in the *cSHMT TT* subgroup (OR 2.7, 95% CI 0.9, 8.8). These data provide a partial replication of the *MTHFR* 677-*cSHMT* 1420 interaction reported in a prior study of men. Lack of cases with the double homozygous genotype is likely due to random variation. Evaluation of this interaction in future studies of folate metabolism and cardiovascular disease is recommended.

Introduction

Cytoplasmic serine hydroxymethyltransferase (cSHMT) is a member of the folate-dependent one-carbon metabolic network that reversibly converts serine and tetrahydrofolate (THF) to glycine and 5,10-methylenetetrahydrofolate (5,10-methyleneTHF) [1]. The one-carbon group of 5,10-methyleneTHF is used in the synthesis of purines or thymidylate or in the remethylation of homocysteine to methionine. A common single nucleotide polymorphism (SNP) in the *cSHMT* gene, L474F (or, equivalently, 1420C→T), has been identified and associated with elevated plasma and red blood cell folate levels[2] . Epidemiologic studies suggest the presence of functional biochemical consequences given associations of this SNP with metabolic disruption and disease risk, including altered homocysteine concentrations[3], decreased risk of leukemia[4], and decreased risk of malignant lymphoma[5]. A recent study reported a gene-gene interaction between *cSHMT* 1420C→T and *MTHFR* 677C→T polymorphisms in relation to CVD risk[6], such that the effect of the *MTHFR* genotype was stronger in the presence of the variant *cSHMT* allele.

MTHFR and cSHMT catalyze two sequential reactions in the folate metabolic network: thus, the reported gene-gene interaction between *cSHMT* 1420C→T and

MTHFR 677C→T polymorphisms in relation to CVD risk is biologically plausible. However, such associations in epidemiologic studies may be explained by methodologic and/or biologic factors, and continued investigation of this gene-gene interaction in new populations is warranted. The first report of an interaction between *cSHMT* 1420C→T and *MTHFR* 677C→T in relation to CVD risk was based on males only[7], making investigation of the interaction in females a high priority. The study reported herein investigates the main and interactive effects of *cSHMT* 1420C→T and *MTHFR* 677C→T in a nested case-control study of coronary heart disease within a cohort of US women. The association of genotype with serum homocysteine level, a marker of folate-dependent remethylation, was also explored in light of the potential mediation of this relation through homocysteine elevation.

Methods

Study design and population

A nested case-control study was conducted within the Nurses' Health Study (NHS), a prospective cohort study of 121,700 female registered nurses in the United States. The cohort study was initiated in 1976, when the participants were aged 30-55[8]. Study participants completed detailed, self-reported questionnaires assessing dietary intake, lifestyle factors, and medical history. Every two years, follow-up questionnaires were mailed to update information on potential disease risk factors and newly-diagnosed disease. The details of selection of cases and controls for this study have been published elsewhere [9]: cases and controls were selected from among the subset of the cohort (32,826 of the initial 121,700) who gave a blood sample. In brief, 248 incident cases of nonfatal MI and fatal CVD between 1990 and 1998 occurred in women who had provided a blood sample and were free of CVD or cancer at blood

draw: these 248 cases comprised about one fourth of all cases that occurred in women initially free of CVD in the full cohort. Nonfatal MI was confirmed by study physicians blinded to participants' exposure status using World Health Organization criteria[10]. Fatal CVD was confirmed by hospital records, autopsy report, or death certificate, if CVD was the most plausible cause, and if evidence of previous CVD was available[11]. Two matched controls for each case were randomly selected from participants free of coronary heart disease at the time the case was diagnosed. Control identification used risk-set sampling[12, 13] and matched on age, smoking, month of blood draw, fasting status, and reports of any problems with blood drawing.

The study was approved by the Institutional Review Board of the Brigham and Women's Hospital, the Harvard School of Public Health Human Subjects Committee Review Board, and the Cornell University Committee on Human Subjects.

Measurement of biochemical and genotype variables

Between 1989 and 1990, a blood sample was requested from all participants of the NHS cohort through mail, and was provided by 32,826 women. Participants who provided blood samples had a similar distribution of cardiovascular risk factors compared to those who did not provide blood. Participants received a blood collection kit and collected whole blood samples in liquid sodium heparin blood tubes. The samples were returned in an enclosed ice pack via overnight mail and were centrifuged, separated, and aliquoted for storage in liquid nitrogen freezers (−130 °C). Plasma folate was measured using a radioimmunoassay kit (Bio-Rad, Richmond, CA), and homocysteine was measured by using HPLC at the Jean Mayer US Department of Agriculture Human Nutrition Research Center on Aging (Tufts University, Boston, MA). Laboratory personnel conducting the assays were blinded to case-control status.

The intra-assay coefficient of variation for folate and homocysteine was 6.8% and 2.9%, respectively[14].

DNA was extracted from buffy coat fractions using the QIAmp Blood Kit (Qiagen, Chatsworth, CA), and *cSHMT* 1420C→T and *MTHFR* 677C→T genotypes were assessed using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Primers and probes are available from the authors on request. Replicate quality control samples were included and genotyped with 100% concordance. Matched case-control sets were incomplete in a few instances due to genotype failures, thus, genotype data were available for 720 women (240 cases and 480 controls).

Statistical analysis

The Student's *t*-test and Wilcoxon's rank sum test were used for comparisons of continuous and nonparametric variables, respectively. The chi-square test was used to compare proportions between cases and controls and between genotype subgroups, and to assess Hardy–Weinberg equilibrium. Conditional logistic regression models were used to estimate relative risks. A genetic model categorizing genotype subgroups for inclusion in the conditional logistic regression model was determined based on previous findings and confirmed as appropriate in this population (additive model for *MTHFR* 677C→T and recessive model for *cSHMT* 1420 C→T)[15]. All analyses were performed using SAS v8.2 (SAS Institute, Cary, NC).

Results

Table 3.1 presents the baseline characteristics of women by case-control status. The matching factors were age, smoking, and sample collection conditions. Cases had

higher body-mass index and a greater proportion with family history of myocardial infarction compared to controls (46% vs. 29%, P<.0001). Cases were more likely to have a history of hypertension (57% vs. 29%, P < .0001) and a higher total cholesterol (P = .0010). Alcohol intake was greater in controls compared to cases (P =.0359) and moderate alcohol intake (between 0.1 and 30 g/day) was reported more often in controls (P = .0040). There was little or no difference in folate-related biomarkers between cases and controls, with the exception of vitamin B6 (mean in controls greater than in cases; P = 0.045).

The genotype frequencies of two polymorphisms are shown by case-control status. The frequency of the *cSHMT* 1420 C→T *TT* genotype was 7.7% in cases and 8.1% in controls. The prevalence of *MTHFR* 677 C→T *TT* was 10% in cases and 9.6% in controls. The joint distribution of the two polymorphisms revealed a complete absence of double homozygotes (*cSHMT TT*/*MTHFR TT*) in the case group (Table 3.2), limiting further analyses.

The *cSHMT* 1420C→T polymorphism (*TT* vs. *CC/CT*) was not associated with coronary heart disease in the unadjusted regression model (OR 0.94, 95% CI 0.5, 1.7) or in covariate-adjusted models (OR 0.98, 95% CI 0.4, 2.3). The addition of *MTHFR* 677C→T genotype to this model had little or no effect on regression coefficients for *cSHMT* genotype in crude or adjusted models. The *MTHFR* 677C→T genotype had little or no association with coronary heart disease in these data (*MTHFR CT* vs. *CC*: OR 1.15, 95% CI 0.8, 1.6 and *MTHFR TT* vs. *CC*: OR 1.13, 95% CI 0.7, 1.9).

The conditional logistic regression model testing the *MTHFR* by *cSHMT* interaction could not estimate the risk of CVD associated with the MTHFR 677C→T polymorphism among women with the *cSHMT* 1420C→T *TT* genotype due to the absence of double homozygote cases (Table 3.2). Thus, the evaluation of gene-gene interaction was limited to evaluating the effect of the *MTHFR* 677C→T *CT* genotype

(vs. *CC*) across levels of the *cSHMT* 1420C→T genotype. There was evidence for an interaction in the crude conditional logistic regression model (P = 0.13 for the interaction coefficient) (Table 3.3). The statistical significance of the interaction coefficient was strengthened when homocysteine was adjusted in the model (P=0.09; Table 3.3). Further adjustment for a comprehensive set of covariates made little or no difference to the effect estimates for genotype, but resulted in a large decrease in sample size and thus are not presented herein (n decreased from 747 to 564). Since the drop in sample size produced a different starting point for the analysis and there is no difference in covariates by genotype groups (data not shown), analyses focused on the crude and homocysteine-adjusted models.

To estimate the effect of the *MTHFR* variant allele in subgroups of women according to their *cSHMT* genotype, the coefficient estimates from Table 3.4 were used to calculate effect estimates and associated 95% confidence intervals. The effect of *MTHFR* 677C→T *CT* (vs. *CC*) on CVD risk differed markedly by the *cSHMT* genotype (Table 3.4). There was little or no association of the *MTHFR* 677C→T *CT* (vs. *CC*) genotype with CVD in the *cSHMT CC/CT* subgroup (OR 1.1, 95% CI 0.8, 1.5), and almost a 3-fold increased risk in the *cSHMT TT* subgroup (OR 2.7, 95% CI 0.9, 8.8) (Table 3.4). In models adjusted further for plasma total homocysteine, the corresponding odds ratio estimates for the effect of *MTHFR* 677C→T *CT* (vs. *CC*) on CVD risk were 1.05 (in *cSHMT CC/CT* subgroup; 95% CI 0.8, 1.5) and 3.3 (in *cSHMT TT* subgroup; 95% CI 0.9, 11.7) (Table 3.4).

The average concentration of homocysteine, a marker of folate-dependent remethylation, was not statistically significantly different across genotype groups (Table 3.5). However, two patterns are worthy of mention. Lower plasma folate levels were observed with increasing number of *MTHFR* variant *T* alleles in women with the

Table 3.1. General baseline characteristics of women with incident coronary heart disease (cases) and matched* controls from a nested case-control study within the Nurses' Health Study cohort.

Covariates[a]	*Cases (N = 249)*	*Controls (N = 498)*	
	Mean ± SD	Mean ± SD	P value
Age (yrs)	60.4 ± 6.5	60.3 ± 6.5	.8107
BMI	25.5 ± 5.5	23.8 ± 3.6	<.0001
Family History of MI (%)	45.8	29.4	<.0001
Hypertension (%)	57.4	29.3	<.0001
Cholesterol (mg/dL)	235.9 ± 40.2	225.5 ± 40.0	.0010
HDL-cholesterol (mg/dl)	51.7 ± 14.6	60.3 ± 17.5	<0.001
LDL-cholesterol (mg/dl)	143 ± 34.5	132 ± 36.4	<0.001
Post-Menopausal Hormone Use at blood draw (%)	30.5	36.4	.1143
Smoking Status (current smokers) (%)	45.8	39.0	.0739
Aspirin Usage (% users)	44.1	41.3	.5051
Alcohol Intake (gms/day)	4.5 ± 9.6	6.2 ± 10.7	.0359
0.1 ≤ 30 g/day (%)	49.8	60.8	.0040
≥ 30 g/day (%)	4.4	4.2	.8983
Physical Activity (Mets/week)	13.9 ± 15.7	14.9 ± 17.0	.4256
Caloric Intake (kcal/day)	1746.4 ± 511.5	1770.2 ± 522.0	.5648
Plasma Folate (ng/mL)	8.8 ± 6.4	8.2 ± 7.2	.2646
Homocysteine (nmol/mL)	11.3 ± 3.8	10.9 ± 5.8	.2360
RBC Folate (ng/g Hb)	1201.8 ± 352.7	1208.7 ± 366.4	.8073
B6 (pmol/ml)	58.05 ± 75.9	73.40 ± 131.7	.0451
B12 (pg/ml)	446.2 ± 175.2	450.1± 184.7	.7816

* Matching criteria were age, smoking, date of blood draw, fasting status at blood draw, and problems with blood draw.

[a] The majority of covariates were measured in a subset of cases and controls, thus reducing sample sizes as follows: BMI (n= 739), cholesterol (n=737), HDL-cholesterol (n=737), LDL-cholesterol (n=718), caloric intake (n= 710), alcohol intake (n= 710), RBC folate (n= 740), Plasma folate (n= 737), B6 level (n=740), B12 level (n= 737), and homocysteine level (n= 738).

Table 3.2. The joint distribution of *MTHFR* 677C→T and *cSHMT* 1420C→T genotypes by cases-control status in the Nurses' Health Study.

Polymorphism	***Cases (n= 249)***			***Controls (n= 498)***		
	n	***CC/CT*** **(%)**	***TT*** **(%)**	**n**	***CC/CT*** **(%)**	***TT*** **(%)**
cSHMT 1420C→T	234	92.3	7.7	467	91.9	8.1
MTHFR 677C→T	240	90	10	481	90.4	9.6
cSHMT 1420C→T stratified by:						
MTHFR 677 *CC*	100	94.0	6.0	220	91.8	8.2
MTHFR 677 *CT*	106	88.7	11.3	193	91.7	8.3
MTHFR 677 *TT*	23	100	0	45	91.1	8.9

Table 3.3. Multivariate conditional logistic regression models of the relation of *MTHFR* 677C→T and *cSHMT* 1420C→T genotypes, and their interaction, to coronary heart disease risk, Nurses' Health Study.

	Crude[1] model			**Homocysteine-adjusted[2] model**		
Model variables:	**β**	**SE**	**P**	**β**	**SE**	**P**
cSHMT 1420C→T (*TT* vs. *CT/CC*)	-0.58	0.48	0.22	-0.84	0.53	0.11
MTHFR 677C→T (*CT* vs. *CC*)	0.06	0.17	0.69	0.048	0.17	0.78
MTHFR 677C→T (*TT* vs. *CC)*	0.13	0.28	0.64	0.08	0.28	0.77
Gene-Gene Interaction[3] *cSHMT* 1420 (*TT* vs. *CT/CC)* and *MTHFR* 677 (*CT* vs. *CC*)	0.94	0.62	0.13	1.15	0.67	0.09

[1] Values are regression coefficient, SE of regression coefficient and P value from crude (unadjusted) models; N=747 in crude model
[2] Model adjusted for homocysteine only, N=738
[3] Due to lack of double homozygous variants among the cases, the second interaction term cannot be estimated in the model.

Table 3.4. Model-based estimates (95% confidence interval) of *MTHFR* 677C→T and *cSHMT* 1420C→T genotype effects, Nurses' Health Study.

		Genotype strata	
		***cSHMT* 1420C→T**	***cSHMT* 1420C→T**
Model	**Effect**	***CC/CT***	***TT***
Crude model[1]			
	MTHFR CT vs. *CC*	1.07 (0.76-1.5)	2.7 (0.85-8.8)
	MTHFR TT vs. *CC*	na*	na
Homocysteine-adjusted model[2]			
	MTHFR CT vs. *CC*	1.05 (0.75-1.47)	3.3 (0.93-11.7)
	MTHFR TT vs. *CC*	na	na

[1] All cases and controls have data, N=747

*There is no model estimate for this interaction due to zero cases in double homozygote group

[2] Cases and controls with homocysteine data, N=738

Table 3.5. Folate-related biomarkers and dietary folate intake in genotype subgroups in cases and controls, Nurses Health Study nested case-control study.

	MTHFR 677 *CC*	**MTHFR 677 *CT***	**MTHFR 677 *TT***
cSHMT* 1420 *CC/CT	**n = 293**	**n = 270**	**n = 64**
Plasma Hhomocysteine (nmol/mL)	11.1 ± 4.2	10.8 ± 3.6	11.0 ± 3.0
Plasma Folate (ng/mL)	8.8 ± 7.6	8.1 ± 6.7	6.8 ± 5.1
RBC Folate (ng/g Hb)	1213.0 ± 382.2	1194.4 ± 338.8	1183.9 ± 319.5
Dietary Folate (w Supp)mcg	429.3 ± 225.2	420.1 ± 242.7	448.6 ± 235.5
Dietary Folate (w/out Supp)mcg	301.9 ± 93.3	303.1 ± 91.4	315.0 ± 107.4
Plasma B6 (pmol/ml)	69.5 ± 94.9	60.9 ± 67.8	99.7 ± 290.1
Plasma B12 (pg/ml)	441.5 ± 171.4	455.2 ± 188.4	450.5 ± 178.1
cSHMT* 1420 *TT	**n = 24**	**n = 28**	**n = 4**
Homocysteine (nmol/mL)	13.5 ± 20.7	12.0 ± 4.9	10.4 ± 4.3
Plasma Folate (ng/mL)	9.8 ± 8.8	9.2 ± 6.6	11.8 ± 9.5
Mean RBC Folate (ng/g Hb)	1343.2 ± 388.9	1167.8 ± 362.4	1411.1 ± 729.8
Dietary Folate (w Supp)	453.9 ± 256.2	447.8 ± 248.4	585.5 ± 471.8
Dietary Folate (w/out Supp)	292.3 ± 111.6	285.8 ± 71.1	384.3 ± 228.1
Plasma B6 (pmol/ml)	66.4 ± 57.3	54.4 ± 93.6	55.9 ± 28.3
Plasma B12 (pg/ml)	469.5 ± 223.8	474.4 ± 228.5	520.1 ± 265

cSHMT CC/CT genotypes (8.8 in *MTHFR CC*, 8.1 in *MTHFR CT*, and 6.8 in *MTHFR TT* women, respectively. In addition, the average homocysteine concentration of women with the *MTHFR CT/ cSHMT TT* genotype was 12.0 compared to 10.8 among women with the *MTHFR CT/ cSHMT CC/CT* genotype.

Discussion

We studied the association of two genetic polymorphisms in the folate metabolic network (*MTHFR* 677C→T and *cSHMT* 1420C→T) with cardiovascular disease in a nested case-control study within the Nurses' Health Study cohort. There was little or no independent association of the *cSHMT* polymorphism with coronary heart disease risk in crude or adjusted models, and similarly little association of the *MTHFR* 677C→T genotype with disease risk. There was evidence of an interaction between these genes such that the effect of the *MTHFR* 677C→T genotype was limited to the subgroup of women with the *TT* genotype for the *cSHMT* 1420C→T polymorphism. Thus, the *MTHFR* 677C→T *CT* genotype (vs. *CC*) increased the risk of CVD about 3-fold in this subgroup, compared to little or no effect of *MTHFR* 677C→T *CT* genotype among women with the *cSHMT* 1420C→T *CC/CT* genotype.

In the first study to report a gene-gene interaction between the *MTHFR* 677 and *cSHMT* 1420 polymorphisms on CVD[16], the increased risk of CVD associated with *MTHFR* 677C→T *CT* and *TT* genotypes was of greater magnitude in men with the *cSHMT* 1420C→T *TT* genotype. The effect size in this study of women is somewhat lower in magnitude compared to the report in men. In the Normative Aging Study[16] the MTHFR 677C→T *CT* genotype (vs. CC) was associated with a 3.6-fold increased risk of CVD (95% CI 1.7, 7.8) among men with the cSHMT 1420C→T *TT* genotype. The smaller effect size reported herein (about a 3-fold increased risk) may relate to the phenomenon of "winner's curse", which suggests that reported magnitude

of a genotype-phenotype association is usually overestimated in the first published report of that association[17]. The complete absence of double homozygote cases (*MTHFR* 677C→T *TT* and *cSHMT* 1420C→T *TT*) in the present study precluded a full replication of the gene-gene interaction. The lack of double homozygote cases may be explained by a severely increased risk of CVD and ensuing premature mortality in the double homozygotes, a highly unlikely scenario given the observed effect sizes, or this may be due to a random phenomenon resulting from chance. The latter explanation is considered more likely given that blood is collected on less than 25% of all cohort members, and given that less than 25% of all incident cases comprise the studied cases. The observation of a gene-gene interaction in the same direction as observed by Lim et al (2005) supports the inference that this interaction has a causal role and is not due to a false positive finding. The true size of the effect remains to be estimated as further findings accrue.

Cytoplasmic serine hydroxymethyltransferase (cSHMT) is a key enzyme of the folate metabolic network, affecting the intracellular homeostasis among folate cofactor forms[18]. cSHMT reversibly converts serine and tetrahydrofolate (THF) to glycine and 5,10-methylenetetrahydrofolate (5,10-methyleneTHF), which is, in turn, used in the synthesis of purines or thymidylate or in the remethylation of homocysteine. The single nucleotide polymorphism (SNP) in the *cSHMT* gene is a C to T substitution at nucleotide 1420, resulting in the amino acid substitution of leucine to phenylalanine at position 474 of the protein[19]. Screening of the coding and regulatory regions of the *cSHMT* gene yielded only 2 other variants: *cSHMT* 1181G→A, an extremely rare variant[20] , and an E to Q substitution at amino acid position 340. The functional biochemical consequences of *cSHMT* 1420 C→T variant are under investigation. The polymorphism is distant from the enzyme's active site: results from molecular modeling demonstrate that the 1420C→T polymorphism is located on the exterior of

the protein on a side chain that faces the bulk solvent (unpublished data, Oppenheim, Szebenyi, and Stover, Cornell University). Further evidence supports the role of the *cSHMT* polymorphism in sumoylation of intracellular proteins and regulation of nuclear transport (Stover P, personal communication). In a recent study, site-directed mutagenesis of the human enzyme expressed in *E. coli* showed that the polymorphism is associated with lower affinity for the pentaglutamate form of the folate ligand (but not for the other forms) and decreased rates of pyridoxal phosphate addition to the enzyme. The polymorphism did not affect the stability of SHMT or the rate at which it converts 5,10-methenyl tetrahydropteroyl pentaglutamate to 5-formyl tetrahydropteroyl pentaglutamate[21]. The polymorphism has also been shown to inhibit thymidylate synthase (TS) and to lead to decreased homocysteine remethylation *in vitro* (Stover P, personal communication).

Evidence supports an association of the *cSHMT* 1420C→T genotype with altered homocysteine level and disease risk in epidemiologic studies of leukemia and neural tube defects[22, 23]. The *cSHMT* 1420C→T genotype had a significant effect on serum homocysteine level: among mothers of children with NTDs, lower plasma homocysteine concentrations were reported in mothers with homozygous variant *TT* genotype (vs. *CC*)[24]. This effect was not observed in children who were affected by NTD, and these effects were not stratified by *MTHFR* genotype. In a study of adult acute lymphocytic leukemia, persons with *cSHMT* 1420C→T *CT* genotype had a 2.1-fold decrease in acute lymphocytic leukemia risk (OR 0.48; 95% CI 0.25, 0.91), whereas those with *TT* genotype had a 3.3-fold reduction in risk (OR 0.31; 95% CI 0.10, 0.90)[23].

The observed interaction between the *cSHMT* and *MHTFR* genotypes is biologically plausible because the two enzymes catalyze sequential steps in the folate metabolic network. Three proposed functions of cSHMT based on isotope tracer

studies are preferential supply of 1-carbon units to thymidylate biosynthesis; glycine-dependent serine synthesis, leading to depletion of 5,10-methyleneTHF for S-adenosyl methionine synthesis; and sequesteration of 5-methyltetrahydrofolate (5-methylTHF), leading to a decrease in S-adenosyl methionine synthesis[25]. Since 5,10-methyleneTHF is the substrate for the MTHFR enzyme, alterations in the substrate's level due to functional consequences of the genetic variant in *cSHMT* gene could affect the availability of the substrate for MTHFR action, and hence affect the supply of 5-methylTHF, and ultimately the homocysteine remethylation reaction. Alteration of homocysteine level is one of the proposed mechanisms through which genotypic variant in genes of the folate metabolic network might exert their effect. The above-mentioned NTD study[22] provides some evidence for the increased turnover of homocysteine in mothers with the variant T allele, but in our study there is no evidence for such an effect. We observed that the *MTHFR-cSHMT* interaction effect size is not decreased by including homocysteine level in the model. On the other hand, inclusion of homocysteine strengthens the interaction effect size, pointing to a different role for homocysteine than traditionally hypothesized.

The prospective design of the Nurses' Health Study cohort is important in minimizing the biases associated with case-control studies of coronary heart disease. The availability of serum biomarker data on folate and homocysteine prior to diagnosis of disease is a strength of the study. The unexpected problem of a complete absence of CVD cases with the double homozygous genotype is attributed to the random process of sampling subjects and not expected to be related to any specific aspect of the study design.

In summary, this study of women partially confirms a prior report of a gene-gene interaction between *cSHMT 1420*C→T and *MTHFR 677*C→T polymorphisms in men. The *cSHMT* genotype was not independently associated with CVD risk, but it

modified the effect of *MTHFR* genotype on disease risk. The observed interaction was not mediated by serum homocysteine levels. Additional studies are needed to replicate this finding and evaluate the role of other functional genetic variants of the folate metabolic network.

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CHAPTER 4

METHIONINE SYNTHASE 2756A→G AND METHYLENETETRAHYDROFOLATE REDUCTASE 677C→T AND 1298A→C GENETIC POLYMORPHISMS AND CARDIOVASCULAR DISEASE RISK IN THE NORMATIVE AGING STUDY

Abstract

Genetic variation in the folate-regulating enzymes may reveal important clues to aid in understanding metabolic and nutritional influences on cardiovascular disease (CVD). Methylenetetrahydrofolate reductase (MTHFR) and methionine synthase (MTR) catalyze sequential steps in folate-mediated remethylation of homocysteine to methionine, thus we hypothesized that variant alleles of these two genes would interact to affect CVD risk. This study investigated the association between three single nucleotide polymorphisms (SNPs) in these 2 genes (*MTHFR* 677C→T, *MTHFR* 1298A→C, and *MTR* 2756A→G) and the risk of CVD. Incident cases of CVD (n=507) were identified in the longitudinal Normative Aging Study, and two controls were selected by matched incidence density sampling. In the simultaneous model, considering all SNPs without interactions, only *MTHFR* 677C→T was associated with CVD risk (*MTHFR* 677C→T *TT* vs. *CC* : odds ratio (OR) 1.4; 95% confidence interval (CI) 1.0, 2.0). Models including all possible gene-gene interactions revealed a statistically significant interaction between *MTHFR* 1298A→C and *MTR* 2756A→G (P=0.02): the effect of *MTHFR* on CVD risk was in opposite directions, depending on the *MTR* genotype. The *MTR* 2756A→G *AA* genotype(vs. *AG/GG*) was associated with a 50% decrease in the risk of CVD (95% CI 0.23, 1.06) in men with the *MTHFR* 1298A→C *CC* genotype. In men with the *MTHFR* 1298A→C *AA/AC* genotype, the

MTR AA genotype increased the risk of CVD (OR 1.30; 95% CI 1.001, 1.69). The observed gene-gene interaction was not mediated by homocysteine: inclusion of serum homocysteine concentration in the model strengthened the interaction. Evaluation of this interaction and similar interactions of sequential steps in the folate metabolic network is recommended in future studies of cardiovascular disease.

Introduction

Genetic variability in genes involved in folate metabolism might affect the cardiovascular risk of individuals by altering serum homocysteine concentrations, by affecting the composition of folate cofactor pools in endothelial and red blood cells, and/or by influencing serum folate levels. The association between variation in some of the folate metabolism genes and cardiovascular disease (CVD) has been extensively studied and biologic mechanisms underlying these associations have been posited: a well-studied case in point is the methylenetetrahydrofolate reductase gene (*MTHFR*)[1]. Other genes are less well-studied, including the methionine synthase (*MTR*) gene, which has only recently been evaluated in the relation to CVD risk. One of the main pathways for the catabolism of homocysteine, a metabolic intermediate proposed to increase the risk of CVD[2], is its remethylation, which is dependent on two enzymes. Methionine synthase (MTR) is a vitamin B12-dependent enzyme which catalyzes the remethylation of homocysteine to methionine. During the remethylation process, MTR uses a methyl group, 5-methyltetrahydrofolate (5-methylTHF) provided through the action of MTHFR. Since remethylation of homocysteine is dependent on the action of MTR (as the catalyzing enzyme) and MTHFR (as the substrate-providing enzyme), common genetic polymorphisms in genes encoding these enzymes may cause elevations in serum homocysteine concentration and/or other consequences of altered folate metabolism leading to an altered risk of clinical consequences.

MTHFR catalyzes the conversion of 5,10-methyleneTHF to 5-methylTHF: 5-methylTHF is the major folate derivative in plasma and provides the methyl group for remethylation of homocysteine[3, 4]. A thermolabile form of MTHFR has been found, which exhibits reduced activity and is caused by a common C677T polymorphism of the *MTHFR* gene[5, 6]. In many studies, the *MTHFR* 677C→T *TT* genotype is associated with increased plasma homocysteine levels and increased cardiovascular risk, especially in subjects with low serum folate levels [7-9]. A second common polymorphism was reported in exon 7 of the *MTHFR* gene, a nucleotide 1298A→C substitution that leads to a glutamate-to-alanine exchange in the amino acid sequence, and also causes reduced MTHFR enzyme activity [10, 11].

A common polymorphism has been identified in the methionine synthase (*MTR*) gene, an A to G transition at nucleotide 2756 (2756A → G) in the open reading frame of the gene resulting in the substitution of a glycine (G) for aspartic acid (D) at codon 919 (D919G), a potentially functional site of the protein [12]. Only a handful of epidemiologic studies, limited by sample size or cross-sectional design, have investigated the effect of this polymorphism on homocysteine levels or cardiovascular disease risk. Mixed findings include reports of an increase [13], decrease [14-16], or no effect on risk [17-20].

Evaluating the functional effect of genetic variation in genes contributing to linked steps in a metabolic network may lead to the discovery of more robust associations between genetic variation and clinical consequences. This approach is likely to be far more informative than focusing on single SNPs, but is hampered by studies with a small sample size, which have limited statistical power to reliably evaluate gene-gene interactions. The objective of this study is to examine the effects of three single nucleotide polymorphisms in the *MTHFR* and *MTR* genes, and their interaction, in relation to the risk of cardiovascular disease.

Methods

Study Population

A nested case-control study was carried out within the prospective Normative Aging Study (NAS) cohort. The NAS was established by the Veterans' Administration in 1961 from a pool of several thousand volunteers in the Boston vicinity[21]. Two thousand two hundred eighty community-dwelling men aged 21-81 (mean 42 years at study entry) were selected based on health criteria. Men with past or current chronic conditions, including coronary heart disease, hypertension, diabetes, cancer, peptic ulcer, gout, asthma, chronic bronchitis, and chronic sinusitis, were ineligible. Since their enrollment in 1961-1968, participants have undergone comprehensive clinical examinations at 3- to 5-year intervals. The response rate was greater than 90% for mailed questionnaires, including food frequency questionnaires, which supplemented on-site examination. As of June 1998, 543 participants (24%) were deceased and about 1600 men (70%) remained under active observation with a mean age of 70 years. The rate of continued participation of NAS men over the follow-up period was excellent, with less than 1% annual attrition for all causes.

From the beginning of the study through 1998, 749 incident cases of cardiovascular disease (CVD) occurred. Cases included incident coronary heart disease (CHD) and stroke that occurred between November 1961 and December 1998. Possible occurrences of CHD, including angina pectoris and nonfatal myocardial infarction (MI), were evaluated based on medical records and physician examination[22]. Angina was defined according to Framingham Heart Study criteria as recurrent chest discomfort lasting up to 15 minutes distinctly related to exertion or excitement that was relieved by rest or nitroglycerin[23]. Nonfatal MI was diagnosed only when documented by unequivocal electrocardiographic changes together with chest discomfort consistent with MI. Possible occurrences of stroke were identified by

report of a neurological deficit of sudden or rapid onset that persisted for 24 hours or longer and confirmed by neurologists who reviewed individual medical records[24]. CHD or stroke mortality was confirmed by death certificates, which were coded according to the eighth revision of the International Classification of Diseases[25]. About six percent of cases had only stroke. DNA was available only for more recent cases, thus 535 incident CVD cases were studied. The cases without DNA were older at study entry, and had slightly higher systolic blood pressure and greater cumulative smoking exposure.

A total of 1,048 matched controls were selected from the cohort by risk set sampling. The controls were matched to each case by age at onset and birth period (in five-year intervals) of the case. By this approach, as each case occurred, all other cohort members at risk (including cases that occurred at later times), under active follow-up, with the same matching conditions, and with DNA available formed a risk set for the incident case. Two men from the risk set were randomly chosen as matched controls. Therefore, cases were eligible to serve as a control in the time period before they were diagnosed, and some individuals, both cases and non-cases, served as a control for more than one case[26]. As a result, the final list of distinct individuals selected for the nested case-control study included 535 cases and 547 non-cases, with a maximum of 2 controls matched to each case. The study was approved by the Brigham and Women's Hospital Human Subjects committee, the Veterans' Administration R & D committee, and the Cornell University Committee on Human Subjects.

Data Collection: Covariates

Extensive data were collected on all participants, including physical examination data, lifestyle factors and blood analyses. Beginning in 1987, men

completed the Willett semi-quantitative food frequency questionnaire (FFQ) on dietary intake in the past year. The questionnaire was mailed to participants and returned during a scheduled examination. Estimations of dietary intake, including B vitamins, methionine, coffee and alcohol, were derived from the frequency and dosage information on the FFQ using software developed by the Nurses' Health Study [27]. At least one FFQ measurement was available prior to the date of diagnosis for about half of the CVD cases (246 out of 535). For the non-cases, 307 out of 547 had at least one FFQ measurement. Certain blood analyses were started after the study baseline as well, including serum HDL cholesterol, which was measured beginning in 1981. The majority of cases and controls had HDL data before the CVD diagnosis of the case.

Data Collection: Genotyping Methods

In 1999, DNA was extracted from stored frozen buffy coat of 7 cc whole blood, using the Qiamp DNA blood kits (Qiagen, Valencia CA): DNA was successfully extracted for 1,584 participants. Genotypes of three polymorphisms (*MTHFR* 677C→T, *MTHFR* 1298A→C, and *MTR* 2756A→G) were determined by the TaqMan procedure using the allelic discrimination technique (ABI Prism 7900 Sequence Detection System, Applied Biosystems, Foster City, CA). The DNA samples for all men (cases and controls) were plated in random order with a mixture of TaqMan Universal PCR Master Mix, primers, and probes. PCR cycling conditions consisted of one two-minute cycle at 50°C, one 10-minute cycle at 95°C, followed by 40 to 46 cycles at 95°C for 15 seconds and at 60°C for one minute. The primers and probes for the two *MTHFR* polymorphisms and the *MTR* 2756A→G polymorphism were created according to standard methods for the TaqMan procedure.

Statistical Analysis

Observed genotype frequencies were compared to those expected in Hardy-

Weinberg equilibrium[28] and tested with the chi-squared statistic. Linkage disequilibrium among the three polymorphic sites was tested using the Likelihood Ratio Test and Fisher's Exact Test[28]. Conditional logistic regression analysis (SAS PHREG; SAS Institute, Cary, NC) was used to analyze the risk set sampled cases and controls[29]. Men homozygous or heterozygous for the 2756A→G variant of *MTR* (*GG and AG*) were compared to homozygous (*AA*) genotypes because it was established that there was little or no risk difference between *GG* and *AG* subgroups in overall and stratified analyses. To test whether the *MTR*-CVD association varied by other polymorphisms and to assess effect modification of the *MTR*-CVD association by other risk factors, product terms were included in the regression model. Effect estimates were derived from these models and confirmed in stratified analyses.

The *MTR*-CVD association was estimated in unadjusted models and in models adjusted for risk factors for CVD. In addition, the *MTR*-CVD association was estimated in the subset of cases and non-cases with dietary data. Once again, further models were considered to assess potential confounding and effect modification.

Results

The overall distribution of cardiovascular risk factors in the cases compared to the controls (Table 4.1) indicates a pattern of greater CVD risk in cases compared to controls, as expected. Given the relation of known risk factors to CVD risk, the association of genotype with these risk factors was considered carefully. Genotype had little or no association with traditional cardiovascular risk factors, except for alcohol use: in men with the *MTR* 2756A→G *AA* genotype the prevalence of drinking more than 2 drinks a day was greater compared to men with *AG/GG* genotype (16.8% vs. 8.6; Table 4.2). The pattern of vitamin intake, as assessed by total dietary intake of folate, vitamin B6, and vitamin B12, was similar between cases and controls, and

mean serum homocysteine concentration was 0.5 μmol/L higher in cases.

The observed genotype frequencies are comparable to reported frequencies in other studies of Caucasian populations. All three polymorphisms are in Hardy-Weinberg equilibrium. We observed statistically significant linkage disequilibrium between *MTHFR* 677C→T and *MTHFR* 1298A→C, manifesting as lack of double homozygotes *(*complete absence of *MTHFR* 677 *TT/MTHFR* 1298 *CC* combination).

In multivariate models considering the relation of genotype to CVD risk, genotype models were determined based on past literature and on empirical considerations. Thus, for *MTHFR* 677C→T, the additive model (*TT* vs. *CC* and *CT* vs. *CC*) best captures the relation of this genotype to CVD risk. For *MTHFR* 1298A→C, the recessive model (*CC* vs. *AA/AC*) was optimal, and for *MTR* 2756A→G, the dominant model (*AG/GG* vs. *AA*) was optimal. The optimal coding of polymorphisms was based on the effect size for heterozygous and homozygous variants compared to homozygous wildtype genotypes, and the associated P values in unadjusted conditional logistic regression models.

In unadjusted conditional logistic regression models with CVD occurrence as the outcome, considering each genotype in separate models, both *MTHFR* 677C→T and *MTHFR* 1298A→C were significantly associated with increased and decreased risk of CVD (Table 4.4), respectively. However, the *MTHFR* 1298 protective effect was completely explained by linkage disequilibrium with *MTHFR* 677, and with both polymorphisms in the model the effect of the 1298 polymorphism is attenuated (odds ratio changes from 0.69 to 0.74). In the overall multi-SNP model without interactions, only *MTHFR* 677 C→T *TT* genotype was associated with increased CVD risk (odds ratio (OR) 1.4; 95% confidence interval (CI) 1.0, 2.0) (Table 4.5). The MTR 2756 polymorphism had little or no association with CVD in the single-polymorphism and overall models.

Table 4.1. Characteristics at study entry of cases and controls, Normative Aging Study, 1961-1998[1].

Variable	Cases	Matched Controls
Questionnaire and Physical Exam[2]		
BMI	26.3 ± 2.8	25.7 ± 2.9
Alcohol intake, usual ≥ 2/day (%)	13.0	11.4
Current smokers (%)	33.3	29.1
Duration of smoking (years)	14.8 ± 12.8	13.0 ± 11.8
Cumulative smoking (pack-years)	19.8 ± 21.4	15.9 ± 18.9
Systolic blood pressure (mmHg)	125.0 ± 13.1	121.4 ± 12.2
Diastolic blood pressure (mmHg)	77.7 ± 8.4	76.4 ± 8.4
Food Frequency Questionnaire[3]		
Folate (µg/d)	423 ± 255	427 ± 221
Vitamin B6 (mg/d)	3.4 ± 5.6	3.4 ± 6.3
Vitamin B12 (µg/d)	9.0 ± 6.4	9.8 ± 7.8
Serum Biomarkers[4]		
Total cholesterol (mg/dL)[5]	211 ± 45	198.8 ± 43.0
HDL cholesterol (mg/dL) [5]	45.0 ± 12.2	48.3 ± 13.3
Triglycerides (mg/dL)	146 ± 69	133 ± 68
Folate (ng/ml)	10.2 ± 5.4	10.5 ± 5.0
Total homocysteine (nmol/L)	10.9 ± 3.2	10.4 ± 3.8
Vitamin B6 (nmol/L)	80 ± 74	88 ± 90
Vitamin B12 (pg/ml)	455 ±182	471 ±272

[1] Values are means±SD or %, total n=1034.
[2] n=979 minimum, due to missing data
[3] n=546 minimum, due to missing data
[4] n=630 minimum, due to missing data
[5] To convert mg/dL to SI units (mmol/L), multiply by 0.0259.

Table 4.2. General Characteristics at Study Entry by Genotype: Controls Only, Normative Aging Study, 1961-1998[1].

	MTR *2756A→G*		**MTHFR *677C→T***			**MTHFR *1298A→C***	
	AA (n=349)	AG/GG (n=178)	CC(n=229)	CT (n= 241)	TT (n= 57)	AA/AC (n=473)	CC (n=50)
Questionnaire and Exam[2]							
BMI	25.7 ± 3.0	25.8 ± 2.7	25.7 ± 3.1	25.7 ± 2.7	26.1± 2.3	25.8 ± 2.9	25.6 ± 2.4
Drinkers, usual ≥ 2/day (%)	8.6	16.8	12.7	10	12.3	11.3	12
Current smokers (%)	38.7	30	27.2	28.8	38.2	28.3	36.7
Duration of smoking (years)	13.0 ± 12.0	13.1 ± 11.3	13.1 ± 12.1	12.4 ± 11.4	15.5 ± 12.1	12.8 ± 11.7	15.2 ± 12
Smoking (pack-years)	15.7 ± 19.3	16.3 ± 18.1	16.1 ± 20.1	14.9 ± 17.7	19.6 ± 18.3	15.5 ±18	20.2 ± 25.5
Systolic BP (mmHg)	121 ± 12.1	121 ± 12.3	122 ± 12.9	120.6 ± 12	120.6 ± 9.9	121 ±11.8	121 ± 15.2
Diastolic BP (mmHg)	76.4 ± 8.6	76.3 ± 7.9	76.3 ± 9.1	76.4 ± 8.1	76.3 ± 6.5	76.3 ± 8.2	77 ± 10.2
Dietary intake (FFQ)[3]							
Folate (µg/d)	424 ± 220	434 ± 226	439 ± 225	428 ± 225	377 ± 192	421 ± 213	490 ± 291
Vitamin B6 (mg/d)	3.48 ± 6.8	3.35 ± 5.2	3.2 ± 4.7	3.9 ± 8.0	2.26 ± 1.02	3.46 ± 6.6	3.3 ± 1.95
Vitamin B12 (µg/d)	9.5 ± 8.2	10.38 ± 6.9	9.4 ± 5.9	10.6 ± 9.6	8.2 ± 4.45	9.7 ± 8.0	10.9 ± 5.0
Serum Biomarkers[4]							
Total cholesterol (mg/dL)[5]	198 ± 44	201 ± 41	198 ± 42	199 ± 45	200 ± 40	200 ± 44	198 ± 33
HDL cholesterol (mg/dL)[5]	48.3 ± 12.5	48.3 ± 14.8	48.2 ± 13.6	48.5 ± 13.8	48.3 ± 9.3	48 ± 13.3	49.1 ± 13.8
Triglycerides (mg/dL)	131 ± 73	137 ± 57	127 ± 52	138 ± 81	137 ± 63	134 ± 70	126 ± 35
Folate (ng/ml)	10.6 ± 5.2	10.4 ± 4.7	11.0 ± 5.2	10.3 ± 5.0	9.7 ± 4.6	10.5 ± 5.0	10.75 ± 5.3
Total homocysteine (nmol/L)	10.5 ± 4.1	10.3 ± 2.9	10.3 ± 3.3	10.3 ± 3.4	11.4 ± 5.9	10.4 ± 3.8	11.1 ± 3.0
Vitamin B6 (nmol/L)	81 ± 77	101 ± 110	93 ± 102	87.5 ± 85.8	69 ± 42	88 ± 91	88 ± 79
Vitamin B12 (pg/ml)	481 ± 304	450 ± 192	470 ± 201	460 ± 200	520 ± 586	471 ± 280	467 ± 184

[1] Values are means ± SD or %, total n=1034. [2] n=979 minimum, due to missing data [3] n=546 minimum, due to missing data
[4] n=630 minimum, due to missing data

Table 4.3. Genotype frequencies of the *MTHFR* and *MTR* polymorphisms in case and control groups, Normative Aging Study, 1961-1998.

Polymorphism		*Cases (n=505)*	*Matched controls*[1] *(n=679)*
MTHFR 677	*CC*	196 (38.7)	291 (42.9%)
	CT	227(44.8%)	301 (44.3%)
	TT	84 (16.6%)	87 (12.8%)
MTHFR 1298	*AA*	242 (47.7%)	318 (46.8%)
	AC	224 (44.2%)	289 (42.6%)
	CC	41 (8.1%)	72 (10.6%)
MTR 2756	*GG*	350 (69.2%)	453 (66.7%)
	GA	138 (27.3%)	200 (29.5%)
	AA	18 (3.6%)	26 (3.8%)

[1] Includes disease-free controls and cases serving as matching controls prior to the disease onset.

Table 4.4. Univariate and multivariate models of the relation of genotype with CVD risk, nested case-control study within the Normative Aging Study Cohort, 1961-1998.

Models	*n**	*Odds Ratio*	*95% CI*
1. Unadjusted single polymorphism models			
a)MTHFR 677 (*CT* vs. *CC*)	1514	1.13	0.90, 1.43
MTHFR 677 (*TT* vs. *CC*)		1.54	1.11, 2.15
b)MTHFR 1298 *(CC vs. AC/AA)*	1514	0.69	0.47, 1.00
c)MTR 2756 *(AG/GG vs. AA)*	1514	0.89	0.70, 1.12
2. Unadjusted simultaneous model, including all 3 SNPs			
MTHFR 677 (*CT* vs. *CC*)	1514	1.04	0.81, 1.35
MTHFR 677 (*TT* vs. *CC*)		1.43	1.02, 2.02
MTHFR 1298 *(CC vs. AC/AA)*		0.74	0.49, 1.11
MTR 2756 (AG/GG vs. AA)		0.89	0.71, 1.12
3. Unadjusted simultaneous model, with all gene-gene interaction			
MTHFR 677 (*CT* vs. *CC*)	1514	1.04	0.77, 1.41
MTHFR 677 (*TT* vs. *CC*)		1.35	0.90, 2.04
MTHFR 1298 *(CC vs. AC/AA)*		0.52	0.31, 0.90
MTR 2756 *(AG/GG vs. AA)*		0.78	0.51, 1.17
MTHFR 677 *(CT vs. CC) by MTR* 2756		1.04	0.60, 1.78
MTHFR 677 *(TT vs. CC) by MTR* 2756		1.22	0.59, 2.53
MTHFR 1298 *(CC vs AC/AA) by MTR* 2756		2.58	1.11, 5.98

Table 4.5. Multivariate models evaluating *MTR* 2756A→G, *MTHFR* 1298A→C genotypes, and their interaction in relation to CVD risk while adjusting for cardiovascular risk factor covariates and serum homocysteine levels, Normative Aging Study, 1961-1998

	Crude			***Covariate-adjusted***[2]			***Covariate and homocysteine adjusted***[3]		
Model variables:	**β**[1]	**SE**	**P**	**β**[2]	**SE**	**P**	**β**[3]	**SE**	**P**
MTR 2756A→G (A*G/GG* vs. *AA*)	-0.21	0.12	0.096	-0.26	0.13	0.049	-0.30	0.20	0.13
MTHFR 1298A→C (*CC* vs. *AA/AC*)	-0.70	0.25	0.005	-0.77	0.27	0.004	-0.77	0.39	0.05
Interaction, *MTR* 2756 and *MTHFR* 1298	0.90	0.40	0.024	0.98	0.42	0.02	1.97	0.66	0.003
Controlling for *MTHFR* 677C→T and *MTR-MTHFR* interaction	**Crude**			**Covariate-adjusted**[2]			**Covariate and homocysteine adjusted**[3]		
MTR 2756A→G (A*G/GG* vs. *AA*)	-0.25	0.21	0.23	-0.44	0.23	0.06	-0.43	0.37	0.25
MTHFR 1298A→C (CC vs.*AA/AC*)	-0.65	0.27	0.02	-0.77	0.29	0.007	-0.73	0.43	0.087
Interaction, *MTR* 2756 and *MTHFR* 1298	0.95	0.43	0.03	1.15	0.46	0.012	2.09	0.74	0.004

[1] Values are regression coefficient, SE of regression coefficient and P value from crude (unadjusted) models.
[2] models adjusted for covariates including body mass index, serum cholesterol and triglyceride levels, drinking more than two drinks per day, current smoking, and a cumulative measure of smoking (pack-years).
[3] models adjusted for covariates listed above, and for homocysteine

Table 4.6. *MTR* 2756A→G genotype effect size, stratified by the levels of *MTHFR* 1298A→C genotype, Normative Aging Study, 1961-1998.

		MTHFR *1298 genotype*	
		MTHFR* 1298**A→C ***CC	***MTHFR* 1298** A→C **AA/AC**
Covariate*-adjusted model*	*MTR* 2756 *AA* vs. *AG*/*GG*	0.49 (0.23-1.06)	1.30 (1.001-1.69)
Covariate + serum homocysteine adjusted model	*MTR* 2756 *AA* vs. *AG*/*GG*	0.19 (0.06-0.65)	1.35 (0.91- 2.0)

*Covariates include body mass index, serum cholesterol and triglyceride levels, drinking more than two drinks per day, current smoking, and a cumulative measure of smoking (pack-years).

Testing of gene-gene interactions revealed a significant interaction between *MTHFR* 1298A→C and *MTR* 2756A→G (P=0.02). The interaction was unchanged in the covariate adjusted model (P=0.02) and was strengthened when both covariates and serum homocysteine concentration were added to the model (P=0.003) (Table 4.6). The interaction was unchanged when *MTHFR* 677 polymorphism and its interaction with *MTR* were added to these models (Table 4.6). We investigated the interaction between the *MTHFR* 677 and *MTR* 2756 polymorphism in the absence of *MTHFR* 1298 polymorphism and found no evidence for an interaction (data not shown).

The effect of *MTR* 2756A→G genotype depended on the *MTHFR* 1298A→C genotype. In the adjusted models, among men with the *MTHFR* 1298A→C *CC* genotype, *MTR* 2756A→G *AA* (vs. *AG*/*GG*) decreased the risk of CVD (OR 0.49; 95% CI 0.23, 1.06), whereas among men with the *MTHFR* 1298A→C *AA*/*AC* genotype, *MTR* 2756A→G *AA* increased the risk of CVD (OR 1.30; 95% CI 0.001, 1.69). Adding serum homocysteine concentration to the covariate-adjusted models strengthened the interaction: among men with the *MTHFR* 1298A→C *CC* genotype, *MTR* 2756A→G *AA* (vs. AG/GG) decreased the risk of CVD (OR 0.19; 95% CI 0.06, 0.65), whereas among men with the *MTHFR* 1298A→C *AA/AC* genotype, *MTR* 2756A→G *AA* increased the risk of CVD (OR 1.35; 95% CI 0.91, 2.0).

Discussion

We studied the association of three genetic polymorphisms in the folate metabolic network (*MTHFR* 677 C→T, *MTHFR* 1298 A→C, and *MTR* 2756A→G) with cardiovascular disease in a nested case-control study of the Normative Aging Study cohort. We investigated the main and interactive effect of a single-nucleotide polymorphism in the *MTR* gene (*MTR* 2756 A→G) on the risk of cardiovascular disease. *MTR* 2756 A→G had little or no independent association with CVD, but an

interaction with the *MTHFR 1298* A→C genotype was evident (P=0.02). The effect of *MTR* on CVD risk was in opposite directions depending on the *MTHFR* genotype: the *MTR* 2756A→G *AG/GG* (vs. *AA*) genotype was associated with an increased risk of CVD in men with the *MTHFR* 1298A→C *CC* genotype, whereas in men with the *MTHFR* 1298A→C *AA/AC* genotype, the *MTR* genotype was associated with a decreased risk of CVD. The interaction was independent of another polymorphism, namely *MTHFR* 677 C→T, which has been shown to be important in one-carbon metabolism in general and in cardiovascular disease pathology in this population [30].

The *MTR* 2756 polymorphism is an A to G transition at nucleotide 2756 (2756A → G) in the open reading frame of the gene resulting in the substitution of a glycine (G) for aspartic acid (D) at codon 919 (D919G) [31]. MTR has been shown to be a modular protein with four functional modules [32]. The polymorphism is located in the activation domain of the protein that is involved in reductive activation. The *A* allele is suggested to decrease the docking of the MTR enzyme and therefore prevent the replenishment of the MTR enzyme through the MTR reductase pathway (Patrick Stover, Personal communication). Such an effect would lead to increased homocysteine level and potentially an increase in CVD risk given hypothesized cardiotoxic actions of homocysteine. Another proposed mechanism for the connection between changes in MTR activity and homocysteine level is through RBC folate increase and changes in type of folate in the RBC. It has been argued that a reduction in MTR activity causes an increase in RBC Methyl-THF, a process referred to as methyl trapping. This might be accompanied by an elevated homocysteine (if the reduction in enzyme activity is severe)[33], or a normal homocysteine level (if the reduction in enzyme activity is mild).

We have previously shown significant effects for folate deficiency and for *MTHFR* 677 polymorphism on homocysteine levels (Chapter 2). We have also

reported a significant gene-nutrient interaction between folate deficiency and the *MTHFR* 677 polymorphism, an interaction that is consistent with other studies [34]. Folate-replete populations (such as North American populations after the institution of folate fortification) show an attenuated relation between *MTHFR* and homocysteine levels[35, 36]. Therefore this sample provides an optimal population for studying folate metabolism and CVD risk.

In these data, no interaction was observed between the *MTHFR* 677 and *MTR* 2756 polymorphisms. *MTHFR* 677 has been one of the most widely studied genetic variants in relation to homocysteine and cardiovascular disease, but the observation that another genetic variant that is in close proximity to *MTHFR* 677 shows an independent and strong interactive effect is interesting. *MTHFR* 1298 was discovered as a "second" polymorphism on the *MTHFR* gene [37], and is in linkage disequilibrium with the *MTHFR* 677 polymorphism. It also causes a reduction in the catalytic activity of the MTHFR enzyme, though to a lesser extent compared to the *MTHFR* 677 polymorphism[38]. The observed effect of *MTHFR* 1298 polymorphism in our study is not attenuated by including the *MTHFR* 677 polymorphism and related interaction terms to the regression models. This suggests that although *MTHFR* 677 is a major determinant of genetic variation on *MTHFR* gene, significant residual genetic variation may only be captured by including additional markers on this gene. Using haplotypes in this setting is the recommended approach, but to construct reliable haplotypes from unphased markers, multilocus genotype data from a number of closely-linked variants is ideal: these data are unavailable for the present study.

The MTR and MTHFR enzymes catalyze sequential steps in the folate metabolic network, leading to the hypothesis of interaction between them in relation to the risk of CVD. MTR is a B12 dependent enzyme that catalyzes the remethylation of homocysteine to methionine. The methyl group for this reaction is provided by 5-

methyl THF, which is a product of the MTHFR enzyme. Given the known effect of *MTHFR* polymorphism on the functionality of the enzyme, it is plausible to hypothesize an interaction between these polymorphisms and a potentially functional polymorphism in *MTR* gene. Other studies have investigated the relation of *MTR* to a variety of outcomes, and some evaluated the role of an *MTHFR-MTR* interaction, but none have detected an interaction between these two polymorphisms. The lack of similar findings from other studies may be due to small sample size or population-specific features of the studies. Studies on the effect of *MTR* polymorphism on homocysteine level or cardiovascular disease risk have had mixed results including associations with increased risk [39], decreased risk [40-42], or no effect on risk [43-46].

In a prospective study of US male physicians, Chen et al. found that the *MTR GG* genotype was associated with a non-significant reduction in MI risk (RR 0.51, 95% CI 0.17, 1.16) compared to individuals with *AA* genotype [47]. The *MTR* polymorphism was associated with decreased homocysteine levels (10.55, 9.87 and 9.57 nmol/ml for *AA*, *AG* and *GG* genotypes, respectively) only among controls [48]. Similarly in a study on subjects from the NHLBI Family Heart Study, a weak positive association was observed between changes in homocysteine after a methionine load and the number of mutant *MTR* alleles (P trend = 0.04), but this was not significant in the overall F test [49]. These studies are similar to our study in having a relatively large sample size (about 500 cases) and therefore have adequate power to detect an association between the variant allele and alterations in homocysteine level of the order of magnitude reported herein. Our results are consistent with these findings, and other reports that fail to see a significant association between the fasting homocysteine concentration and the *MTR* 2756 polymorphism.

Serum homocysteine may partly or wholly mediate the relation of the *MTHFR* and *MTR* polymorphisms with CVD risk. The further addition of serum homocysteine

to regression models increased the magnitude of the gene-gene interaction coefficients. This is in contrast to the expectation of attenuation of coefficients that would be expected if homocysteine mediated the genotype effects. Therefore our findings suggest that the observed gene-gene interaction is not mediated through homocysteine. The view that mild hyperhomocysteinemia is a causal factor in atherogenesis has been challenged by two recent homocysteine-lowering randomized controlled trials showing no effect of lowering homocysteine levels through vitamin administration on cardiovascular disease risk or recurrence[50, 51]. Since homocysteine is part of a larger metabolic network that provides substrates for many biological functions (methylation reactions, nucleotide biosynthesis, provision of folate cofactor forms), functional genetic polymorphisms in the genes of the network might exert their effect through pathways other than methionine biosynthesis.

The Normative Aging Study is a prospective cohort study that has collected extensive lifestyle and medical data on its participants. The prospective nature of the NAS cohort minimizes the biases associated with case-control studies of cardiovascular disease. A potential weakness of this study is the availability of biomarker data on a subset of the full cohort only.

In summary, we have investigated the role of the *MTR* 2756A→G and *MTHFR* 677C→T polymorphisms in relation to serum homocysteine level and cardiovascular disease risk. There is some evidence for a gene-gene interaction such that the effect of the *MTHFR* 1298A→C genotype is limited to men with the *MTHFR* variant genotype. The observed effects are independent of homocysteine. Evaluation of this interaction in future epidemiologic studies and inclusion of multiple markers on MTHFR gene including the *MTHFR* 1298, are recommended.

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