EFFECT OF THE METHYLENETETRAHYDROFOLATE REDUCTASE 677C→T POLYMORPHISM ON FOLATE STATUS AND DNA METHYLATION RESPONSE IN YOUNG WOMEN

By

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This dissertation is dedicated to my aunt Nellie J. Mendéz whose successful career as a dietitian inspired me to study nutrition. Although her career ended early as a result of health complications, she continues to incorporate good nutrition into her daily life. I hope that one day I will leave my mark in nutrition research as she has done in community nutrition.
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<td>SNP</td>
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<td>tCyt</td>
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<td>TE</td>
<td>tris EDTA</td>
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<td>tetrahydrofolate</td>
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<td>venous thromboembolism</td>
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Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in folate metabolism. A C→T substitution at base pair 677 of MTHFR may impair folate status when folate intake is marginal, a particular concern for women of reproductive age. Folate status response to a controlled folate depletion-repletion feeding protocol (14 wk) was investigated in young women (20 to 30 y) with (TT) (n = 19) and without (CC) (n = 22) the MTHFR 677C→T variant. Subjects consumed a moderately folate-deplete diet (115 µg DFE/d) for 7 wk, followed by folate repletion for 7 wk with the current RDA for this population (400 µg DFE/d).

Overall serum folate decreased (P < 0.0001) during depletion and increased (P < 0.0001) during repletion with lower (P = 0.03) post-depletion serum folate in women with the TT versus CC genotype (LS mean 15.3 ± 1.2 vs 19.5 ± 1.3 nmol/L, respectively). Folate status was low (serum folate < 13.6 nmol/L) in more women with
the TT (59%) versus the CC (15%) genotype post-depletion. Red blood cell folate for all subjects decreased during depletion (P < 0.0001) and repletion (P = 0.02) with lower (P = 0.04) red blood cell folate in women with the TT versus the CC genotype post-repletion (LS mean 1036 ± 53 vs 1203 ± 53 nmol/L, respectively). Homocysteine increased (P < 0.0001) during depletion and decreased for subjects with the CC (P = 0.02) but not the TT (P = 0.47) genotype during repletion. Homocysteine tended to differ by genotype post-depletion [10.5 ± 3.3 (TT) vs 8.9 ± 1.9 (CC) (P = 0.09)] and post-repletion [8.9 ± 1.6 (TT) vs 7.2 ± 1.9 (CC) (P = 0.08)]. Overall percent change in [³H]methyl group acceptance tended to increase during depletion (P = 0.08). Women with the TT genotype had an increase in raw and percent change in methylated cytosine during repletion (P < 0.05). Except for global DNA methylation, these data suggest that young women with the MTHFR 677 TT genotype respond more negatively to folate depletion and less positively to repletion with the current RDA compared to women with the CC genotype.
CHAPTER 1
INTRODUCTION

Folate is an essential vitamin that functions to accept and transfer one-carbon units in nucleotide synthesis, amino acid interconversions including methionine regeneration, and the formation of S-adenosylmethionine (SAM)—the primary methylating agent in the body. An important reaction in folate metabolism is the reduction of 5,10-methylenetetrahydrofolate (5,10-methyleneTHF) to 5-methyltetrahydrofolate (5-methylTHF) by the 5,10-methylenetetrahydrofolate reductase (MTHFR) enzyme. This reaction generates folate in the form of 5-methylTHF, essential for the remethylation of homocysteine to methionine. The conversion of homocysteine to methionine is essential for the synthesis of SAM, which is the methyl donor in > 100 physiological reactions, including methylation of DNA, RNA, and membrane phospholipids as previously reviewed (1).

Emerging science involving single-nucleotide polymorphisms offers new insight and a more precise understanding of how individual genetic variations influence folate-dependent metabolic pathways (2). Single-nucleotide polymorphisms, genetic variations present in > 1% of the population, can act alone or synergistically with nutritional deficiencies to accelerate and accentuate metabolic pathologies. A very common polymorphism and one that has been studied extensively is a C-to-T substitution at base pair 677 in the gene that codes for MTHFR (3). The MTHFR 677C→T variant is prevalent in the overall population: ~12% homozygous (TT); and ~50% heterozygous (CT) (4). This variant results in an alanine-to-valine substitution in the enzyme resulting in
in a marked reduction in enzyme stability that can be enhanced by the addition of 5-methylTHF (5). In individuals who are homozygous for the MTHFR 677C→T variant (TT) the impaired enzyme stability is associated with reduced methylated blood folate (6) and elevated plasma homocysteine concentration that can be significantly improved in response to improved folate status (7).

Different studies have provided evidence that methylation of DNA plays a role in genome stability and gene expression (8,9). The potential for impaired folate status coupled with the MTHFR 677 TT genotype to negatively influence genome stability (10,11), including DNA methylation (12,13), provided one incentive for the present study. The effect of folate inadequacy in individuals with the TT genotype on indicators of global DNA methylation has not been previously evaluated in a metabolic study in which nutrient intake was carefully controlled. The present study was designed to monitor changes in DNA methylation by MTHFR genotype in conjunction with other folate status indicators in response to a folate depletion-repletion protocol.

An estimated 120,000-150,000 infants are born with a major birth defect in the US each year, representing more than 3% of all live births (14). Emerging scientific evidence involving single nucleotide polymorphisms such as the MTHFR 677C→T variant, including data from the present study, may lead to a precise understanding of how individual genetic variations influence folate-dependent metabolic pathways and potentially increase birth defect risk. Various studies have shown that the MTHFR 677 TT genotype is a significant risk factor for neural tube defects when folate intake is not sufficient to maintain metabolic homeostasis (15,16). Since the combined presence of the MTHFR 677C→T variant and low folate status has been associated with increased risk
for birth defects, the present study was designed to address the specific aim in females of reproductive age.

**Hypotheses**

1. Consumption of a low-folate diet will result in more significant impairment in indicators of folate status and global DNA methylation in young women with the homozygous MTHFR 677C→T TT genotype compared to those with the CC genotype.

2. Consumption of the current Recommended Dietary Allowance for folate (400 µg dietary folate equivalents/d) following a low-folate diet will not be sufficient to significantly improve folate status or global DNA methylation in females with the TT compared to the CC genotype.

**Specific Aim**

The specific aim of the present study is to determine the combined effects of the MTHFR 677C→T variant and dietary folate depletion-repletion on indicators of folate status response (serum and red blood cell folate and plasma homocysteine concentrations) and global DNA methylation ([$^3$H]methyl group acceptance and methylcytosine/total cytosine ratio) in women of reproductive age.
CHAPTER 2
BACKGROUND AND LITERATURE REVIEW

Folate

Chemistry

Folate is a general term that refers to naturally occurring food folate and synthetic folic acid found in supplements and fortified foods. Synthetic folic acid is yellow with a molecular weight of 441.4. Folate is made up of three parts that are all necessary for vitamin activity. A pteridine bicyclic ring is linked by a methylene group to para-aminobenzoic acid whose carboxy group is peptide bound to the α-amino group of glutamate to form folate (17) (Figure 2-1).


Dietary Sources

Mammals are unable to synthesize folate de novo and must therefore obtain it from the diet. Folate is not considered to be prevalent in the food supply. Two different types of folate are available for consumption: folate found naturally in the food supply and synthetic folic acid added to enriched food products and supplements.
**Naturally occurring food folate.** Some good sources of naturally occurring food folates are orange juice, dark green leafy vegetables, asparagus, strawberries, peanuts, and legumes such as kidney and lima beans (18). Folate concentration in raw foods is higher than in cooked foods due to the folate losses incurred by cooking (19). Variability of losses associated with cooking is attributable to differences in oxygen exposure, ascorbic acid content, and the amount of water present (19). The folates that occur naturally in food are the fully reduced tetrahydrofolates and usually have 5 to 7 glutamates in the polyglutamate side chain (19).

**Folic acid in fortified foods.** Another major source of folate is synthetic folic acid used in enriched foods and fortified, ready-to-eat breakfast cereals. Effective January 1, 1998, the U.S. Food and Drug Administration (FDA) mandated the fortification of all cereal grain products labeled as enriched (e.g., bread, pasta, flour, rice) with 140 µg folic acid per 100 g of product (20). Thousands of food items have been affected under this mandate (21). A large number of ready-to-eat breakfast cereals contain folic acid in varying amounts. Most ready-to-eat cereals are fortified to provide 25% the Daily Value of folate, with some brands providing four times this amount (18).

**Bioavailability**

Folate bioavailability refers to the overall efficiency of utilization of the vitamin, including absorption, transport, metabolism, catabolism, and excretion (1,22). The bioavailability of food folate differs greatly from the bioavailability of synthetic folic acid.

**Food folate bioavailability.** The bioavailability of folate in different foods varies considerably because of differences in digestion, absorption, and metabolism. Some possible digestion/absorption issues that can affect folate bioavailability include altered
pH, conjugase activity, and transit time (22). The effect of different food components on folate utilization further complicates the issue by trapping the folate in the food matrix or inhibiting conjugase activity (22). Food processing can cause oxidative damage of folates (22) and can account for 50 to 95% of any folate lost (23). Alcohol and pharmaceuticals also can inhibit the absorption of folate (22). All of these factors attribute to the high variability of folate bioavailability, which can be as high as 96% and as low as 25% (24).

**Synthetic folic acid bioavailability.** Synthetic folic acid is much more bioavailable than food folate. When folic acid is administered without food it is 100% bioavailable (25). Sauberlich et al. (23) estimated that when compared to synthetic folic acid, the bioavailability of food folate is no more than 50% available. Pfeiffer et al. (26) examined the bioavailability of $^{13}$C$_5$-labeled folic acid administered in apple juice when given with or without food. Consumption of the folic acid supplement with food slightly decreases its bioavailability by approximately 15% (26). The Dietary Reference Intake Committee of the Institute of Medicine (IOM) used these data to derive an estimated bioavailability of folic acid when consumed with food (85%) (27). This estimate of folic acid bioavailability was used by the IOM as a basis for the new term, dietary folate equivalents (DFEs), which was used to express the 1998 Dietary Reference Intakes (DRIs).

**Dietary folate equivalents (DFEs).** The DFEs convert all forms of dietary folate, including synthetic folic acid in fortified products, to an amount that is equivalent to food folate (28). The rationale for the DFEs is that folic acid consumed with food is only 85% bioavailable and naturally occurring food folate is only 50% bioavailable. Using these
estimates of folate bioavailability, folic acid consumed with food is estimated to be 1.7 times (i.e., 85/50) more available than natural food folate (27). The total folate content can be calculated by multiplying the micrograms of synthetic folic acid by 1.7 and then adding this number to the number of micrograms of food folate present in the meal (18). To compare folic acid with food folate the following conversions are used: 1 µg DFE = 1 µg food folate = 0.5 µg folic acid taken on an empty stomach = 0.6 µg folic acid taken with meals. When estimating food folate intake only, no adjustment is needed (27).

**Absorption**

Before absorption of ingested food folate can occur, it must be hydrolyzed to the monoglutamate form by glutamate carboxypeptidase II (EC 3.4.17.21) (29) also called pteroylpolyglutamate hydrolase or folate conjugase. This enzyme is located in the jejunal brush border membrane and has an optimum pH of 6.5 to 7.0 (30). Under normal conditions, monoglutamyl folate is transported across the intestinal membrane by a saturable, pH-dependent carrier-mediated intestinal folate carrier (31). The expression of this intestinal folate carrier may be upregulated in response to folate deficiency (32). When folate concentrations are high, a nonsaturable mechanism involving passive diffusion transports folate across the intestinal membrane. This mechanism may be more important in the absorption of supplemental folic acid as opposed to food folate (22).

Most folate is converted to the reduced forms, dihydrofolate (DHF) or tetrahydrofolate (THF), by dihydrofolate reductase before portal blood entry. This mechanism is saturable and large amounts of oxidized folic acid have been found in the plasma and urine of individuals ingesting 400-800 µg/d of folic acid (22). Further metabolism to a methylated or formylated form of folate also may occur in the mucosal cells before portal blood entry (22).
Transport

Once the folate is absorbed in the monoglutamate form, it travels in the portal circulation, mostly as 5-methylTHF, to the liver where it is reduced and conjugated for retention. The major forms of folate in the liver are 5-methylTHF and 10-formylTHF, which can be secreted into the bile and reabsorbed via enterohepatic circulation (33). Approximately two-thirds of folates in the plasma are protein bound, 50% of which are bound to albumin. The remaining one-third is tightly bound to folate binding protein (34).

Folate binding proteins (FBPs). Membrane associated FBPs, also known as folate receptors, transport folate across membranes from circulating blood into cells, are highly localized and expressed in specific tissues and cells (35), and have a higher affinity for oxidized folic acid than for reduced folates (36). The FBPs are essential for normal embryonic development and can cause embryonic lethality if the gene that codes for FBP is knocked out in mice (37). The FBP knockout mice have been brought to term with folinic acid supplementation and were born with normal phenotypes (35). Although the FBP has been characterized, little is known about how cells obtain their folate. Antony (38) reviewed two possible mechanisms by which cells take up folate. The first is the well-known process of endocytosis, and the second is referred to as potocytosis. In potocytosis the FBPs are gathered in clusters at the plasma membrane, which move into caveolae and concentrate circulating folates. Following sudden acidification of the caveolae, the folates dissociate from the folate receptors and are transported into the cytoplasm by anion channels. This is not the only mechanism for folate transport (38). This membrane-associated FBP has extensive homology with the plasma FBP (17).
**Reduced folate carrier (RFC).** A RFC also exists that transports reduced folates into the cell (36). The RFC protein is the same folate carrier protein that is expressed in the intestine. The RFCs are located in all tissues and cells (35) and have a higher affinity for reduced folates than oxidized folic acid. They function to form channels in the plasma membrane through which reduced folates, mostly 5-methylTHF, can cross into the cytoplasm of the cell (39). Zhao et al. (40) determined the consequence of inactivation of the RFC in embryonic RFC knockout mice who died *in utero*. They also observed that near normal development of RFC knockout mice could be attained with daily supplementation of the heterozygous pregnant dams with folic acid, but these pups died within 12 d of birth. Zhao et al. (40) concluded that the folic acid supplementation was sufficient *in utero*, but that there was probably insufficient folate in the mother’s milk to sustain life.

A polymorphism of the RFC gene that results in a G→A substitution at base pair 80 replaces arginine with histidine (36) in the protein. This polymorphism has not been found to affect plasma folate or homocysteine concentrations in adults but may be more important to the developing embryo (36). De Marco et al. (41) observed that neural tube defect (NTD)-affected Italian children with the homozygous variant genotype (GG) had a significantly higher risk for NTDs than control children with the normal genotype (AA). In addition, mothers with the GG genotype had a higher risk of giving birth to an NTD-affected child compared to mothers with the AA genotype (40).

Folates enter cells in the monoglutamate form and if in the oxidized form must be reduced by dihydrofolate reductase to THF and polyglutamated by folylpoly-γ-glutamate synthetase. Conjugation retains folates in the cells and is required for participation in
one-carbon metabolism. The different coenzymatic forms of folate are all derivatives of THF and can contain a methyl (CH$_3$), methylene (-CH$_2$-), methenyl (-CH=), formyl (-CH=O), or formimino (-CH=NH) group (1). Tetrahydrofolate accepts the one-carbon moieties, which become bonded to the N$_5$ or N$_{10}$ atoms or both. The polyglutamate folates must be hydrolyzed before release from the tissues into circulation with γ-glutamylhydrolase converting the folates back to the monoglutamate form (17). The total body content of folate is approximately 15 to 30 mg with the liver containing 50% of the body’s folate stores (42).

**Biochemical Functions**

Folate’s main biochemical functions are to accept and transfer one-carbon units involved in amino acid metabolism, purine and pyrimidine synthesis, and the formation of SAM, the main methyl donor in > 100 reactions. Tetrahydrofolate is the main acceptor molecule and ultimately ends up in pathways required for nucleotide biosynthesis or methylation reactions. An overview of the different folate pathways is presented in Figure 2-2.

**Amino acid metabolism.** Folate functions as an intermediate in the metabolism of the amino acids serine, glycine, methionine, homocysteine, and histidine. The metabolism of serine, glycine, methionine, and homocysteine are closely related. Tetrahydrofolate is converted to 5,10-methyleneTHF via serine hydroxymethyltransferase (SHMT) (Figure 2-2, reaction 3). Serine is converted to glycine in the process, and both reactions are reversible. Pyridoxal phosphate is required as a cofactor for this reaction. The degradation of glycine requires THF and NAD+. The β-carbon of serine provides the most one-carbon units in all mammalian systems. These
DNA Methylation

1. dihydrofolate reductase
2. thymidylate synthase
3. serine hydroxymethyltransferase
4. cyclohydrolase
5. formate-activating enzyme
6. transformylases
7. methylenetetrahydrofolate reductase
8. methionine synthase
9. S-adenosylmethionine synthase
10. cellular methyltransferases
11. S-adenosylhomocysteine hydrolase
12. cystathionine β-synthase
13. betaine:homocysteine methyltransferase
14. cystathionase

Purine Synthesis + THF

- DNA Synthesis
- DHF
- THF
- 5-methylTHF
- 5,10-methyleneTHF
- 10-formylTHF
- 5,10-methylenetetrahydrofolate reductase
- dTMP
- dUMP
- Serine
- Glycine
- 10-formylTHF

DNA Methylation

- Methionine
- SAM
- SAH
- Cystathionine
- Cysteine

one-carbon units get transferred to THF (43). After 5,10-methyleneTHF is formed, it is reduced to 5-methylTHF by the irreversible reaction requiring the enzyme MTHFR (Figure 2-2, reaction 7). The substrate and cofactor for methionine synthase, the enzyme that remethylates homocysteine to methionine, is 5-methylTHF (Figure 2-2, reaction 8) and requires vitamin B12, or cobalamin, as a coenzyme. A vitamin B12 deficiency can lead to a secondary folate deficiency that investigators have termed the “methyl trap”. Methionine synthase depends on vitamin B12 as a coenzyme and its activity is reduced during a vitamin B12 deficiency. This results in the accumulation of 5-methylTHF, which gets “trapped” in this form and results in the decreased availability of THF and all of the other forms of folate. This can lead to megaloblastic anemia due to insufficient folate coenzymes available for DNA synthesis (17).

Homocysteine can also be remethylated to methionine by betaine:homocysteine methyltransferase (BHMT) (Figure 2-2, reaction 14), which is a betaine-dependent reaction found in the pathway of choline oxidation. Choline is a methyl rich compound present in food that is first oxidized to betaine and then demethylated by BHMT (44). Choline also can be synthesized by the body in the SAM dependent methylation of phosphatidylethanolamine to form phosphatidylcholine, which can be further catabolized to choline. Choline oxidation primarily takes place in the liver and kidney. Choline is first oxidized to betaine aldehyde via choline oxidase. Betaine aldehyde is then oxidized to betaine via betaine aldehyde dehydrogenase (44). Betaine can then be used in the BHMT reaction. The interdependence of choline and folate has been demonstrated in a variety of studies with rats. Rats fed a choline or choline and methionine deficient diet
for 2 wk to 12 mo had decreased hepatic folate stores (45-47). Adverse effects from a choline deficiency were reversed within 2 wk with adequate dietary choline (47).

Histidine is the other amino acid dependent on folate metabolism. The deamination of histidine produces urocanic acid, which becomes formiminoglutamate (FIGLU) after further metabolism. Formiminoglutamate loses the formimino group via formimino transferase to THF to produce \( N^5 \)-formiminoTHF. Glutamic acid is the final product of this reaction. The pathway of histidine metabolism is shown in Figure 2-3.

![Figure 2-3. Histidine metabolism.](image)

**Purine and pyrimidine synthesis.** The nucleotide biosynthesis pathway utilizes the 5,10-methyleneTHF coenzyme. Pyrimidine synthesis involves the formation of deoxythymidylate monophosphate (dTMP) from deoxyuridylate monophosphate (dUMP) via thymidylate synthase (Figure 2-2, reaction 2), which requires 5,10-methyleneTHF as a coenzyme. This is the rate-limiting step in the cell cycle and allows DNA replication to continue. Once thymidylate synthase utilizes 5,10-methyleneTHF as a coenzyme, it is oxidized to DHF and then regenerated to THF via dihydrofolate reductase (Figure 2-2, reaction 1). Purine synthesis depends on the conversion of 5,10-methyleneTHF to
10-formylTHF via 10-formylTHF synthetase (Figure 2-2, reaction 4). The folate coenzyme is used to donate formyl groups to different positions of the purine ring and depends on a formate activating enzyme (Figure 2-2, reaction 5).

**S-Adenosyl methionine (SAM) synthesis.** The formation of SAM is dependent on the metabolism of homocysteine and methionine. Under normal conditions, methionine synthase transfers the methyl group from 5-methylTHF to vitamin B12 and then to homocysteine. In this reaction, 5-methylTHF is reconverted to THF. The newly synthesized methionine can then be converted to SAM by SAM synthase (Figure 2-2, reaction 9) when combined with adenosine. S-Adenosylmethionine can then act to donate methyl groups in over 100 different methylation reactions, including DNA and RNA methylation. Once a methyltransferase uses the methyl group provided by SAM, it is converted to S-adenosylhomocysteine (SAH) (Figure 2-2, reaction 10).

S-Adenosylhomocysteine is hydrolyzed to homocysteine and adenosine via the reversible SAH hydrolase (Figure 2-2, reaction 11). Homocysteine can be remethylated to methionine or converted to cysteine through the transulfuration pathway where cystathionine β-synthase (CBS) (Figure 2-2, reaction 12) converts homocysteine to cystathionine, a reaction that is dependent on pyridoxal phosphate and serine. Cystathionase (Figure 2-2, reaction 13) converts cystathionine to cysteine.

Cytosolic folate metabolism differs from mitochondrial folate metabolism. The composition of folate pools within the cytosol and mitochondria varies among different tissues (1). In the cytosol, one source of one-carbon groups is provided by formate from the mitochondria and cytosol that is converted to 10-formylTHF by 10-formyl synthetase (Figure 2-4, enzyme C3). It serves as a one-carbon source in the cytosol for purine
Table 2-1. Compartmentalization of enzymes and reactions.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1:M1</td>
<td>5,10-MethyleneTHF dehydrogenase</td>
</tr>
<tr>
<td>C2:M2</td>
<td>5,10-MethyleneTHF cyclohydrogenase</td>
</tr>
<tr>
<td>C3:M3</td>
<td>10-FormylTHF synthetase</td>
</tr>
<tr>
<td>C4</td>
<td>Glycine N-methyltransferase</td>
</tr>
<tr>
<td>C5:M5</td>
<td>Serine hydroxymethyltransferase</td>
</tr>
<tr>
<td>C6</td>
<td>Methionine synthase</td>
</tr>
<tr>
<td>C7</td>
<td>Glycinamide ribonucleotide transformylase</td>
</tr>
<tr>
<td>C8</td>
<td>Phosphoribosylamino-imidazole carboxamide</td>
</tr>
<tr>
<td></td>
<td>transformylase</td>
</tr>
<tr>
<td>C9</td>
<td>5,10-MethyleneTHF reductase</td>
</tr>
<tr>
<td>C10</td>
<td>Thymidylate synthase</td>
</tr>
<tr>
<td>M11</td>
<td>Dimethylglycine dehydrogenase</td>
</tr>
<tr>
<td>M12</td>
<td>Sarcosine dehydrogenase</td>
</tr>
<tr>
<td>M13</td>
<td>Glycine cleavage system</td>
</tr>
<tr>
<td>C14:M14</td>
<td>10-FormylTHF dehydrogenase</td>
</tr>
</tbody>
</table>


synthesis. 10-FormylTHF can be further reduced to 5,10-methenylTHF by

5,10-methenylTHF cyclohydrolase (Figure 2-4, enzyme C2) and reduced once again by

5,10-methyleneTHF dehydrogenase to 5,10-methyleneTHF for pyrimidine synthesis.

The enzyme MTHFR (Figure 2-4, enzyme C9) reduces 5,10-methyleneTHF to

5-methylTHF for homocysteine remethylation to methionine in the cytosol. The

interconversion of serine and glycine not only provides one-carbon units to the cytosol,

but also to the mitochondria. The difference between cytosolic and mitochondrial folate

metabolism can be explained by the form of SHMT (Figure 2-4, enzymes C5:M5), the

enzyme used to catalyze this reaction, since cytosolic SHMT is different than

mitochondrial SHMT. Glycine cleavage occurs only in the mitochondria. The final

folate-dependent steps of choline catabolism occur in the mitochondria.
The biochemical functions of folate are collectively referred to as one-carbon metabolism (1). Although each function is independently important, they are all dependent on the cellular concentration of THF and each other to function.

**Folate–Related Polymorphisms**

Single nucleotide variations in the genome can result in genes that code for enzymes with different activity. If a genetic variation is present in > 1% of the population, it is considered a genetic polymorphism (48). Polymorphisms affecting one-carbon metabolism are of special interest to investigators due to their frequency in the population and their effects on disease risk and developmental abnormalities. The focus of folate research has shifted from evaluating the effect of severe deficiency on clinical indicators and blood folate concentrations to identifying functional indicators of disease risk and how they are affected by genetic polymorphisms. The next section will include a synopsis of recent research findings and key research questions related to polymorphisms affecting folate metabolic function.

**Methylenetetrahydrofolate Reductase (MTHFR)**

Although folate metabolism involves over 30 genes, enzymes, and transporters, the most extensively studied polymorphism affects MTHFR, the enzyme that catalyzes the reduction of 5,10-methyleneTHF to 5-methylTHF (3). In 1988 Kang and colleagues (49) discovered a thermolabile variant of MTHFR that was positively correlated to increased cardiovascular risk and increased homocysteine concentrations. In 1994, Goyette et al. (50) isolated a complementary DNA (cDNA) copy of human MTHFR that was 22 kilobases long consisting of 11 exons. Isolation of this cDNA enabled them to identify nine mutations in the gene in patients with severe MTHFR deficiency (50,51). A more recent report increased this number to 33 severe mutations identified in patients with
severe MTHFR deficiency (52). Frosst et al. (3) identified a homozygous variant of this gene that resulted in decreased enzyme activity and increased thermolability *in vitro*. This autosomal recessive variation results in a C→T substitution at base pair 677 that replaces alanine with valine in the enzyme. Any decrease in MTHFR activity from the 677C→T variant will result in lower 5-methylTHF available to donate a methyl group for the remethylation of homocysteine to methionine. This can result in elevated homocysteine concentrations in individuals with the homozygous variant (TT) genotype in contrast to the heterozygous variant (CT) or homozygous normal (CC) MTHFR 677C→T genotype. The homozygous TT variant is termed “thermolabile” because of a significant decrease in residual activity compared to CC controls after heat inactivation at 46°C for 5 min (49). Frosst et al. (3) compared specific activity and residual activity after heating for 5 min at 46°C. They found that subjects with the TT genotype had a specific activity < 50% of that of the subjects with the CC genotype and a residual activity < 35% after heating. Rozen (53) found a 50-60% reduction in residual activity among individuals with the TT genotype at 37°C and a 65% decrease at 46°C compared to individuals with the CC genotype.

Matthews et al. (54) reported that the MTHFR gene codes for an enzyme with two identical 77 kDa subunits, each consisting of a 40 kDa N-terminal domain and a 37 kDa C-terminal domain. In addition, Frosst et al. (3) found a 70 kDa subunit, which they attributed to the presence of isozymes. Sumner et al. (55) discovered that SAM binds to the C-terminal domain, which led them to implicate this domain as the regulatory region because SAM is an allosteric inhibitor of MTHFR. Matthews et al. (56) reviewed the similarities between the N-terminal domain of the human MTHFR and smaller proteins.
of enteric bacteria that catalyze the same reaction. They explained that since SAM does not regulate the bacteria proteins, the N-terminal domain must be the catalytic region. This is important because the C→T substitution occurs in exon 4 of the N-terminal domain (57). Matthews et al. (56) purified MTHFR from *Escherichia coli*. They reported that the enzyme was a flavoprotein with flavin adenine dinucleotide (FAD) as its cofactor, which is reduced by NADH and NADPH and then reduces 5,10-methyleneTHF to 5-methylTHF. Guenther et al. (58) characterized the normal (CC) and variant (TT) MTHFR enzymes from *Escherichia coli*, which revealed that the variant enzyme loses its flavin cofactor more readily than the normal enzyme. They reported that the catalytic domain shared by all MTHFRs is a barrel that binds FAD, and that the variant does not cause decreased activity of the enzyme directly but instead decreases enzyme stability, which facilitates the dissociation of FAD and decreases enzyme activity. They found that folate supplementation confers protection by increasing the affinity of MTHFR for FAD. Matthews (59) hypothesized that the exact mechanism of this protection lies in the positioning of the folate cofactor with respect to the barrel. Folate may bind with the pteridine ring stacked above the flavin, thus stabilizing the enzyme and preventing FAD dissociation. Yamada et al. (5) were the first to characterize human MTHFR by using a baculovirus expression system. They identified important differences between the mammalian enzyme and the prokaryotic enzyme. For example, the prokaryotic enzyme lacks a regulatory domain and uses NADH as a reductant while the mammalian enzyme has a regulatory domain and uses NADPH as a reductant. Another difference is that prokaryotic MTHFR is a tetramer in contrast to the mammalian MTHFR, which is a dimer. Their results agree with the previous findings of Guenther et al. (58) that the
decreased activity of the enzyme is due to decreased protein stability resulting from FAD dissociation. This loss of FAD leads to dissociation of the dimer into monomers and reduction in activity (59) and has been found to occur 10 times faster in the homozygous variant (TT) compared to the normal (CC) enzyme (58). Yamada et al. (5) concluded that individuals with the TT genotype for the MTHFR 677C→T polymorphism are at higher risk for increased homocysteine concentrations if their folate concentrations are low. This increased risk is due to less remethylation of homocysteine to methionine as a result of the decreased MTHFR stability. Decreased MTHFR stability will lead to even more dissociation of FAD and result in less enzyme activity. The in vitro studies performed by this group showed that increasing folate, SAM, and FAD concentrations protected against loss of activity in the affected human MTHFR enzyme.

The association between riboflavin status and homocysteine concentration has been evaluated. Hustad et al. (60) reported a significant inverse dose-response association between plasma riboflavin and homocysteine concentrations in their combined MTHFR 677 CT/TT group. When plasma riboflavin was separated into quartiles, they observed a 1.4 µmol/L higher homocysteine concentration in the lowest compared to the highest quartile. They concluded that riboflavin is an independent determinant of plasma homocysteine concentration regardless of folate status. McNulty et al. (61) separated the riboflavin status of their population into tertiles and found that among subjects in the lowest tertile, the mean homocysteine concentration of subjects with the TT genotype was approximately twice the concentration in either the CT or CC genotype groups. There were no significant differences in mean homocysteine concentrations between genotype groups in either the medium or high tertiles of riboflavin status. They
concluded that unlike folate status, riboflavin status is only a predictor of homocysteine concentration in subjects with the TT genotype, and that a riboflavin-dependent mechanism may contribute to the interrelationship between folate and plasma homocysteine concentrations in this group (61). Jacques et al. (62) further clarified the relationship between riboflavin and homocysteine concentrations by reporting that the association between these indices in their population was only significant in those with lower folate status. When further stratified by genotype, the interaction between folate and riboflavin was only significant in subjects with the TT genotype. Only those with low folate status and the TT genotype showed a significant association between riboflavin and homocysteine concentrations. In contrast to the previously described studies, Moat et al. (63) found an inverse association between riboflavin and homocysteine concentrations in individuals with both the CC and TT genotypes, but further studies are warranted to clarify this discrepancy. Rozen (64) suggested that additional studies to determine the role of folate in the riboflavin-homocysteine association would be useful before drawing conclusions.

An extensive review by Botto and Yang (4) provides a description of the population frequency for the MTHFR 677C→T polymorphism. They estimated that approximately 22% of Hispanics, 12% of Caucasians, 11% of Asians, and 1 to 2% of African Americans are homozygous for the variant. The variant has been found in ~35% of alleles (3,65). The frequency of the homozygous variant varies among racial and ethnic groups. The lowest prevalence is among blacks and Africans (~2% and 0%). Stevenson et al. (66) reported the frequency of the TT genotype in 151 whites and 146
blacks from South Carolina and found a frequency of 13% and 0%, respectively, which agrees with other reports (4).

The effect of the TT genotype can be quantitated by evaluating the change in cellular composition of the folate derivatives (6). If 5,10-methyleneTHF is not reduced to 5-methylTHF, the methylene group of 5,10-methyleneTHF can be donated to dUMP to form dTMP or oxidized to 5,10-methenylTHF, which ultimately is converted to 10-formylTHF for the synthesis of purines (6). One would expect a decrease in MTHFR activity to result in an increase in formylated folates and a decrease in methylated folates. Bagley and Selhub (6) provided data to support this hypothesis by evaluating the effect of the MTHFR genotype on the form of folate within red blood cells. Of eight subjects with the CC genotype, all had 100% methylated folates. Of the 10 subjects with the TT genotype, eight had formylated folates ranging from 0 to 59% of the total folate content. Genotype did not affect total red blood cell folate concentration. Zittoun et al. (67) also evaluated the effect of the TT genotype on methylfolates and reported that in subjects with the CC genotype, 71% of the red blood cell folate concentration was methylfolate compared to 66% and 27% in individuals with the CT and TT genotypes, respectively. After separating their study population into high and low red blood cell folate status, Friso et al. (13) found significantly less methylated folates in subjects with the TT genotype, regardless of folate status. These data support the findings of Bagley and Selhub (6).

Another more recently discovered MTHFR polymorphism, 1298A→C, results in an A→C base pair substitution at base pair 1298 that replaces glutamic acid with alanine in the C-terminal regulatory domain of the enzyme resulting in a homozygous variant
(CC), heterozygous variant (AC), or normal (AA) genotype (57,68). The MTHFR 1298A→C polymorphism was first discovered by Viel et al. (69) in patients with ovarian carcinomas, but was not characterized until later (57,68). Yamada et al. (5) were unable to distinguish between the properties of the variant and normal enzymes after baculovirus expression. They determined that the homozygous variant enzyme did not affect catalytic function alone or in combination with the 677 TT genotype, and that it appeared thermostable and unaffected by changes in in vitro folate concentration. This is in contrast to the 60% decrease in activity observed in lymphocyte extracts with the homozygous variant genotype (57).

This MTHFR 1298A→C polymorphism was reported to affect 10% of the Canadian population with an allele frequency of 33%. Neither individuals with the CC variant nor individuals with the heterozygous variant genotype have higher homocysteine concentrations or lower folate concentrations (68,70-72) than normal individuals. The risk appears when the 677C→T variant is combined with the 1298A→C variant. Individuals heterozygous for both polymorphisms have been reported to have reduced MTHFR activity based on observed reductions in folate concentration and increased homocysteine concentration in population groups (68).

An association between vitamin B12 status and the 677C→T and 1298A→C MTHFR genotypes was recently reported by Bailey et al. (73). Individuals with the homozygous MTHFR 677C→T variant had higher plasma homocysteine concentrations than any other genotype combination, including those heterozygous for both mutations. They reported a negative association between plasma vitamin B12 concentration and serum folate concentration with plasma homocysteine concentration, which was
dependent on MTHFR genotype. Plasma homocysteine concentration significantly
decreased as vitamin B12 concentration increased in individuals heterozygous for both
MTHFR polymorphisms in contrast to the individuals with the normal genotypes whose
plasma vitamin B12 concentration was not associated with changes in homocysteine
concentration. This study was the first to report an inverse correlation between plasma
vitamin B12 concentration and plasma homocysteine concentration in individuals
heterozygous for the MTHFR 1298A→C polymorphism even when vitamin B12 was
within the normal range.

The population frequency of the MTHFR 677C→T and 1298A→C polymorphisms
in the MTHFR gene far exceeds the percentage required to be defined as a genetic
polymorphism (i.e., > 1% of the population) (48) and has been reported to affect
individual folate and homocysteine concentrations. Further study of these genetic
variations in different populations may determine whether individuals with the
homozygous variants require higher intakes of different vitamins.

**Folate, MTHFR, and homocysteine.** When Kang et al. (49) tested the
thermolability of MTHFR, they found that two of their six subjects who had the
thermolabile enzyme had hyperhomocysteinemia and deficient plasma folate
concentrations, while the other four subjects had normal homocysteine and folate
concentrations. This is the first study to show a possible relationship between deficient
folate status and hyperhomocysteinemia in subjects with the thermolabile enzyme.
Several subsequent studies have provided data that individuals with the TT genotype
have higher homocysteine concentrations than the individuals with the CT or CC
genotypes (65,74-83). Frosst et al. (3) concluded that the MTHFR TT genotype is the
most important genetic determinant for moderate hyperhomocysteinemia. Jacques et al. (7) clarified that hyperhomocysteinemia associated with the MTHFR 677C→T variant is dependent on folate status, and results from Kauwell et al. (84) support this conclusion. Folate status also has been shown to be impaired in individuals with the TT genotype compared to controls (67,77,85). Specifically, pregnant and nonpregnant women homozygous for the MTHFR 677C→T variant have been found to have lower red cell folate concentrations than women with the CC genotype (86). Kauwell et al. (84) demonstrated using a controlled folate dietary protocol with elderly women that subjects with the TT genotype had significantly lower folate concentrations and higher homocysteine concentrations after 7 wk of depletion than subjects with the CT or CC genotypes. Subjects with the TT genotype had the greatest reduction in homocysteine concentration after 7 wk of folic acid supplementation with 415 µg/d than subjects with the CT or CC genotype. Guinotte et al. (87) used a similar dietary protocol in a metabolic study of Mexican American women. Subjects with the TT genotype had lower serum and red blood cell folate concentrations throughout depletion (7 wk) and repletion (7 wk) with 400 µg DFE/d. Homocysteine concentrations did not differ between genotypes throughout depletion, but subjects with the TT genotype had higher homocysteine concentrations throughout repletion with 400 µg DFE/d.

Ashfield-Watt et al. (81) examined the influence of the MTHFR 677C→T genotype on folate status response in adults aged 18 to 65 who were given dietary intake advice. Subjects were assigned to one of three dietary intake advice groups for 4 mo each: advice to exclude folate-rich and fortified foods, advice to consume a folate-rich diet (400 µg folate/d), and advice to take a daily folic acid supplement (400 µg folic acid/d). Subjects
with the TT genotype had the greatest increase in homocysteine and decrease in folate concentrations after 4 mo of folate exclusion. Folate supplementation with fortified foods or supplements were effective in lowering plasma homocysteine concentrations of subjects with the TT genotype to a significantly greater extent than that of the subjects with the CT and CC genotypes. Dietary advice to consume a folate-rich diet and advice to take a daily folic acid supplement effectively increased folate concentrations in subjects with the TT genotype above the baseline concentrations of subjects with the CC genotype, but the response was not significantly greater than that observed in the other genotype groups. The investigators recommended folate intakes between 400 and 600 µg folate/d (575 to 830 µg DFE/d) for individuals with the TT genotype to maintain normal homocysteine concentrations. Although this was not a controlled metabolic study, it demonstrated the beneficial effects of folate intake on folate status and homocysteine concentrations.

In a recently published observational study, de Bree et al. (82) determined plasma folate and homocysteine concentrations by genotype in a Dutch population. They observed that subjects with the TT genotype had lower plasma folate and higher plasma homocysteine concentrations than subjects with the CT and CC genotypes. The difference in folate status indicators between MTHFR genotypes is well established in observational data.

**MTHFR and chronic disease.** Kang et al. (49) were the first to suggest that the homozygous MTHFR 677C→T variant may be a risk factor for cardiovascular disease. Since then conflicting results have been reported. Some studies have found a positive association between MTHFR, hyperhomocysteinemia, and vascular disease (74,75,88-92)
while others have not found this association (76,93,94). Brattström et al. (65) conducted a meta-analysis to determine the relationship between cardiovascular disease risk and MTHFR genotype. They reported that although individuals with the TT genotype had an average 25% higher homocysteine concentration than individuals with the CC genotype, the variant is not associated with an increase in cardiovascular disease risk. A possible reason for the lack of association between cardiovascular disease risk and individuals with the TT genotype is that there was insufficient power in the individual studies.

Chen et al. (95) developed a MTHFR knockout mouse model to evaluate the \textit{in vivo} pathogenic mechanisms of MTHFR deficiency. Plasma homocysteine concentrations were 1.6-fold and 10-fold higher in heterozygous and homozygous knockout mice compared to controls. Both the heterozygous and homozygous knockout mice had significantly decreased SAM concentrations and/or increased SAH concentrations and abnormal lipid deposition in the aorta. Homozygous knockout mice were developmentally retarded with cerebellar pathology. Although this study evaluated the effect of MTHFR deficiency in mice, it is reflective of possible abnormalities in humans with the TT genotype.

Only one study has linked homozygosity for the 1298A→C variant with a higher risk for early-onset coronary artery disease independent of homocysteine concentration (94). Rothenbacher et al. (96) did not find an association between the MTHFR 1298 CC genotype and risk for coronary heart disease. Most other studies suggest that this polymorphism is benign unless it is combined with the homozygous MTHFR 677C→T variant (97).
MTHFR and cancer. Various research groups have evaluated the effect of genetic interactions and cancer. Chen et al. (98) reviewed their previously published studies with respect to MTHFR and colorectal cancer. They found an inverse association between the homozygous TT variant and risk of colorectal cancer in two different case-control studies conducted in the Health Professional’s Follow-up Study (99) and Physician’s Health Study (100). This protective effect was diminished by a high alcohol and low methionine intake. Ma et al. (100) found that there was no protective effect if folate status was low, but if folate status was adequate, risk for colorectal cancer was reduced by 50% in individuals with the TT genotype versus the CC genotype. Toffoli et al. (101) reported a significantly reduced risk of developing proximal colon cancer in Italian subjects with the TT genotype compared to the CC or CT genotypes. Other studies only have observed a protective effect in subjects with the TT genotype with high plasma folate concentrations compared to subjects with the CC or CT genotypes with low plasma folate concentrations (102,103). Studies also have shown a protective effect of the homozygous TT mutation related to acute lymphocytic leukemia (ALL) in children and adults (104-106). In contrast, the homozygous TT variant appears to be associated with an increased risk for breast (107-110), endometrial (111), gastric (112,113), and esophageal (114) cancers in certain populations, although folate status of the subjects was not reported in any of the studies.

Few studies have investigated the influence of the MTHFR 1298A→C polymorphism on cancer risk. Chen et al. (115) reported that subjects with the homozygous variant (CC) had a decreased risk for colorectal cancer compared to the normal genotype (AA), but it was a less substantial independent risk factor than the
MTHFR 677C→T polymorphism. Sharp et al. (109) investigated the role of the MTHFR 1298A→C polymorphism on breast cancer risk and observed a reduced risk in individuals with the CC genotype compared to the AA genotype. No effect was reported for the CC genotype on gastric cancer risk (113).

Matthews et al. (59) hypothesized that the reason a potentially harmful polymorphism is so common in humans is this protective effect against colon cancer and certain ALLs. The reason for this protective effect is hypothesized to be that under low folate conditions, the polymorphism decreases the flux of 5,10-methyleneTHF into 5-methylTHF and instead this coenzyme can be used to convert dUMP to dTMP, which is the rate limiting step in cell synthesis (99). Crott et al. (116,117) tested this hypothesis in vitro and did not find a significant decrease in uracil misincorporation (116) or micronuclei formation (117) in human lymphocytes taken from individuals with the TT genotype compared to those with the CC genotype. The investigators argued that their results are due to differences between in vitro and in vivo conditions.

Folate, MTHFR, and neural-tube defects (NTDs). Neural tube defects are congenital abnormalities resulting from the malformation of the brain and/or spinal cord or from a failure of the skeleton to cover them, resulting in a protrusion (118). Development of these malformations occurs prior to day 28 of gestation, before many women know they are pregnant. Most groups studying NTDs generally only include anencephaly (failure of the brain to develop) or spina bifida (exposure of the spinal cord due to defective closure of the neural tube), but NTDs also include encephalocele, craniorachischisis, and iniencephaly. Each year anencephaly or spina bifida occur in 1 in 1,000 pregnancies in the US and roughly 300,000 or more births worldwide (119). This
section will focus on key studies linking folate status, MTHFR, and homocysteine with NTD risk.

During the 1960’s Hibbard suggested that folate deficiency might be a cause for congenital birth defects (120). This hypothesis was supported by many subsequent studies. Smithells et al. (121) and Laurence et al. (122) conducted studies to evaluate the possible protective effect of folic acid supplementation against NTDs in high-risk mothers who already had an NTD-affected pregnancy. In both studies, the risk for a subsequent NTD-affected pregnancy was significantly decreased in the supplemented compared to that of the nonsupplemented mothers. These preliminary findings prompted a large randomized placebo-controlled intervention trial to determine whether it was folic acid alone that reduced the risk of NTDs. The Medical Research Council (MRC) Vitamin Study launched this trial in 1983 and ended it early because they found that 72% of NTDs were prevented with 4 mg folic acid supplementation daily in women who had a previous NTD-affected pregnancy (123). Czeizel and Dudas (124) found that periconceptional folic acid supplementation (800 µg/d) could prevent the first occurrence of NTDs in a Hungarian population group compared to subjects receiving no folic acid. Berry and colleagues (125) investigated the effect of periconceptual folic acid supplementation in Chinese women from areas with high rates of NTDs (northern region of China) and low rates of NTDs (southern region of China). They observed that 400 µg/d of folic acid reduced the occurrence of NTDs from 4.8 to 1.0 per 1000 pregnancies in the northern region of China and 1.0 to 0.6 in the southern region of China. Kirke et al. (126) evaluated blood samples from 56,049 pregnant women on their first clinic visit. Of this group 81 had NTD-affected babies and their blood folate concentrations were
compared to 247 control females with normal pregnancy outcomes. The median plasma vitamin B12 (pmol/L), serum folate (ng/ml), and red blood cell folate (ng/ml) concentrations in the mothers with NTD-affected pregnancies compared to the control mothers were 243 and 296, 3.5 and 4.6, and 269 and 338, respectively. They concluded that both vitamin B12 and folate were independent risk factors for NTDs. Using data from the Kirke et al. (126) study, Daly et al. (127) were able to stratify the serum and red blood cell folate concentrations into quintiles. They found a greater than 8-fold increase in risk of NTDs in those women with red blood cell folate concentrations < 150 ng/ml versus those with red blood cell folate concentrations > 906 ng/ml. They concluded that NTD risk is reduced as red blood cell folate concentrations increase even in the normal range. Moore et al. (128) observed that NTD risk declines in a dose-responsive manner according to supplemental folic acid intake, dietary folate intake, and total folate intake. They reported that for every additional 500 µg DFE/d consumed, the prevalence of NTDs decreased by 0.78 cases per 1000 pregnancies. In addition, compared to women in the lowest quintile of folate intake (0 to 149 DFE/d), the prevalence of NTDs decreased by 34%, 30%, 56%, and 77% among the offspring of women consuming 150 to 399, 400 to 799, 800 to 1199, and ≥ 1200 DFE/d, respectively (128).

This strong link between NTDs and folate has prompted investigations to determine whether polymorphisms of genes that code for enzymes involved in folate metabolism have an effect on NTD risk. van der Put et al. (15) were one of the first groups to associate the MTHFR 677C→T variant with NTD risk. Botto and Yang (4) provided an extensive review of case-control studies evaluating the association between the homozygous MTHFR 677C→T variant and risk for spina bifida. Shields et al. (16)
obtained blood samples from a large number of NTD-affected Irish individuals and their parents to determine if there was any genetic association with NTDs. They concluded that the homozygous TT genotype in embryos is the most important risk factor for NTD pregnancy outcome in this population. They also stated that the homozygous TT genotype might not play such a large role in other populations with more adequate folate status. Despite the many positive associations found between individuals with the TT genotype and NTD risk, not all study results support this correlation (129-133). Lucock et al. (133) hypothesized that another genetic insult of folate metabolism may underlie the condition. Recently, Rampersaud et al. (134) reported a significantly higher frequency of the TT genotype in 175 Caucasian NTD-affected subjects compared to controls, but the investigators suggested that an additional gene may be responsible for an increase in NTD risk. After careful consideration of published data related to the MTHFR 677C→T polymorphism and NTD risk, the IOM concluded that only approximately 15% of NTD risk is attributable to the homozygous variant of MTHFR (27). Molloy et al. (85) investigated the role of maternal MTHFR TT genotype on the risk of having an NTD-affected pregnancy. Although they found a greater frequency of mothers of affected children with the TT genotype than control mothers, they did not find an effect of the TT genotype on red blood cell folate concentration. Therefore they concluded that maternal risk factors for NTDs are not explained by the TT genotype for MTHFR. Instead, maternal folate status may be the most important risk factor.

Few studies have evaluated the association between the 1298A→C polymorphism and risk of NTDs (4). van der Put et al. (68) did not find a significant risk for NTDs in individuals with the 1298A→C homozygous or heterozygous variant genotypes;
however, they observed a significantly increased risk for individuals with combined heterozygosity for the 1298A→C and 677C→T polymorphisms. They concluded that this combined heterozygosity accounts for a proportion of NTDs not explained by the TT genotype and should therefore be considered a risk factor for NTDs. Parle-McDermott et al. (135) did not find an association between NTDs and the MTHFR 1298A→C polymorphism. Because most of the MTHFR and NTD studies have focused on living infants and parents, Isotalo et al. (136) collected fetal tissue samples from spontaneously aborted and terminated pregnancies to compare genotypes to neonatal umbilical cord samples for controls. They found all possible genotype combinations including the 677CT/1298CC and 677TT/1298CC genotypes in the fetal tissue, which were absent in the neonatal control group. The presence of NTDs could not be determined in the fetal tissue group, but due to the presence of the 677CT/1298CC and 677TT/1298CC genotypes in fetal tissue, they concluded that the combined MTHFR variants contributed to decreased fetal viability. Volcik et al. (137) found a higher rate of the homozygous 1298A→C variant in mothers of an NTD-affected pregnancy in a Hispanic population, although it was not significant. Their results also contrast those of Isotalo et al. (136) in that they identified all possible genotype combinations in living individuals, including the 677CT/1298CC and 677TT/1298CC genotypes. It is possible that ethnic differences between the populations studied resulted in different genotypes in the population and, therefore, differences in results between the two studies.

Homocysteine concentrations also have been found to be higher in mothers of NTD-affected infants (138,139), but the mechanism is unknown. Based on epidemiological studies, Eskes et al. (140) and Steegers-Theunissen et al. (141)
hypothesized that homocysteine may have a direct teratogenic effect on the embryo. To
test this hypothesis, Rosenquist et al. (142) treated avian embryos with incremental
physiologic doses of homocysteine and observed the development of NTDs. They
concluded that homocysteine is in fact teratogenic to the embryo. In contrast,
homocysteine exposure to mammalian embryos (rat or mouse) did not increase the
incidence of NTDs (143-145). Greene et al. (145) concluded that elevated homocysteine
concentrations in NTD-affected pregnancies are more likely a marker of abnormal folate
or methionine metabolism. More recently, high homocysteine concentrations have been
found in NTD-affected individuals with the TT genotype. Bjorke-Monsen et al. (130)
found significantly higher homocysteine concentrations in NTD-affected patients versus
controls, but this increase was confined to patients with the CT or TT genotype.
Wenstrom et al. (146) collected amniotic fluid from NTD-affected pregnancies and
controls and found significantly more patients with TT genotypes in the NTD-affected
fluid versus controls. They also found significantly higher amniotic fluid homocysteine
concentrations in fluid from cases compared to controls. High homocysteine
concentrations due to a polymorphism in the gene coding for MTHFR may be a risk
factor for NTDs (139,147).

The association of folate status, MTHFR polymorphisms, and homocysteine
concentrations with NTD risk has been widely studied. Although results have been
conflicting, the general opinion is that each factor plays a role in the occurrence of NTDs
with folate status being the greatest contributor to risk. MTHFR polymorphisms may
affect folate status and increase homocysteine concentrations resulting in an increased
risk associated with the MTHFR polymorphism. Future studies need to further evaluate
this possibility in order to achieve the goal of preventing the maximal number of folic acid preventable NTDs.

**Folate, MTHFR, and fetal malformations.** Although there is significant evidence indicating a protective effect of folic acid supplementation in the prevention of NTDs, studies are beginning to link poor folate nutriture with other fetal malformations. Folate is involved in DNA synthesis and methionine production, both of which are crucial for normal embryonic development. Low folate status may contribute to embryonic malformations for the following reasons: decreased cell division, homocysteine-associated vascular events, impaired maternal-to-fetal folate transfer, or homocysteine-associated neurotoxicity, as reviewed by Moyers and Bailey (97). An estimated 120,000 to 150,000 infants with fetal malformations are born in the United States yearly (97). This section will focus on the possible role folate status and MTHFR polymorphisms may have on the risk for cleft lip and palate, congenital heart defects, and Down syndrome.

Cleft lip with or without cleft palate is one of the more common malformations seen at birth and occurs in every 1/500 to 1/1000 births (148). Early studies documented the beneficial effect of high dose folic acid supplementation (10 mg) in the reduction of recurrent cleft lip with or without cleft palate (149). Two to 3 mg of folic acid also have been shown to significantly protect against cleft lip with or without cleft palate in a Hungarian population (150). The dose of folic acid used is important because 0.8 mg of folic acid in a multivitamin was not found to be protective against cleft lip with or without cleft palate in a Hungarian population (124,151). Hayes et al. (152) did not find a protective effect of periconceptional folic acid supplementation of mothers against the
risk of oral clefts in infants in a North American population. The conflicting data on whether or not folic acid supplementation confers a protective effect against oral clefts in the North American population are reviewed by Moyers and Bailey (97). Wong et al. (153) determined the prevalence of hyperhomocysteinemia in mothers of infants with orofacial clefts and concluded that maternal hyperhomocysteinemia is a risk factor for having a child with an orofacial cleft. Mills (154) listed some possible limitations to the study by Wong et al. (153), including unexpectedly lower homocysteine concentrations in the controls compared to cases, differences in vitamin status between cases and controls, and the vitamin status of the population during the study compared to pregnancy. Mills (154) also listed some possible explanations including a metabolic defect in vitamin B6 and folate metabolism. Additional studies are needed to further define the relationship between maternal homocysteine concentrations and orofacial clefts.

Because MTHFR is directly involved in folate metabolism, there may be an association between the homozygous MTHFR 677C→T variant and cleft lip with or without cleft palate. An Irish group determined the prevalence of the TT genotype in their population with cleft lip with or without cleft palate. They found that the homozygous 677C→T variant was significantly more prevalent in an Irish population with cleft palate only; however, periconceptional maternal vitamin use was not reported (155). In contrast, Gaspar et al. (156) did not find an increased prevalence of individuals with the TT genotype in their cleft lip patients with or without cleft palate. The presence of the TT genotype in mothers of children affected with cleft lip with or without cleft palate also has been shown to be significantly more prevalent when compared to controls.
A subsequent study reported a significantly higher frequency of the TT genotype in mothers with affected children who were also affected themselves compared to healthy mothers with affected children (158). In addition, Shotelersuk et al. (159) did not observe an association between patient genotypes and occurrence of cleft lip or palate, but did detect a significantly higher frequency of compound heterozygotes (MTHFR 677CT and 1298AC) in mothers of patients. In a series of studies, an American group determined that infants with the TT genotype are not at increased risk for cleft lip (160) or cleft palate (161). Neither study indicated an interaction between infant genotype and maternal multivitamin use on the malformation occurrence. Reanalysis of the data by Wyszynski et al. (148) revealed significant differences in the risk of clefting between patients with the TT compared to the CC genotypes in patients whose mothers were not supplemented. The effect of the TT genotype of patients with supplemented mothers decreased the risk for orofacial clefts slightly, but not significantly. They concluded that based on their reanalysis of the data that periconceptional vitamin supplementation may protect against clefting (148). In another series of studies in an American population, no association was observed between familial (162) or isolated (163) nonsyndromic cleft lip and palate and the TT genotype. The relationship between cleft lip with or without cleft palate and MTHFR needs further study.

Birth defects involving the heart include transposition of the great arteries, conotruncal heart defects, atrial septal defects, and others. Conotruncal heart defects are one of the more prevalent defects and occur in every 4/10,000 births. They are disorders that involve the neural crest cells that are ultimately incorporated into the aortic arch vessels, truncal outflow tract, and vessel walls, as reviewed by Moyers and Bailey (97).
There are three main observational studies that provide evidence regarding the relationship between folate and heart defects (150,164,165). Briefly, high doses (2-3 mg) and low doses (i.e., multivitamin or fortified cereal) of folic acid supplementation significantly reduced the risk for heart defects in one Hungarian and two American studies. Boot et al. (166) attributed the development of conotruncal heart defects to the abnormal differentiation of neural crest cells in the presence of high homocysteine concentrations in a recently published in vitro study. Although the inverse association between folate supplementation and reduced risk for heart defects has been the focus of investigations, the role of MTHFR on conotruncal heart defect risk has been evaluated in only one study. Storti et al. (167) determined the MTHFR 677 and 1298 genotypes in an Italian population of affected children and their parents. Although increased odds ratios for heart defects were detected for different combinations of MTHFR polymorphisms in mothers and affected children, none of the odds ratios were significant. More studies evaluating the role of MTHFR polymorphisms on the risk of conotruncal heart defects are needed.

Down syndrome is a genetic disorder that results from the presence of three copies of chromosome 21 (trisomy 21). This extra chromosome is a result of abnormal chromosome segregation during meiosis, also known as meiotic nondisjunction. Ninety-five percent of Down syndrome cases are maternal, with nondisjunction occurring in the oocyte before conception. Down syndrome occurs in every 1/600 live births and in 1/150 conceptions. It is estimated that approximately 80% of all trisomy 21 conceptions results in spontaneous abortion. It is a major public health concern and is the leading cause of mental retardation (168). James and colleagues (168) hypothesized that altered folate
metabolism in mothers of affected children with the TT genotype may affect their DNA methylation and result in nondisjunction that leads to Down syndrome. They found that the presence of the MTHFR 677C→T variant on one or both alleles in these mothers significantly increased the risk of having a child with Down syndrome. Their results were supported by Hobbs et al. (169). Other studies have not reported an association between maternal MTHFR genotype and Down Syndrome (170-172). Differences in the ethnicity among population groups may have contributed to the differences in results. Controlled intervention clinical trials will need to be performed before preconceptional folic acid supplementation is recommended to reduce the risk of Down syndrome.

The possibility of preventing major malformations with folic acid supplementation has directed the focus of research to the influence of folate status and MTHFR genotype on fetal malformations. More studies are needed to develop concrete evidence in support of folic acid supplementation as a preventive measure against fetal malformations.

**Homocysteine, MTHFR, and pregnancy outcome.** Although the main focus of folate research involves NTDs and fetal malformations, evidence has been reported for the role of homocysteine and MTHFR polymorphisms in preeclampsia and early pregnancy loss. Preeclampsia and recurrent early pregnancy loss are very serious complications of pregnancy. Some recent findings for preeclampsia and early pregnancy loss will be discussed in this section.

Preeclampsia is defined as pregnancy-induced proteinuric hypertension with onset of clinical symptoms beyond 20 wk gestation (173). The cause of preeclampsia is thought to be due to increased resistance to uterine artery blood flow (174) and has been associated with elevated homocysteine concentrations (139). López-Quesada et al. (175)
observed a 7.7-fold increased risk for preeclampsia in pregnant women with hyperhomocysteinemia in the third trimester (> 10.5 µmol/L) compared to normal pregnant controls. The role of homocysteine as a risk factor for vascular events has been well established (176). Because the homozygous MTHFR 677C→T variant is associated with elevated homocysteine concentrations in individuals with low folate status (3), investigators have implicated this polymorphism in mothers as a risk factor for preeclampsia. There have been positive associations (174) and negative associations (177) found between mothers with the homozygous TT variant and the development of preeclampsia. Pramusinto et al. (173) did not associate this polymorphism in mothers or infants with an increased risk for preeclampsia. Although reported data are not consistent, possible explanations for the variation include the differences in the population groups and subsequent variation in the frequency of the MTHFR polymorphism.

Recurrent early pregnancy loss is another serious problem in pregnancy. Although still unresolved, the mechanism responsible for this pregnancy complication has been a topic of intense study. There is evidence to support the hypothesis that abnormal procoagulant activity may be a causative factor for early pregnancy loss (178). As mentioned previously, high homocysteine concentrations are associated with various cardiovascular diseases, including increased prothrombotic tendency (179). Since high homocysteine concentrations may promote thrombotic events, they also may play a significant role in early pregnancy loss. Nelen and colleagues (180,181) concluded that elevated homocysteine concentrations were a risk factor for recurrent early pregnancy loss in a case-controlled study (180) and a meta-analysis (181). Any factor that may
increase homocysteine concentrations also may increase the risk for early pregnancy loss. Results from different studies have supported an association between the MTHFR 677 TT genotype and risk for recurrent early pregnancy loss (80,182). Zetterberg et al. (183) determined the genotype of spontaneously aborted embryos for the 677C→T and 1298A→C MTHFR polymorphisms and found a high prevalence of MTHFR polymorphic genotypes. Regardless of the population, homozygosity for the 677C→T variant has been found to be a risk factor for early pregnancy loss (80,182,184). Other studies have been unable to confirm this association in different populations (184-186). More studies are needed on the 1298A→C polymorphism to draw any conclusions.

In summary, risks for preeclampsia and recurrent early pregnancy loss are additional examples of pregnancy complications that may be reduced with folic acid supplementation. The possible increased need for folate in individuals homozygous for the MTHFR polymorphisms needs to be substantiated further prior to recommending doses of folic acid for the prevention of these pregnancy complications.

**Methionine Synthase (MS)**

Another widely studied enzyme involved in folate metabolism is methionine synthase (MS), which catalyzes the conversion of homocysteine to methionine. This enzyme requires 5-methylTHF and cobalamin to function properly. Bacterial MS has been characterized and studied extensively (187). It consists of 3372 nucleotides and a molecular weight of 123,640. *Escherichia coli* MS studies have shown the enzyme to be a modular protein consisting of four different regions. The module residing on the N-terminal is the one responsible for binding homocysteine. The second module binds 5-methylTHF, the third binds cobalamin, and the fourth binds SAM (56). Leclerc et al.
(188) were the first to clone human MS cDNA, which shares approximately 58% identity with *Escherichia coli*, and to describe specifics about its localization, expression, and partial characterization. They reported on a gene that codes for a protein containing 3795 base pairs encoding a polypeptide of 1265 amino acids located near the telomeric region of the long arm on chromosome band 1q43. They also identified an A→G transition at base pair 2756 that converted an aspartic acid into a glycine in patients with MS activity deficiency. They hypothesized that this MS deficiency could be associated with mild hyperhomocysteinemia.

**MS and chronic disease.** The role of the MS 2756A→G variant in chronic disease has been investigated. Individuals with the homozygous 2756A→G variant have been reported to have lower fasting plasma homocysteine concentrations (189-191). Jacques et al. (192) found no evidence for an association between homocysteine and the MS 2756A→G variant. van der Put et al. (193) sequenced the entire coding region of MS in eight individuals with hyperhomocysteinemia (four NTD patients and four vascular disease patients) to determine whether mutations in this gene were involved in homocysteine-related diseases. They reported that there was no association between the MS 2756A→G substitution and hyperhomocysteinemia in their population. In fact, they also detected a slightly lower homocysteine concentration in patients homozygous for the variant compared to heterozygotes, as described previously. It was hypothesized that when the strong helix breaker glycine was present in the enzyme instead of the moderate helix breaker aspartic acid at the position near the vitamin B12 binding site, enzyme function may be modified. In contrast, Harmon et al. (194) reported that this polymorphism was associated significantly with homocysteine concentrations in their
population of Irish males, with the AA genotype having higher homocysteine concentrations. Silaste et al. (190) investigated the effect of two 5 wk dietary interventions of low and high folate intake to determine whether genetic variations in enzymes involved in homocysteine metabolism affect the responsiveness of folate status to naturally occurring food folate. They reported that individuals with the G allele for the MS variant had a greater decrease in homocysteine concentrations and lower homocysteine concentrations during the high folate period than individuals with the normal enzyme. Since hyperhomocysteinemia also is a risk factor for venous thromboembolism (VTE) an association with the MS 2756A→G variant was investigated. den Heijer et al. (194) and Salomon et al. (195,196) did not find an association between the GG variant and VTE. In contrast, Yates and Lucock (197) reported a protective effect for the presence of the G allele in relationship to risk for VTE.

Although the association of the MS 2756A→G variant and cancer has been investigated, no significant correlation was found (198). Swanson et al. (199) developed a MS knockout mouse model to study the pathophysiology associated with a severe MS deficiency. Heterozygous knockout mice had a 50-60% decrease in enzyme activity and slightly elevated plasma homocysteine and methionine concentrations while complete omission of MS resulted in embryonic lethality, proving that MS activity is essential for early embryonic development in mice.

**MS and NTDs.** The possible association between a polymorphism affecting MS and risk for NTDs has been the focus of investigations. van der Put et al. (193) did not find an increased prevalence of the MS 2756 GG genotype in NTD cases compared to
controls or in mothers of children with NTDs compared to controls. Unlike the homozygous MTHFR 677C→T variant, it is hypothesized that the MS 2756 GG genotype confers some sort of protection against NTDs compared to the normal enzyme. Christensen et al. (200) did not find the MS 2756 GG genotype in any of their cases, but 10% of their controls had the 2756 GG genotype. In contrast, Zhu et al. (201) observed a higher frequency of the G allele in NTD cases compared to controls. The GG genotype was detected in very low frequencies in cases. Doolin et al. (202) found an increased risk for spina bifida-affected pregnancy in mothers with the GG genotype compared to the AA genotype. They emphasized the importance of considering maternal and embryonic genotypes when evaluating risk for spina bifida. The role of the MS 2756A→G polymorphism on NTD risk is not well defined and needs further investigation.

**Methionine Synthase Reductase (MSR)**

Associated with MS is MSR, a flavoprotein responsible for the reductive activation required for the maintenance of MS once cobalamin becomes oxidized over time. Leclerc et al. (203) isolated the cDNA clone for the human MSR gene and found that it had three binding sites for FMN, FAD, and NADPH, which are required for the reduction of MS. The gene consists of 2094 base pairs encoding a polypeptide of 698 amino acids with a molecular mass of 77.7 kDa. It is localized on human chromosome 5. Wilson et al. (204) discovered a variation in the gene coding for MSR in homocystinuric patients with severe MS deficiency. This polymorphism results in a A→G transition at base pair 66, which converts an isoleucine to methionine in the protein. Individuals can have the homozygous variant (GG), heterozygous variant (AG), or normal (AA) genotype for this MSR 66A→G variation.
**MSR and NTDs.** The association between the MSR 66A→G variant and risk for NTDs has been evaluated. Wilson et al. (205) found that cases and mothers of cases had almost a 2-fold increase in risk for spina bifida compared to controls, although not significant. These investigators observed that when low cobalamin status was coupled with the MSR GG genotype, it conferred an even greater risk for NTDs in the children and mothers. In addition, a combination of homozygous MTHFR 677C→T and MSR 66A→G variants in both children and mothers conferred a 4-fold increase in risk, a greater increase than for each individual variant. These results were supported by Zhu et al. (201) who observed that the G allele was associated with increased risk for spina bifida. Doolin et al. (202) reported that the risk of having a child with spina bifida increased with increasing maternal G alleles. These results link the homozygous MSR 66A→G variant with an increased risk of spina bifida, but more studies are needed to make any concrete associations.

**Other Polymorphisms**

Although much of folate research focuses on the preceding polymorphisms, other polymorphisms have been identified that have not received as much attention. Devlin et al. (206) identified a polymorphism in the gene coding for the conjugase enzyme located in the intestine that results in 53% less activity than the normal enzyme. This enzyme termed GCPII is responsible for cleaving polyglutamates into monoglutamates. Glutamate carboxypeptidase II can have a C→T transition at base pair 1561, which replaces histidine with tyrosine at codon 475, codes for a 750 amino acid polypeptide, and is located on chromosome 11. Devlin et al. (206) associated the 1561C→T transition in GCPII with low serum folate concentrations and higher homocysteine concentrations.
in a healthy English population. More studies are needed to corroborate these findings because dietary intakes were not assessed at the time of sample collection, and recent intakes of folate, vitamin B6, vitamin B12 can affect folate and homocysteine concentrations. Any supplements taken by the subjects containing any of these B vitamins also could have had an effect. Vargas-Martinez et al. (207) concluded that the polymorphism is not associated with plasma folate or homocysteine concentrations after determining the GCPII 1516C→T genotype in subjects from the Framingham Offspring Study. They took many factors into account known to affect folate and homocysteine concentrations and, after adjusting for these factors, still did not find an association. A possible explanation for variations between these studies are the factors accounted for by Vargas-Martinez et al. (207). Vargas-Martinez et al. (207) concluded that this polymorphism has no effect on folate and homocysteine concentrations when confounding factors are taken into account. Födinger et al. (208) observed that GCPII was a predictor of red blood cell folate but not homocysteine concentrations in chronic dialysis patients.

Other polymorphisms that may have an impact on folate status are the thymidylate synthase promoter region polymorphisms (209,210) and folate receptor polymorphisms (37,211). Multiple studies have illustrated the complexity of the problem of how a genetic variation associated with different diseases and birth defects can be modified by nutrient intake. A perfect example of this comes from the previously discussed study by Jacques et al. (7), which reported that homocysteine concentrations were higher in individuals with the homozygous MTHFR 677C→T variant only when plasma folate concentration was below the median. There was no difference in homocysteine between
individuals with the TT and CC genotype with plasma folate concentrations at or above the median. Adequate folate status also can provide protection against certain cancers in individuals with the TT genotype, while inadequate folate status can add to the risk for these cancers (100).

**DNA Stability**

For years scientists have known that exposure to chemical mutagens and carcinogens can cause gene mutations and chromosome damage. Only recently has there been more and more evidence linking dietary factors to similar damage (212). Currently, a large body of evidence supports the conclusion that folate deficiency has a negative impact on DNA stability. The proposed mechanisms by which folate deficiency impairs DNA structure include: DNA methylation, uracil misincorporation, DNA strand breaks, and micronuclei formation (11,213-215). This section will summarize research findings related to each mechanism to provide a basis for understanding the links between folate status and DNA stability.

**DNA Methylation**

Until recently, not much was known about gene regulation. It is now recognized that it is not just the primary sequence of the DNA that determines whether or not a gene is expressed. Gene transcription depends on modifications referred to as epigenetics, which is defined as any DNA modification that regulates gene activity without changing the primary DNA sequence and can persist through one or more generations (216). It is these epigenetic modifications that allow cells to adapt to external stimuli from the environment and from regulatory molecules within the body without having to change the primary DNA sequence (216). DNA methylation is one epigenetic modification that is critical for normal development of cells and organs (217). Li et al. (218) reported that a
mutation of a key methylating enzyme, Dnmt1, produced a recessive, lethal phenotype. A few years later, Okano et al. (219) confirmed these findings in a study that targeted two other DNA methyltransferases, Dnmt3a and Dnmt3b. Deletion of Dnmt3a produced mice that appeared normal at birth but failed to grow normally and died after 4 wk. In contrast, deletion of Dnmt3b did not produce any viable mice at birth. Deletion of both DNA methyltransferases produced an embryonic lethal phenotype. These series of studies illustrate the importance of methylation for the normal development of mammals.

Before the function of DNA methylation can be discussed, the specifics of methylation will be reviewed briefly. As reviewed by Costello and Plass (217), the majority of 5’-methylcytosine is present in cytosine-guanine (CpG) dinucleotides. Clusters of CpGs also exist and are referred to as CpG islands. Gardiner-Garden and Frommer (220) originally defined these CpG islands as regions of DNA from 200 base pairs to several kilobases in length with a CpG frequency approximately five times greater than the whole genome. They also determined that these CpG islands comprise 1 to 2% of the genome. It is estimated that 3 to 6% of total mammalian cytosine bases are methylated and that 70% of mammalian CpG dinucleotides are methylated (8). The exact mechanism that determines the pattern of DNA methylation is unknown. Methylation assays using polymerase chain reaction amplification have shown that mouse sperm DNA is primarily methylated at all non-CpG island sites located throughout the genome in contrast to mouse oocyte DNA, which is primarily unmethylated at these same nonCpG island sites (221). Early in the life of the embryo, the CpG sites of germ and somatic cells are demethylated and then de novo methylation reestablishes a methylation pattern, although differently in germ cells versus somatic cells (221). Different
mechanisms can change DNA methylation patterns during different developmental stages. The spontaneous deamination of a methylated cytosine produces thymine, which can be replicated and result in a TG base pair mismatch and subsequent TA transition. This transition is more difficult for the cell to repair (222) and can result in the loss of heritable methylation patterns (8).

There are three DNA methyltransferases that have been discovered which transfer a methyl group from SAM to the cytosine in CpG dinucleotides: Dnmt1, Dnmt3a and Dnmt3b (223). These enzymes are necessary for embryonic development (218,219) and possess de novo methylation capabilities (i.e., methylation of a CpG sequence opposite an unmethylated CpG sequences) (224). Dnmt1 has a higher affinity for hemimethylated DNA and therefore works mostly as a maintenance enzyme (225). The exact mechanism as to how the 5-position of cytosine becomes methylated is still unknown. It is hypothesized that cytosine is everted from the DNA helix and inserted deep within the active site of the DNA methyltransferases where a methyl group is transferred (226). The mechanism of DNA methylation involves the methylated parent strand as a guide. The daughter DNA strand is methylated by one of the maintenance DNA methyltransferases shortly after replication, resulting in the exact methylation pattern present in the parent strand. DNA methylation patterns are preserved throughout many rounds of replication by DNA methyltransferases (8).

DNA methylation has many functions. Stabilization of DNA by preventing cleavage by nucleases is one proposed function for DNA (8). The primary function, however, is as a gene silencer. Genes make up a small portion of the genome, while the rest is made up of introns, repetitive elements, and potentially active transposable
elements. In order for genes to be successfully expressed, all of the noncoding DNA needs to be silenced. Mammals appear to have evolved to use methylation as a mechanism to silence this DNA. The post-transcriptional addition of a methyl group to the 5-position of cytosine alters the DNA-protein interactions, which in turn keeps the DNA from being transcribed (9). CpG islands often contain the promoter regions near the 5’ end of genes. If these promoter regions are unmethylated, this denotes active transcription (227). If they are methylated, transcription is suppressed. As technology has improved, scientists have determined that CpGs located within these CpG islands are mostly unmethylated while CpGs located outside these islands are generally methylated (217). Costello and Plass (217) suggested that these patterns of methylation may act to separate the genome into transcriptionally active and inactive areas. These specific methylation patterns are upheld through DNA replication in order to promote and maintain the transcription of specific genes (228). Methylation within these promoter regions can stop transcription. Cooney et al. (229) reported that dietary methyl group supplementation of rats has a significant effect on DNA methylation and their subsequent methylation-dependent phenotype by successfully changing the coat color of the yellow agouti mouse.

Kass et al. (228) reviewed how methylation in the promoter region represses transcription. The most obvious mechanism would be to prevent the transcription factors and proteins from binding to the DNA. However, this cannot be the only explanation because there is transcription machinery that will bind to DNA despite its methylation. Jones et al. (230) reported that the binding of the methyl CpG binding complex 2 to methylated promoter regions recruits transcriptional repressors. These complexes contain
histone deacetylases (HDAC1, HDAC2), which function to deacetylate lysine residues in histone tails, which are associated with DNA and result in the compaction of chromatin. Once the chromatin becomes compacted, it is transcriptionally inactive. Therefore, DNA methylation also has a repressive effect on chromatin, serves to inactivate one of the two X chromosomes in females during development, and determines the expression of imprinted genes (217). Similar machinery is used to establish methylation patterns.

Robertson (231) reviewed two models for how DNA methylation patterns may be established in somatic cells. The first model incorporates the use of HDACs, ATP-dependent remodeling complexes, and DNA methyltransferases. Histones destined for silencing are first deacetylated by HDACs. The chromatin remodeling enzymes can now move the nucleosomes that are wrapped by DNA from side to side in an ATP-dependent manner to allow the DNA methyltransferases access to its target DNA sites. These enzymes also may create a specific area on the DNA that is recognized by the DNA methyltransferases. Once DNA is methylated, the methyl-CpG binding proteins bind the methylated cytosines and further repress transcription as described above. The second model involves the maintenance methylase (DNMT1) and the retinoblastoma protein, Rb, which is a protein involved in transcriptional regulation of chromatin (231). In resting cells, Rb is associated with DNMT1 and inhibits its catalytic activity to prevent any aberrant methylation of the genome. Early in cell division (S phase) both proteins colocalize with the replication foci. In late S phase Rb is no longer colocalized with the replication foci and, instead, HDAC colocalizes with DNMT1, which is now active. It is possible that Rb departs from the replication foci when hypermethylated regions are replicated (231).
The main methyl donor used by the DNA methyltransferases is SAM. DNA methylation is dependent on the availability of SAM; therefore, anything that affects the supply of SAM may have an effect on DNA methylation. In humans, the main dietary sources of methyl groups that are transferred to SAM are folate, choline, and methionine (232). SAM is the main methyl donor in over 100 reactions. The methyl group requirement normally exceeds the supply available in food, but the difference is usually made up by the synthesis of methyl groups utilizing folate coenzymes (213). Most of the 5-methylTHF is regenerated through the one-carbon cycle illustrated in Figure 2-2, but a small amount is lost through urinary excretion, skin, bile, and catabolism. If this folate is not replaced, it could decrease the methylation cycle resulting in lower amounts of SAM available for methylation and increased homocysteine concentrations (233).

Early studies investigating the role of folate in methylation used rats as a mammalian model. Balaghi and Wagner (213) found that after 4 wk of feeding a folate deficient diet to rats, methylation of hepatic DNA was significantly reduced in folate depleted rats compared to controls. Alonso-Aperte and Varela-Moreiras (234) observed that administration of the folate antagonist methotrexate in rats produced a folate deficiency compared to controls and was associated with significant hypomethylation of brain DNA. Kim et al. (235) investigated global and protooncogene specific (c-myc) DNA methylation. As a model for conditions preceeding colorectal neoplasia in rats and humans, the effect of moderate folate depletion over a longer period of time was evaluated. After 15 and 24 wk, the folate deplete rats had a significantly lower plasma folate concentration than the control rats. There was no significant difference in global methylation after 15 or 24 wk. They also did not find a significant difference in
methylation of \(c\text{-}myc\) between the two groups after 15 or 24 wk of folate depletion. It was hypothesized that the SAM/SAH ratios were not low enough, the strain of rat used was resistant to hypomethylation, or the betaine pathway compensated for any folate loss and prevented significant hypomethylation. Kim et al. (214) investigated the effect of a more severe folate deficiency resulting from antibiotic treatment on global DNA methylation and strand breaks and methylation and strand breaks within a specific sequence of the p53 tumor suppressor gene. Folate deficient rats at 4 and 6 wk had significantly lower plasma folate concentrations than the controls and significantly more strand breaks within the p53 gene than control rats. They also had reduced DNA methylation of this gene that obtained significance at 6 wk. The investigators concluded that a dietary folate deficiency could have a negative effect within critical regions of the p53 tumor suppressor gene. Deficient rats fed for 6 wk also had significantly higher global strand break accumulation than controls and rats fed for 4 wk, indicating a time dependence of strand breaks. They did not find a significant difference between global methylation between the two groups at 4 or 6 wk, strengthening their previous observations.

Although the preliminary rat studies were important in making a connection between folate status and DNA methylation, the links to clinical implications in humans were unclear. Studies correlating low folate status in humans with increases in micronuclei formation and uracil misincorporation were reported, which supported using them as new folate status indicators in addition to the traditional blood folate values (11,236). Jacob et al. (237) evaluated DNA methylation as a potential new biomarker for folate status. This study was designed to assess the correlation between impaired folate
status and DNA methylation in humans. Ten, healthy postmenopausal women lived in a metabolic unit for the 13 wk of this study and consumed a low folate diet and varying amounts of synthetic folic acid to provide intakes ranging from 56 to 516 µg/d during this depletion-repletion study. Days 6 to 41 were designed to provide a moderately-deplete folate diet to evaluate the effect of low folate intakes not associated with overt clinical signs of deficiency. Mean plasma folate concentrations dropped significantly from baseline to post-depletion. Lymphocyte DNA hypomethylation was determined using a methyl acceptance assay, whereby acceptance of $[^3]H$methyl groups is inversely associated with methylation. The investigators observed that the marginal folate deficiency induced in these postmenopausal women was associated with reduced DNA methylation, which was reversed with folate supplementation. This was the first study to show that folate intake affects DNA methylation in humans and reflects the results seen in the aforementioned rat studies (213,214). Rampersaud et al. (238) investigated the effects of controlled folate intake on global genomic DNA methylation in leukocytes of elderly women. Thirty-three healthy, elderly women consumed a depletion-repletion diet consisting of a low folate diet (118 µg/d) for 7 wk and repletion with 200 or 415 µg/d for 7 wk. Blood samples were taken weekly and leukocyte DNA methylation was determined using the methyl acceptance assay (213). Moderate folate depletion in elderly women was severe enough to be associated with increased $[^3]H$methyl incorporation in vitro, which reflects decreased methylation in vivo. Decreased methylation was evident in these women at wk 7, when they were also found to have the lowest folate status. DNA methylation did not increase after 7 wk of repletion with 200 or 415 µg folate/d, which suggests that the repletion period may not have been long
enough to increase the methylation. The results from this study show that low folate status may significantly reduce DNA methylation and support the use of DNA methylation as a functional folate status indicator. The investigators stressed that results related to DNA methylation status within specific cells may not relate to whole body methylation.

Epidemiological data also support a correlation between folate status and DNA methylation. Fowler et al. (239) found a significant, inverse correlation between cervical tissue folate concentration and DNA methylation and serum folate concentration and DNA methylation. An inverse correlation between folate status and DNA methylation has not been found by all investigators. Fenech et al. (240) measured the folate and lymphocyte DNA methylation status of young Australian men and women. Volunteers were required to consume their normal diets and were assigned to eat a bowl of fortified or unfortified bran cereal once/d plus a placebo or a vitamin supplement for 24 wk. The folate group received 2700 µg folic acid/d and 27 µg vitamin B12/d (cereal + tablet), while the placebo group received no folic acid. No significant differences in DNA methylation were detected after treatment relative to baseline for either group or any correlations between DNA methylation and folate status. This is not surprising because this was not a folate depletion protocol, and all volunteers had normal status throughout the study. It is only when folate status is impaired that the availability of methyl groups decreases and there is a decrease in genomic methylation (233).

The effect of folate on methylation and subsequent transcription was evaluated in cell culture experiments. Jhaveri et al. (241) performed experiments with human nasopharyngeal epidermoid carcinoma KB cells to determine whether folate deplete
media affects the transcription of genes. They found that eight genes responded to a variation of folate concentrations. Three genes were upregulated and five genes were downregulated in cells grown in folate deficient media. H-cadherin, a protein involved with cell adhesion molecules, was one of the genes down regulated in folate deficient media. This down regulation was associated with hypermethylation of the CpG island that contained the promoter region. These data indicated that decreased folate positively and negatively influences the expression of certain genes, so that folate deficiency affects specific genes rather than global methylation. Regardless of whether experiments were \textit{in vitro} or \textit{in vivo}, results support a role for folate in DNA methylation and transcription.

Methionine is an essential amino acid that is the immediate precursor to SAM in the methionine cycle and is required for protein synthesis (242). It is well known that methyl deficient diets (i.e., choline and methionine deficient) can cause global hypomethylation in rats (243,244) and that it can occur within 1 wk of starting the diet (244,245). Choline deficient diets also have been reported to induce significant hypomethylation of brain DNA compared to controls (234).

\textbf{SAM/SAH regulation of DNA methylation.} Once SAM is used as a substrate for methylation, SAH is formed within the active site of the methyltransferase enzyme. Most methyltransferases have a higher binding affinity for SAH than SAM; therefore, excess SAH can result in strong product inhibition of these methyltransferases, which may lead to decreased DNA methylation (246). In a review by James and colleagues (247), three defense mechanisms against toxic SAH concentrations are discussed. They suggest that SAH can be bound to proteins, exported into the plasma, or hydrolyzed by SAH hydrolase, which degrades SAH into homocysteine and adenosine.
In order for methyltransferases to work efficiently, SAH concentrations need to be regulated intracellularly, which is primarily accomplished by SAH hydrolase. Homocysteine can then be remethylated to form methionine or shuttled down the transulfuration pathway (248). Previous studies have implicated decreased availability of SAM as a limiting cofactor for methyltransferases, and therefore, decreased cellular methylation (249).

The SAM/SAH ratio has been used to predict reduced cellular methylation, with SAM as the main effector (249). Yi et al. (248) estimated whether SAM or SAH had a greater impact on global DNA methylation in healthy, adult women. They found that an increase in homocysteine concentration in these women correlated with a significant increase in SAH concentration but had no relationship with SAM. A strong, correlation between homocysteine concentration and the SAM/SAH ratio was detected, and this decreased ratio was associated with an increased SAH concentration rather than decreased SAM concentration. Lymphocyte DNA hypomethylation increased significantly with increasing concentrations of SAH, but had no relationship with SAM. This study was the first to show that moderate elevations in homocysteine concentration are in fact associated with increases in SAH and decreases in lymphocyte DNA methylation. These investigators suggested that there might be another mechanism of homocysteine pathogenicity via SAH inhibition of DNA methyltransferases. Instead of limiting DNA methyltransferase activity and subsequently causing reduced DNA methylation, it was hypothesized that low SAM concentrations may instead affect DNA methylation by decreasing thymidine and purine synthesis via increased activity of MTHFR (248).
A combined genetic and dietary approach in a mouse model was used by Caudill et al. (250) to investigate the effect of changes in plasma homocysteine and intracellular SAM, SAH, and the SAM/SAH ratio on global DNA methylation in different tissues. Mice were normal or heterozygous for CBS deficiency and were randomized into a methyl-deficient diet group or the control group for 24 wk. The combined results for different tissues indicated that an increase in SAH alone or in conjunction with a decrease in SAM was associated with a decrease in DNA methylation. A decrease in SAM alone was not sufficient to affect DNA methylation. In addition, a decrease in the SAM/SAH ratio was associated with reduced DNA methylation only when associated with an increase in SAH. The negative effects increased SAH concentrations and decreased DNA methylation may have on altered gene expression and chromatin formation have been reviewed (251,252). James et al. (247) hypothesized that SAH-mediated reduced DNA methylation as a result of increased homocysteine concentrations may increase DNA damage from homocysteine-induced free radicals. Further research is needed to determine whether this is a possibility.

**Genetic polymorphisms and methylation.** Individuals homozygous for the MTHFR 677C→T variant have diminished methylation capabilities. It has been shown that the distribution of folate forms was altered in the red blood cells of individuals with the TT genotype (6). These individuals had formylated folates in addition to methylated folates in contrast to individuals with the CC genotype who exclusively had methylated folates. Genotype did not affect total red blood cell folate content. This finding prompted Stern et al. (12) to use a methyl acceptance assay to evaluate whether this decrease in methylated folates affected DNA methylation in these individuals. They
found that individuals with the TT genotype had significantly decreased DNA methylation compared to individuals with the CC genotype, and that this methylation was directly correlated with red blood cell folate concentration. This was the first study to report that the TT genotype may be associated with epigenetic alterations. These findings were supported by a second observational study by the same research group in a larger population. Friso et al. (13) evaluated the effect of folate status and MTHFR $677C \rightarrow T$ TT genotype on DNA methylation in an Italian population. They directly measured methylated cytosines using a liquid chromatography/mass spectrometry method. DNA methylation in individuals with the TT genotype was approximately 50% that of individuals with the CC genotype when plasma folate concentration was below the median for this population group. There was no significant difference in methylation between the individuals with the TT and CC genotypes when plasma folate concentration was above the median. An inverse relationship between DNA methylation and homocysteine concentration also was observed in this study. An important limitation of this study is that dietary folate intake was not controlled. These results have been corroborated using a MTHFR knockout mouse model. Chen et al. (95) found lower global DNA methylation in heterozygous and homozygous knockout mice compared to control mice. This body of evidence supports the conclusion that the homozygous MTHFR $677C \rightarrow T$ variant is associated with reduced DNA methylation when folate status is low.

**Methylation and cancer.** Recent evidence has shown that cancer is a process that is modified by DNA mutations and epigenetic mechanisms (253). In normal cells, CpG islands are hypomethylated and located in the promoter regions of 40-50% of genes.
Many cancer genes are being discovered that are hypermethylated in the promoter region (253). The role of hypermethylation of DNA in cancer involves the silencing of genes by hypermethylation of promoter regions. Although the information on hypermethylation is extensive, a review by Ehrlich (254) argues that there has been inadequate attention given to global hypomethylation of DNA in cancer. The evidence for global hypomethylation in carcinogenesis is considerable. Rats fed methyl deficient diets had hypomethylated liver DNA that was associated with increased mRNAs for protooncogenes (255). After 1 mo of consumption of a diet adequate in methyl groups, a reversal in methylation and protooncogene expression was observed (255). Rats fed methyl deficient diets also had hypomethylated p53 tumor suppressor genes (10,214), which could enhance tumor production. Sibani et al. (256) reported that reduced DNA methylation in preneoplastic intestinal cells was directly associated with tumor multiplicity, which was increased under low folate conditions. It was hypothesized that if hypermethylation and hypomethylation of DNA can be altered, there may be an opportunity to influence tumor production.

Caution is warranted when interpreting data regarding hypermethylation and hypomethylation of DNA. These terms denote more or less methylation of DNA relative to some standard. When studying cancer, normal tissue is the standard (254). A different standard has to be developed for each tissue studied because methylation is species and tissue-specific. Cell types also have to be considered because tissues are comprised of a mixture of cells (254). As reviewed by Ehrlich (254), DNA hypomethylation has been found in leukemia, liver and prostate, and cervical cancer, and DNA hypermethylation has been found in colon, kidney, esophageal, and pancreatic cancer.
DNA Strand Breaks

Another measure of genomic stability is the number of strand breaks in the DNA. Strand breaks were initially found to be associated with reduced DNA methylation resulting from a methyl group deficiency. Pogribny et al. (10) fed rats a diet deficient in the methyl donors methionine, choline, and folate and found that genomic strand breaks increased with increasing DNA hypomethylation. They also showed that increased methylation protected the DNA from enzyme-induced strand breaks. These results were supported by a subsequent study from the same group (214) who found increased DNA strand breaks with prolonged folate deficiency. They hypothesized that DNA hypomethylation secondary to folate deficiency may induce strand breaks by changing the conformation of the chromatin and protein-protein interactions. These changes make the DNA more susceptible to DNA-damaging agents or endonucleases (214).

Although methyl group deficiency was the first explanation for strand breaks, recent research also has implicated uracil misincorporation as a source for strand breaks. As seen in Figure 2-2, the enzyme thymidylate synthase requires 5,10-methyleneTHF as a coenzyme for the conversion of dUMP to dTMP for DNA synthesis and repair. A folate deficiency will limit the amount of coenzyme available for this conversion and cause a buildup of dUMP with subsequent dUTP misincorporation into DNA by DNA polymerase resulting in a UA base pair (11). Uracil is excised from DNA by uracil deglycosylase, which can cause breaks in the DNA if there is insufficient dTMP available for repair. If two breaks are located within 12 base pairs from each other on the DNA it could result in a double-stranded DNA break (257). Another mechanism of uracil misincorporation involves spontaneous deamination of nonmethylated cytosine residues to uracil, which can result in a UG base pair and a cytosine to thymine transition mutation.
if the uracil is not excised before replication. Uracil misincorporation is not a problem unless the capacity for uracil excision is exceeded (258).

Uracil excision is an important DNA repair mechanism involving different enzymes. Uracil DNA glycosylase recognizes a conformational change produced by the misincorporated uracil and binds to the DNA. The uracil is flipped out of the double helix into the active site of the enzyme and cleaved. This leaves an apyrimidinic (AP) site, which is cleaved by an AP endonuclease. Deoxyribophosphodiesterase removes the 5'-phosphate group, DNA polymerase inserts the correct nucleotide, and DNA ligase seals the gaps in the DNA (258).

Cell culture studies have confirmed that folate deficiency causes increased uracil misincorporation and DNA strand breakage. Duthie and Hawdon (259) used single cell gel electrophoresis with uracil deglycosylase on human lymphocytes to determine DNA damage in cells in a variety of experiments. Stimulation of cells in folate deficient media resulted in no growth compared to cells in folate-rich media that grew 6-fold in 8 d. The effect of folate deficiency was graded for cell growth, with cells growing normally in 100 ng/ml, poorly in 10 ng/ml, and not at all in 1 ng/ml. Uracil misincorporation and DNA strand breakage was significantly greater in cells grown in a folate deficient medium compared to cells grown with folic acid. Finally, cells grown in folate deficient media for 5 d were unable to repair oxidant-induced DNA damage as efficiently as controls. Melnyk et al. (260) used Chinese hamster ovary cells to determine the effect of folate on DNA damage. Cell growth was inhibited in folate deficient media. They expanded the experiments and measured intracellular nucleotide concentrations, finding significantly increased dUTP and decreased dTTP concentrations and an increased dUTP/dTTP ratio.
during folate deprivation. Uracil misincorporation, AP sites, and DNA strand breaks all increased with increasing duration of folate depletion. These cell culture studies supported an association between poor folate status and increased DNA damage.

Pogribny et al. (258) found significantly increased uracil misincorporation and AP sites after just 3 wk of feeding rats a methyl deficient diet low in methionine, choline and folate. DNA strand breaks also were increased but progressed more slowly with significant results appearing after 9 wk of feeding and continued to increase with prolonged feeding. Duthie et al. (261) separated their rats into a control group, a folate deficient group, a choline/methionine deficient group, and a folate/choline/methionine deficient group and determined DNA integrity at 4, 8, and 10 wk. Lymphocyte DNA strand breaks were higher in all groups compared to controls after just 4 wk. The greatest amount of strand breakage was seen in the folate/choline/methionine deficient group. The choline/methionine deficient group had more DNA strand breaks than the folate deficient group at 4 wk, indicating that methyl group deficiency has a greater effect on strand breaks than folate deficiency alone. Uracil misincorporation was highest in the folate deficient group, with no uracil misincorporation seen in the choline/methionine group, indicating the specificity of this biomarker for folate deficiency. The investigators concluded that DNA strand breaks are more affected by methyl-donor status than folate status, and that uracil misincorporation is more affected by folate status than methyl-donor status.

The extent of uracil misincorporation in humans has been evaluated. After separation of subjects in normal and deficient folate groups at baseline, Blount et al. (11) supplemented all subjects with 5 mg of folic acid for 8 wk. DNA uracil concentrations
were reduced in all subjects after supplementation with folic acid. The greatest decrease was seen in subjects with the lowest folate status at baseline. Folate deficient subjects had the greatest chromosome breakage as measured by micronucleated reticulocytes and erythrocytes at baseline compared with controls. Folate supplementation reduced chromosome breakage in folate deficient subjects but had no effect on folate replete controls.

Independent of whether strand breaks are formed from DNA hypomethylation, low methyl-donor status, or uracil misincorporation, they lead to chromosome damage and are associated with increased risk for cancer. Adequate folate intake is essential for the prevention of chromosome damage and may reduce cancer risk.

**Micronuclei Formation**

DNA strand breaks ultimately lead to micronuclei, which are formed by the loss of whole chromosomes or portions of chromosomes from daughter nuclei at mitosis and form small, independent nuclei within the cytoplasm of a cell (262). Although micronuclei appear in almost every cell type, cells from the hematopoietic system are most widely used because of their ease of collection (262). Micronuclei formation is a marker of genetic damage that is used to assess different risk factors for their genotoxic capabilities. The role of folic acid in initiating the formation of micronuclei was first investigated in early *in vitro* experiments that were able to induce micronuclei formation with folic acid deficient media (263). They also found dose-dependent protection from micronuclei formation with increasing folic acid concentrations in the media of cultured lymphocytes (263). Everson et al. (264) supported these findings by reviewing a case study of a subject with an elevated frequency of micronucleated cells, which returned to normal after folic acid supplementation.
Many subsequent studies have substantiated the role of folic acid in micronucleated cells (215,236,265,266). Differences have been found between men and women, with folate having an effect on micronuclei formation in women, but not in men (267). Fenech et al. (268) evaluated the association between folate status and micronuclei formation in older men and found a significant increase in micronuclei formation in men with a folate deficiency without any clinical manifestations. They also conducted an intervention study in older men to determine whether folic acid supplements could affect the genetic damage rate in lymphocytes. They did not find a decrease in micronuclei formation with folic acid intakes of up to 10 times the DRI for 16 wk (268). Homocysteine also has been reported to be an independent risk factor for micronuclei formation, although more studies are needed to confirm these results (269,270).

Quantifying micronuclei is an easy and fast method to measure genetic damage. It is an assay that can be used in vitro and in vivo to evaluate the influence of factors like environmental toxins and radiation, on cell carcinogenicity (262). This method, combined with other quantitative and qualitative techniques, can provide good insight relative to the etiology of cancer in mammals.

**Dietary Reference Intakes (DRIs)**

The DRIs are recommendations for intakes of specific nutrients and include the Recommended Dietary Allowances (RDA), Estimated Average Requirements (EAR), Tolerable Upper Limits (UL), and Adequate Intakes (AI). The most recent recommendations were published in 1998 by the National Academy of Science IOM (27). Previous recommendations were established to prevent clinical deficiencies of each nutrient. The basis for the 1998 IOM DRI recommendations changed from preventing clinical deficiencies to ensuring optimum health (27). The EAR for folate for women
between the ages of 19 to 50 is 320 µg DFE/d and is calculated as the amount of folate needed to meet the requirements of 50% of this population. The RDA for this group, which is based on the EAR, is 400 µg DFE/d and is set to cover the needs of 97 to 98% of these individuals (27). The IOM also recommends that all women of childbearing age consume 400 µg/d of folic acid in the form of supplements and/or fortified foods in addition to the daily diet to reduce the risk of NTDs. An UL of 1,000 µg/d of synthetic folic acid was set for this age to avoid a delay in the diagnosis of a vitamin B12 deficiency that could otherwise lead to neurological damage from a masked vitamin B12 deficiency (27).

Folate plays an important role in genomic stability. Fenech (271) argues that because DNA damage increases the risk for degenerative diseases and aging, the basis of dietary intake recommendations should shift to defining optimal intakes of nutrients to prevent DNA damage. Vitamin B12 and folate are the two nutrients with the greatest effects on DNA stability (239). Fenech et al. (240) performed a dietary intervention study to evaluate DNA damage and concluded that an intake of 700 µg folic acid/d and 7 µg vitamin B12/d was sufficient to minimize chromosome damage, which are amounts that greatly exceed the current DRIs for these nutrients. The results of different intervention studies in humans that indicate DNA damage is minimized when red blood cell folate concentration is > 700 nmol/L were evaluated in a review by Fenech (272). This red blood cell folate concentration is associated with folate intakes greater than the current DRIs. This review also lists different intakes recommended by various investigators to minimize genomic instability that range from 228 to 10,000 µg folic
Fenech (271) concluded that there is a need for an international collaboration to establish DRIs for nutrients to enhance genomic stability.

**Folate Status in Women of Reproductive Age**

**Effect of Fortification**

Earlier research associating the intake of folic acid with NTD risk reduction prompted the Public Health Service in 1992 to recommend that all women of childbearing age consume 400 µg/d of folic acid (273). Since then there have been monumental efforts to help women in this age group meet this recommendation, including the mandate by the FDA in the US in 1996 to fortify all enriched cereal grain products with folic acid.

The fortification of the food supply with folic acid has had a positive effect on the folate status of the population as a whole. In order to assess the benefit of fortification, the CDC (274) compared serum and red blood cell folate concentration for women of childbearing age who participated in the 1999 National Health and Nutrition Examination Survey (NHANES 1999) to women of childbearing age who participated in the Third National Health and Nutrition Examination Survey (NHANES III, 1988-1994). The serum and red blood cell folate concentrations from the NHANES III were 6.3 and 181 ng/ml and increased significantly to 16.2 and 663 ng/ml, respectively, in NHANES 1999. Choumenkovitch et al. (275) also evaluated the effect of fortification in a cross-sectional study with participants in the Framingham Offspring Cohort. They compared the mean red blood cell folate concentration of subjects before fortification to red blood cell folate concentration of subjects after fortification and observed a 38% increase post fortification.
Fortification also has had a positive effect clinically. Honein et al. (276) reported a 19% reduction in NTD prevalence in the United States, although they concede that other factors also may have contributed to this decrease. The FDA predicted that fortification would increase the average folate consumption by 100 µg/d. Studies by Choumenkovitch et al. (277) and Quinlivan and Gregory (278) determined that intakes are approximately twice the predicted level.

**Effect of Ethnicity**

Ethnicity may affect the folate status and homocysteine concentration of certain populations. Specifically, certain Hispanic groups may be the most affected. The Office of Management and Budget’s revised minimum standards for reporting race, which have been adopted by the NIH, define two ethnic categories: Hispanic and non-Hispanic (279). Hispanic ethnic groups can be further divided into the subcategories Cuban, Mexican, Puerto Rican, South or Central American, or other Spanish culture or origin, regardless of race (279). Ford and Bowman (280) published data from NHANES III on serum and red blood cell folate concentration in non-Hispanic white, non-Hispanic black, and Mexican American men and women. Both Mexican American men and women had significantly lower serum and red blood cell folate concentrations compared to non-Hispanic white men and women even after controlling for dietary folate intake. In addition, Jacques et al. (281) published data from NHANES III on homocysteine concentrations in non-Hispanic white, non-Hispanic black, and Mexican American men and women. Mexican American women had significantly lower homocysteine concentrations compared to non-Hispanic white and non-Hispanic black women. In response to the publication of these two studies, Baggott (282) questioned how lower serum folate concentrations could lead to lower homocysteine concentrations in Mexican Americans, which is inconsistent with
a large body of literature that reports that lower serum folate concentrations are associated with higher homocysteine concentrations. Jacques et al. (283) responded by suggesting that lifestyle factors and racial and genetic differences may influence the homocysteine concentration of this population, and that the lower homocysteine concentrations are not a result of higher serum folate and vitamin B12 concentrations. The basis for the lower serum and red blood cell folate and homocysteine concentrations in Mexican Americans needs further study.

**Folate Deficiency**

There is a progressive sequence of events associated with the development of folate deficiency. The first event is a reduction in serum folate concentration, which can occur within 1 to 3 wk. Serum folate concentration is considered to be a short-term indicator of folate status because it is most indicative of recent intake. After a longer period of folate deficiency, red blood cell folate concentration will begin to drop. Red blood cell folate concentration is considered a long-term indicator of folate status. Homocysteine concentration begins to increase around the same time that red blood cell folate concentration decreases (284).

After blood folate status is compromised for a period of time, the clinical manifestations of folate deficiency develop. Neutrophil hypersegmentation causes a state of macrocytosis without anemia. Eventually, macrocytic, megaloblastic anemia will develop with decreases in hemoglobin, hematocrit, and red blood cell folate concentrations (284). A decrease in folate availability will cause a reduction in the DNA cycle, ultimately decreasing cell division, and resulting in the formation of the large, immature red blood cells observed in megaloblastic anemia (233).
Folate Status Assessment

Folate status assessment can be separated into quantitative assessment and functional assessment. The two quantitative indicators are serum and red blood cell folate concentrations. The functional indicators include plasma homocysteine, SAM/SAH ratio, DNA methylation, uracil misincorporation, and DNA strand breaks.

Quantitative measures of folate status can provide an indication to the clinician of the patient’s folate status. Because serum folate is a more sensitive indicator of recent intake, an isolated serum folate measurement may not differentiate between a temporary reduction in serum folate and chronic folate deficiency (27). Repeated serum folate measurements over time will provide more information as to the status of the individual. A serum folate concentration less than 3 ng/ml (6.8 nmol/L) is reflective of a negative folate balance in the individual at the time the sample was taken (285). Red blood cell folate concentration is the primary indicator chosen to determine folate status because it reflects tissue stores of the vitamin (27). Red blood cells only take up folate during erythropoiesis and have a lifespan of 120 d; therefore, they are indicative of long-term folate status (27). The IOM recognizes a red blood cell folate concentration of < 140 ng/ml (305 nmol/L) as a deficient red blood cell folate concentration and has based this number on a review of studies in which this value was associated with clinical indicators of deficiency (27). Although red blood cell folate concentration is a better indicator of folate status than serum folate concentration, both serum and red blood cell folate concentrations taken together can provide a good measure of folate status.

The functional indicators of folate status become affected as a result of changes in the quantitative indicators. Plasma homocysteine concentration becomes elevated during a folate deficiency because methionine synthase, which requires folate as a cofactor, is
unable to remethylate homocysteine to methionine. As mentioned previously, different studies have provided evidence that homocysteine concentration rises as folate concentration decreases. Specifically, Jacob et al. (286) reported that folate depletion in young men led to a rise in homocysteine concentration. O’Keefe et al. (287) found an inverse relationship between serum and red blood cell folate concentrations and plasma homocysteine concentration in women. Although there are differences in the cut off values for plasma homocysteine, any value > 14 µmol/L is generally considered high (27). Ubbink et al. (288) suggested a reference range of 4.9 to 11.7 µmol/L. Regardless of gender, low folate concentrations result in increased homocysteine concentrations. High homocysteine concentrations lead to an increase in SAH concentration via the reversible enzyme SAH hydrolase (Fig. 2-2, reaction 11). Increases in SAH without a concurrent increase in SAM results in a decreased SAM/SAH ratio. Balaghi and Wagner (213) found significantly decreased SAM/SAH ratios in rats fed a folate deficient diet for 4 wk compared to controls. There are currently no published reports related to SAM/SAH ratios in humans fed folate-deficient diets.

DNA methylation, uracil misincorporation, and DNA strand breaks are also functional indicators of folate status but cannot be used in the same way as plasma homocysteine concentration. Each indicator has been discussed in detail under the DNA stability section. Briefly, a folate deficiency can affect DNA methylation by limiting the amount of methionine available for the production of SAM. Low folate status causes a decrease in the formation of dTTP from dUTP and results in uracil misincorporation. Finally, uracil misincorporation ultimately leads to DNA strand breaks as a result of DNA repair. There are no generalized norms for these indicators because of individual
variation in DNA and variation in the assessment of these methods. These indicators are only useful when comparing subjects to a control group or to themselves after some kind of treatment within the same study.

**Analytical Methodology**

A variety of methods have been used to assess folate status. Not all research groups use the same methods, and even within a given method, protocols may vary between labs. These differences limit the ability to directly compare results from different studies without an evaluation of interlaboratory differences (289). Although there are many methods used, this section reviews some of the more commonly used methods.

**Blood Folate Analysis**

Serum and red blood cell folate concentrations can be measured using a microbiological assay or a radiobinding assay. The microbiological assay is the most widely accepted method for determining folate concentrations in blood, urine, tissue, and food samples. The test organism used is *Lactobacillus casei* subspecies rhamnosis. This organism metabolizes the greatest number of folate derivatives, including polyglutamated residues with up to three glutamates (290). The growth of the organism is calculated by measuring the turbidity of the medium, which is directly proportional to the amount of folate in the sample. This assay cannot be used on samples containing antibiotics or methotrexate because they will inhibit the growth of the organism.

The radiobinding assay (RA) also is used to measure folate concentrations of samples because of its speed and the fact that it is not affected by pharmaceuticals. A folate binding protein attached to microbeads and [125I]-labeled folic acid or methyltetrahydrofolate are used to quantitate serum or red blood cell folate concentration.
Unlabeled folate competes with labeled folate for binding to the folate binding protein. Samples are centrifuged and bound folates and microbeads precipitated. The supernatant is discarded and bound labeled folate is counted in a scintillation counter. The decrease in radioactivity is proportional to the folate concentration in the sample. There have been problems associated with using RA assays. Folate values for the first 4 y of the NHANES III were determined using a RA, and results had to be adjusted to correct values after it was determined that the RA results were 30% too high due to the problems with the standards produced by the manufacturer of the assay kit (27).

Although the microbiological assay tends to be time consuming and tedious, it has been the preferred method for quantitative analysis of folate status. The RA is a quicker method and tend to be lower than values obtained using the microbiological method (291). A round robin comparison of different lab techniques for the measurement of serum and red blood cell folate concentration was conducted by the CDC (289). Five different analytical techniques were used by 20 different laboratories worldwide to assess the folate concentrations of six serum and six whole blood pools. They reported overall CVs for the serum folate pools and the whole blood pools of 27.6% and 35.7%, respectively. They also reported a 2- to 9-fold difference in concentrations of the pools within the different methods. These results support the fact that folate values cannot be compared between labs unless interlaboratory variations are considered.

**Plasma Homocysteine and SAM/SAH Ratio Analysis**

Measurement of plasma homocysteine concentration is generally limited to total homocysteine concentration. Before total homocysteine concentration can be measured, the disulfides must be chemically reduced. The preferred approach to homocysteine quantitation is an HPLC method because of the relatively low cost of chemicals and
solvents, the availability of equipment, and the existence of a fully automated assay (292). Some other automated methods include gas chromatography-mass spectrometry, liquid chromatography electrospray tandem mass spectrometry, and immunoassay (293). HPLC methods for homocysteine utilize reversed-phase columns, which have hydrophobic chains that protrude and retain the hydrophobic molecules of interest. These molecules are eluted in order of hydrophobicity using a mobile phase. Once eluted, homocysteine can be detected fluorescently, electrochemically, or colorimetrically. A similar technique also can be used to measure SAM and SAH concentrations (294).

**DNA Stability**

The most common ways to assess genomic stability is by evaluating DNA methylation, uracil misincorporation, and DNA strand breaks. Because analysis of these indicators differs between laboratories, results cannot be compared between different studies. Comparisons can be made only within studies between controls and experimental subjects.

**DNA methylation.** The original assay used to determine DNA methylation involved the use of $[^3]H$SAM and a bacterial DNA methylase that only methylates at the 5-position of cytosines. Incorporation of labeled methyl groups is inversely related to the extent of DNA methylation in the sample (213). Some limitations of this method are that damaged DNA can interfere with the methylase, and that DNA strand breaks or abasic sites can give a false positive (295). Pogribny et al. (295) developed a cytosine-extension method based on the use of a methylation-sensitive restriction enzyme that leaves a 5’guanine overhang followed by single nucleotide primer extension with $[^3]H$dCTP. The extent of $[^3]H$dCTP incorporation also is inversely proportional to the DNA methylation in the sample. The cytosine-extension method is less subject to error than the methyl
acceptance assay because the integrity of the DNA does not influence the method, which can be applied to nanogram quantities of DNA (295). Fujiwara and Ito (296) made one modification of the cytosine extension protocol to circumvent the use of radioactivity. Biotinylated dCTP was added to the digested DNA by Taq polymerase, which was visualized with a streptavidin-alkaline phosphatase reaction.

Friso et al. (13) developed a liquid chromatography/mass spectrometry method that allowed direct quantitation of methylated cytosine residues. DNA is enzymatically hydrolyzed with sequential digestion to nucleotides and separated into the four bases. Methylated cytosine elutes 2 min after cytosine and can be quantitated. This method is more accurate than the previous two methods in determining DNA methylation because it is quantitative rather than semiquantitative, it measures methylated cytosines directly rather than indirectly, and it has small intra- and inter-assay CVs. A similar method by Cooney et al. (297) involves the direct measurement of 5-methyldeoxycytidine after sequential enzymatic digestion and HPLC separation. A less utilized method involves bisulfite-induced modification of genomic DNA to convert cytosine to uracil but methylated cytosines remain nonreactive (298).

**Uracil misincorporation.** A high degree of uracil misincorporation can lead to DNA instability when DNA repair enzymes remove the uracil and leave single-strand breaks that could result in the less repairable double-stranded breaks (11). The original assay used to determine uracil misincorporation employed gas chromatography/mass spectrometry in negative chemical ionization mode after DNA was digested with uracil deglycosylase. This allowed for direct measurement of uracil in the DNA sample.
The comet assay can also be used to determine uracil misincorporation (259). The comet assay involves the use of a microscope slide covered with agarose and cells. These slides are washed with uracil deglycosylase to excise the uracil. Slides are subjected to electrophoresis and stained. The DNA sample resembles a comet, with the intact DNA in the head, and any DNA fragments in the tail. Uracil misincorporation is related to the fluorescence in the tail. More recent assays convert misincorporated uracils to DNA strand breaks with addition of the endonuclease Exo III after uracil excision with uracil deglycosylase. DNA strand breaks can then be quantified using a comet assay (299) or by random oligonucleotide-primed synthesis (ROPS) assay (258,260).

**DNA strand breaks.** There are many different methods available to quantify DNA strand breaks. Some of the older methods include alkali elution, DNA unwinding assays, and unscheduled DNA synthesis. Agarose gel electrophoresis, terminal deoxynucleotide transferase, nick translation, and the ROPS assay are more current methods of detection. Most of these assays do not distinguish 3’OH from 5’OH ends and require large amounts of DNA (300). Basnakian and James (300) developed a ROPS assay based on random oligo-nucleotide synthesis catalyzed by Klenow fragment polymerase in order to detect low frequency 3’OH DNA strand breaks. After denaturation and renaturation of the DNA, the single-stranded DNA serves as its own template for extension using [\(^{32}\)P]-labeled dNTPs. Incorporation of labeled dNTPs is proportional to the number of strand breaks in the DNA sample. The strength of this assay is that it only requires nanogram concentrations of DNA and it can detect single-stranded and double-stranded DNA breaks.
Genotype Determination

With the discovery of single nucleotide polymorphisms (SNPs), determining subject genotypes for these SNPs has become common practice. The most common method of genotyping involves polymerase chain reaction (PCR) to amplify the desired region of DNA. Once the region is amplified, specific restriction enzymes are added depending on the SNP being studied. Fragments are then separated by electrophoresis on an agarose gel. There are several SNPs associated with folate metabolism. In order to simplify the search for multiple SNPs in one sample, Barbaux et al. (301) developed a method that allows genotyping of four SNPs on one gel. This “heteroduplexing” method involves the use of a heteroduplexing generator instead of restriction enzymes. The generator is a synthetic DNA molecule identical in sequence to the SNP of choice with a microdeletion adjacent to the polymorphic site (301). The generator combined with PCR technology enables multiple genotyping in a single-tube reaction that can be separated on a single gel. Recently, Ulvik and Ueland (302) developed a method utilizing real-time PCR to genotype for multiple folate related SNPs in whole blood or serum in one tube with the goal of eliminating the DNA purification step. Advances in technology keep improving current methods and will one day enable all SNPs associated with folate metabolism to be identified in the most efficient and cost friendly manner.

Research Significance

The MTHFR 677C→T polymorphism affects a large percentage of the population with an estimated frequency of ~12% for the TT genotype with considerable variation between different ethnic groups (4,303). Blood folate concentrations are reduced (80,86), homocysteine concentrations increased (7,65,67,82), and DNA methylation diminished (13) in individuals with the homozygous TT genotype for the MTHFR 677C→T variant.
It is well recognized that impaired folate status is associated with abnormal fetal growth and development (304) and increased risk of pregnancy complications (139), and that periconceptional folic acid supplementation significantly reduces the risk of NTDs (119). The metabolic basis of these observations has not been definitively established but may relate to folate’s role in nucleotide biosynthesis (1), DNA methylation (218,219), and/or maintenance of normal homocysteine concentrations (142). Since the combined presence of the MTHFR 677C→T variant and low folate status has been associated with increased risk for birth defects, the present study was designed to address the specific aim in females of reproductive age. Guinotte et al. (87) recently published a metabolic study in this age group with a similar design as the present study to investigate differences in response to folate depletion and repletion with the RDA by MTHFR genotype in young women of Mexican American descent. An important difference between these studies is the ethnicity of the study groups, a factor that has been shown to significantly affect folate status and homocysteine concentration (280,281). The present study is the first controlled metabolic study performed in predominantly non-Hispanic women of childbearing age to determine whether there are differences in response to folate depletion and repletion between MTHFR genotypes. In addition, the effect of controlled folate intake on DNA methylation in women with the CC or TT MTHFR 677C→T genotype has not been reported in this age group. Although DNA methylation was found to be significantly reduced after depletion and reversed with folate supplementation in post-menopausal women fed a folate-controlled diet (237), these researchers did not evaluate the effect of genotype on methylation. Similarly, the effect of MTHFR genotype on DNA methylation was was not considered in a group of elderly women fed a
folate-controlled depletion diet (238). The present study is the first to report the effect of folate depletion-repletion on global DNA methylation in women of childbearing age based on MTHFR 677C→T genotype. In addition, there are no genotype-specific DRI recommendations, and data are insufficient to determine whether individuals with the TT genotype require more folate to maintain normal folate status than individuals with the CC genotype. Data from this study can be considered when making future revisions of the RDA for folate.
CHAPTER 3
STUDY DESIGN AND METHODS

Subject Screening and Description

After approval of the study protocol by the University of Florida Institutional Review Board, nonpregnant, healthy women 20 to 30 y old were recruited from the Gainesville, FL area by distributing flyers and placing announcements in local papers. Inclusion criteria were normal blood chemistry, blood folate concentrations, body weight (within 120% of ideal body weight), and health status as determined by medical history. Exclusion criteria were chronic alcohol consumption or use of tobacco or any medications. Approximately 3500 women were screened initially over the phone by a research nurse. Women who seemed to meet the initial screening criteria (n = 379) reported to our lab to have their blood drawn to determine genotype status for the MTHFR 677C→T polymorphism. Only women with the normal CC or homozygous variant TT genotypes were eligible for the study. Forty-six women were selected to participate in the study. All subjects screened and enrolled in the study provided signed informed consent and agreed to participate for the duration of either a 7 wk or 14 wk study and to comply with the study protocol. This study was performed in conjunction with another study whose subjects did not complete the repletion phase of the study. Forty-one women (22 CC, 19 TT) completed the depletion phase of the study (7 wk), and 20 women (10 CC, 10 TT) completed the entire depletion-repletion protocol (14 wk). Serum and red blood cell folate, and plasma vitamin B12, pyridoxal phosphate, and
homocysteine concentrations were normal at baseline for all subjects (i.e., \( \geq 7 \text{ nmol/L} \), \( \geq 317 \text{ nmol/L} \), \( \geq 125 \text{ pmol/L} \), \( \geq 20 \text{ nmol/L} \), and \( \leq 14 \text{ \( \mu \text{mol/L} \)} \), respectively).

**Study Design**

Subjects adhered to a depletion-repletion feeding protocol divided into two consecutive periods of 49 d (7 wk) each (Fig. 3-1). Subjects consumed a low-folate diet providing \( 115 \pm 20 \mu g \text{ dietary folate equivalents (DFE)/d} \) during the first 7 wk of the study. The repletion diet consisted of a combination of the depletion diet plus folic acid and provided 400 \( \mu g \text{ DFE/d} \) \([115 + 285 \mu g \text{ DFE} (168 \mu g \text{ folic acid} \times 1.7 = 285 \mu g \text{ DFE})]\) (27). This controlled metabolic feeding study was conducted in the General Clinical Research Center (GCRC) at Shands Hospital at the University of Florida in Gainesville, FL.

![Figure 3-1. Study design.](image)

Fasting blood samples were obtained at weekly intervals to determine changes in serum and red blood cell folate and homocysteine concentrations. A blood chemistry
profile was performed at baseline (wk 0), post-depletion (wk 7) and post-repletion (wk 14) to monitor health status (Quest Diagnostic Laboratories; Gainesville, FL). Leukocytes were collected at baseline (wk 0), post-depletion (wk 7), and post-repletion (wk 14) for DNA extraction. Complete blood counts with differentials (CBC-D) were performed biweekly and these measurements were evaluated throughout the study to monitor hematological indices. In the event of a reduction in hematocrit to < 30%, 50 mg of iron as ferrous fumarate from a time-released Ferro-Sequels® caplet (Inverness Medical, Inc; Waltham, MA) was provided with dinner until values reverted to > 30%. Additionally, biweekly samples were obtained for quantitative analysis of serum human chorionic gonadotropin (HCG) (Quest Diagnostic Laboratories; Gainesville, FL) in order to detect a pregnancy very early during the folate depletion phase and throughout the repletion phase. Subjects were instructed to use barrier methods for contraception if they were sexually active, and they were informed of the potential risks to maternal and fetal health posed by consumption of the low-folate diet.

**General Clinical Research Center (GCRC) Protocol**

Breakfast was consumed at the GCRC between 6:30 am and 7:30 am for the duration of the study. Subjects were provided with a “take-out” lunch and snacks each day and returned to the GCRC between 5:00 pm and 6:00 pm for dinner. They were required to eat all foods and only those foods provided to them for the duration of the study. Subjects were permitted to take all meals for 2 d away from the GCRC and were supplied with all of the menu items packed for transport with detailed instructions on reheating food items. Compliance with the protocol was monitored through close daily contact by the research team and weekly evaluation of changes in serum folate concentration.
Dietary Treatment and Supplementation Description

An experimental diet that provided a limited quantity of folate using a variety of palatable entrees and accompaniments was developed and analyzed. The diet consisted of a 5-d menu cycle as shown in Table 3-1. Many commercially prepared food items could not be used in this study as a consequence of the 1996 FDA mandate that all enriched foods be fortified with folic acid (20). To keep the folate content to a minimum, customized recipes were developed and prepared with unenriched flour obtained from Kansas State University (Manhattan, KS) and other low-folate ingredients. Recipes were modified in the GCRC kitchen and taste-tested at laboratory meetings to select the recipes that were the most palatable. Foods made with the unenriched flour included waffles, pancakes, blueberry muffins, pita bread, biscuits, brownies, cookies, cakes, toppings, and pizza crust. A limited selection of canned, low-folate vegetables were used in this study, and each of these was boiled 3 times with the cooking liquid being discarded after each boiling to help leach endogenous folate from the food (305). All foods were weighed to ± 0.05 g to ensure that each subject received equal amounts of food and that portion sizes remained constant throughout the study.

The macronutrient and micronutrient contents of the diet, excluding folate content, were estimated using the Minnesota Nutrient Data System (Version 4.03; Nutrition Coordinating Center at the University of Minnesota; Minneapolis, MN). According to this analysis, the diet provided 2358 kilocalories distributed as 11% protein, 62% carbohydrate, and 27% fat. Folate content was determined by laboratory analysis involving a trienzyme extraction procedure followed by a microbiological assay using \textit{Lactobacillus casei} (\textit{L. casei}) as described below. Analyses indicated that the diet contained an average of 115 ± 20 µg DFE/d.
Table 3-1. Five-day cycle menu

<table>
<thead>
<tr>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Breakfast</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shredded wheat</td>
<td>Waffle*</td>
<td>Cornflakes</td>
<td>Pancake*</td>
<td>Blueberry</td>
</tr>
<tr>
<td>Skim milk</td>
<td>Syrup</td>
<td>Skim milk</td>
<td>Syrup</td>
<td>muffin*</td>
</tr>
<tr>
<td>Raisins</td>
<td>Peaches, canned</td>
<td>Raisins</td>
<td>Hash browns</td>
<td>Skim milk</td>
</tr>
<tr>
<td>Brown sugar</td>
<td>Cranberry juice</td>
<td>Apple juice</td>
<td>Applesauce</td>
<td>Pears, canned</td>
</tr>
<tr>
<td>Grape juice</td>
<td></td>
<td></td>
<td>Cranapple juice</td>
<td></td>
</tr>
<tr>
<td><strong>Lunch</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sandwich, Pita pocket*</td>
<td>Tuna salad</td>
<td>Sandwich, Biscuit*</td>
<td>Sandwich, Pita pocket*</td>
<td>Baked stuffed potato</td>
</tr>
<tr>
<td>Honey ham</td>
<td>Crackers</td>
<td>Baked ham</td>
<td>Turkey breast</td>
<td>Turkey ham</td>
</tr>
<tr>
<td>Cheese</td>
<td>Corn chips</td>
<td>Cheese</td>
<td>Cheese</td>
<td>chunks</td>
</tr>
<tr>
<td>Mayonnaise</td>
<td>Soda</td>
<td>Peaches, canned</td>
<td>Mayonnaise</td>
<td>Fruit cocktail</td>
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<tr>
<td>Applesauce</td>
<td></td>
<td>Popcorn</td>
<td></td>
<td>canned</td>
</tr>
<tr>
<td>Doritos</td>
<td></td>
<td>Soda</td>
<td>Corn chips</td>
<td>Popcorn</td>
</tr>
<tr>
<td>Soda</td>
<td></td>
<td></td>
<td>Soda</td>
<td></td>
</tr>
<tr>
<td><strong>Dinner</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enchiladas, Chicken</td>
<td>Chicken pot pie, Cheese</td>
<td>Tacos, Seasoned beef</td>
<td>Pizza, Crust*</td>
<td>BBQ chicken, Chicken</td>
</tr>
<tr>
<td>Cheese</td>
<td>Potatoes‡</td>
<td>Taco sauce</td>
<td>Sauce</td>
<td>BBQ sauce</td>
</tr>
<tr>
<td>Enchilada sauce*</td>
<td>Carrots‡</td>
<td>Sour cream</td>
<td>Mozzarella cheese</td>
<td>Margarine</td>
</tr>
<tr>
<td>Corn tortillas</td>
<td>Green beans‡</td>
<td>Cheese</td>
<td></td>
<td>Mashed potatoes</td>
</tr>
<tr>
<td>Green beans‡</td>
<td>Margarine</td>
<td>shells</td>
<td></td>
<td>Green beans‡</td>
</tr>
<tr>
<td>Blueberry tart</td>
<td>Crust</td>
<td>Carrots‡</td>
<td>Marinated green beans‡</td>
<td>Chocolate pudding</td>
</tr>
<tr>
<td>Ice cream</td>
<td>Green beans</td>
<td>Orange sherbet</td>
<td>Apple crisp*</td>
<td>Shortbread bar</td>
</tr>
<tr>
<td>Cranberry juice</td>
<td>Orange sherbet</td>
<td>Cranberry juice</td>
<td>Cranberry juice</td>
<td>Cranapple juice</td>
</tr>
<tr>
<td><strong>Snacks</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fruit cocktail, canned</td>
<td>Applesauce</td>
<td>Fruit cocktail, canned</td>
<td>Pears, canned</td>
<td>Peaches, canned</td>
</tr>
<tr>
<td>Ginger cookies</td>
<td>Shortbread</td>
<td>Chocolate chip cookies*</td>
<td></td>
<td>Oatmeal</td>
</tr>
<tr>
<td>Caramel popcorn</td>
<td>Popcorn</td>
<td>Snickerdoodle cookies*</td>
<td>Caramel</td>
<td>raisin cookies*</td>
</tr>
<tr>
<td>Apple juice</td>
<td>Apple juice</td>
<td>Brownie*</td>
<td>Popcorn</td>
<td></td>
</tr>
<tr>
<td>Skim milk</td>
<td></td>
<td></td>
<td>Grape juice</td>
<td>Pound cake*</td>
</tr>
</tbody>
</table>

*Menu item prepared with unenriched flour
‡Menu item boiled three times to minimize folate content
Subjects consumed a custom-formulated, folic acid free supplement (Westlab Pharmacy; Gainesville, FL) with breakfast and dinner to provide the 1998 Recommended Dietary Allowance (RDA) for all other nutrients except choline. The vitamin-mineral supplement composition is presented in Appendix A. The composition of the custom-formulated supplement was determined by comparing the computerized micronutrient analysis of the low-folate diet to the 1998 RDA for all nutrients except choline. Additionally, the micronutrient analysis was adjusted for the loss of water-soluble vitamins due to boiling the vegetables 3 times by assuming 100% loss and subtracting those amounts from the total value obtained by computer analysis. Nutrients other than folate and choline present in the diet at less than 100% the 1998 RDA were included in the supplement. The choline content of the diet was analyzed as discussed below and found to provide 283 mg/d, which is 67% of the Adequate Intake (AI) for choline (425 mg/d) (306). In addition, a separate calcium supplement (Citracal® Mission Pharmacal; San Antonio, TX) provided 200 mg of calcium as calcium citrate to provide calcium that was not included in the supplement.

Subjects’ body weights were maintained within 5% of baseline. Subjects who lost more than 5% of their initial body weight were provided with foods with relatively little or no nutrient value aside from calories (i.e., margarine, candy, Jello®, and sweetened beverages). If weight loss was not sustained by these measures, the carbohydrate-based caloric supplement Moducal® (Mead Johnson Nutritionals; Evansville, IN) was added to beverages. For weight gain exceeding 5% of initial body weight, margarine was excluded from food preparation, and only unsweetened beverages were permitted.
Caffeinated beverages were limited to two 12 oz carbonated beverages or one cup of coffee/d plus one 12 oz beverage.

**Sample Collection and Processing**

Weekly fasting blood samples were collected throughout the 14 wk study by a registered nurse using a # 23 gauge needle and a 1/2 inch butterfly (Vacutainer Blood Collection Sets; Becton Dickinson, Vacutainer Systems; Franklin Lakes, NJ). All blood samples were processed within 1 h of collection, as previously described (238,307). A total of 15 blood collections per subject were obtained during the 14 wk study period. Blood samples were treated with extra precaution during collection, processing, and storage to ensure protection from light by wrapping blood collection tubes in foil, processing under yellow lights (Sylvania Gold; Danvers, MA), and storing in cardboard boxes, respectively.

Blood for serum samples was collected in 8.3 ml SST gel and clot activator tubes (Vacutainer®; Becton Dickinson, Rutherford, NJ) and kept at room temperature for 30 to 60 min to allow time for clotting. Serum was obtained by centrifuging the SST gel clot activator tubes at 650 x g for 15 min at 21°C (International Equipment Company; Model HN-S II Centrifuge, Needham Heights, MA). Supernatant sera were mixed with sodium ascorbate (1 mg/ml), aliquoted into 200 µl samples, and stored at −30°C until analysis.

Whole blood was collected in 7 ml tubes containing K₃ ethylenediaminetetraacetic acid (EDTA) (Vacutainer®; Becton Dickinson, Rutherford, NJ). Blood for plasma homocysteine was kept on ice until processing. A small aliquot of whole blood held at room temperature was used for hematocrit determination and another portion diluted 20-fold in 1 mg/ml ascorbic acid was aliquotted into 200 µl samples and frozen for
measurement of red blood cell folate concentration. The iced blood was centrifuged at 2000 x g at 4°C for 30 min (Astel Enterprises, Inc.; Model ALC 4237R Refrigerated Centrifuge, Winchester, VA). The plasma from these samples (500 µl/sample) was frozen and used to measure the plasma homocysteine concentration. Following removal of the plasma, approximately 1.0 ml of peripheral leukocytes were carefully removed with a transfer pipette and stored at −30°C. These samples were subsequently used to extract DNA.

**Analytical Methods**

**Food Folate Extraction**

In order to determine the folate content of the study diet, folate was extracted from the diet using a tri-enzyme extraction method (308). Each meal and its related snacks were prepared in the GCRC kitchen using standardized procedures, homogenized in a blender, and stored frozen for 3 d in sealed plastic bags prior to lyophilization (Vertis Company; Gardner, NY) for 3 d. Weights of the freeze-dried meals were recorded. Duplicate 2 g samples from each meal were mixed with 20 ml boiling extraction buffer (6.0 g HEPES, 5.2 g CHES, 10 g sodium ascorbate, 0.4 ml β-mercaptoethanol in 500 ml deionized water; pH 7.85) and boiled for 10 min. After homogenization with a tissue tearer homogenizer (Biospec Products, Inc.; Bartlesville, OK) at a setting of 5 for 1 min, samples were incubated with 33,000 U α-amylase (Sigma; St. Louis, MO) and 18 U protease (Sigma; St. Louis, MO) for 1 h at 37°C to aid in the release of folate from the carbohydrate and protein matrix, respectively. This was followed by incubation with rat plasma conjugase purified from purchased rat plasma (Pel Freeze; Rogers, AZ) at 37°C for 4 h to deconjugate polyglutamyl folates. Samples were placed in a boiling water bath
to inactivate the enzymes and placed on ice for 10 min. After centrifugation at 17000 X g for 20 min at 4°C (DuPont Instruments; Sorvall RC-5 Superspeed refrigerated centrifuge, Newton, CT), the supernatant was filtered through Whatman #1 paper in a graduated cylinder. Pellets were resuspended in 5 ml Hepes-Ches extraction buffer and recentrifuged. Additional supernatant was added to the previous filtrate, diluted to the greatest sample volume with Hepes-Ches extraction buffer, and aliquotted into 2-ml microcentrifuge tubes. Extracted samples were sparged with nitrogen for ~20 s to guard against folate degradation and stored at −20°C. Each meal was analyzed using the microbiological assay with L. casei subspecies rhamnomis, as described below. The trienzyme extraction and folate determination were performed 4 times on different meal samples during the course of the study period to verify uniform folate content over time.

**Food Choline Analysis**

Choline content of the 5-d menu cycle was determined twice by Dr. Steve Zeisel, at the University of North Carolina, Chapel Hill. All forms of choline and betaine were quantitated in the diet by liquid chromatography/electrospray ionization-isotope dilution mass spectrometry (LC/ESI-IDMS). Choline was extracted and partitioned from the sample using methanol and chloroform and analyzed directly by LC/ESI-IDMS (309). Choline content of the diet is presented in Appendix B.

**Supplemental Folic Acid**

Commercially available folic acid (Sigma; St. Louis, MO) was used to prepare the folic acid supplements containing 84 µg folic acid administered in 10 ml apple juice for use in the repletion phase of the study. Each subject consumed two folic acid supplements daily for a total of 168 µg folic acid (285 µg DFE/d). Stock solutions were
prepared by weighing 10 mg folic acid, dissolving in 200 µl 0.1 N NaOH, and filling to 100 ml with deionized water for a final concentration of 100 µg/ml. Two hundred microliters of the stock solution were further diluted to 10 µg/ml by adding 1.8 ml phosphate buffer (pH 7.0). The exact concentration of the diluted stock solution was determined spectrophotometrically at 282 nm using a molar absorptivity coefficient at pH 7.0 of 27,600 L · (mol · cm)^{-1} (310). In order to prepare the supplements, 850 µl of the 100 µg/ml stock solution was added to 10 ml of apple juice in a 15 ml conical tube, wrapped in foil to protect it from light, and frozen immediately at –30°C.

**Microbiological Assay**

**Serum Folate.** Serum folate concentrations were determined using a modified microtiter plate adaptation of the microbiological assay (311). The microorganism *L. casei* subspecies rhamnosis (American Type Culture Collection; Manassas, VA), which requires folate for growth, was used as the test organism and was grown in Folic Acid Casei Medium (Difco Laboratories; Detroit, MI). The main folate coenzyme in the serum is 5-methylTHF, which supports the growth of *L. casei* subspecies rhamnosis (ATCC # 7469) (312).

The assay was performed in a 96-well, flat-bottomed, sterile, microtiter MICROTEST tissue culture plate (Becton Dickinson Labware; Franklin Lakes, NJ). For each assay, 20 µl aliquots of 10 ng/ml folic acid standard, control serum, and 3 subject samples were added in duplicate to 130 µl potassium phosphate buffer. Serial dilutions were made with 150 µl potassium phosphate buffer in each well. All wells were inoculated with 150 µl of microplate medium containing the *L. casei* and incubated at 37°C for 18 h. Cell growth was determined by absorbance at 650 nm using a microtiter
plate reader interfaced with a computer running SOFTmax for Windows (Version 2.35; Molecular Devices; Sunnyvale, CA). A log-linear plot of absorbance against folate standard concentration was used to interpolate unknown folate concentrations of samples and control. The control serum was used to compare values among assays and determine the interassay coefficient of variation (CV). The interassay CV for this study was less than 15%. Intra-assay variation between duplicate samples on each plate also was less than 15%.

**Red Blood Cell Folate.** Red blood cell folate concentration also was determined using the microbiological assay as described above. In order to allow the conjugase in the blood to cleave the polyglutamate form of the reduced folate to the mono-, di-, and tri- glutamate forms, blood was held at room temperature for approximately 1 h prior to processing. Red blood cell folate concentrations were determined by multiplying the whole blood folate concentration by the dilution factor of 20 and using these values in the following equation:

\[
\text{[whole blood folate]} - \left(\text{[serum folate x (1 – hematocrit / 100)]} \right) / (\text{hematocrit / 100})
\]

**Plasma Homocysteine Concentration**

Plasma homocysteine concentration was analyzed in duplicate using a modified high performance liquid chromatography (HPLC) method of Vester and Rasmussen (313). Standards and samples were prepared identically with the addition of 0.1 M borate buffer. The homocysteine disulfide bonds were reduced by adding 20 µl tri-n-butylphosphine in dimethylformamide (Sigma; St. Louis, MO) and incubating at
4°C for 30 min. Proteins were removed by precipitation with perchloric acid (Sigma; St. Louis, MO). Homocysteine was derivitized with ammonium-7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (Wako Chemicals USA; Richmond, VA) to allow for homocysteine quantitation after separation from other sulfide compounds on a Zorbax 4.6 x 250 mm column (Amersham Pharmacia Biotech; Piscataway, NJ) using an HPLC system (Dionex Corporation; Atlanta, GA) with fluorescence detection. A standard curve was prepared using known L-homocysteine (Sigma; St. Louis, MO) concentrations.

**DNA Extraction**

DNA was extracted from the buffy coat layer or whole blood using a genomic DNA extraction kit (Genomic DNA Isolation Kit; Bio-Rad Laboratories; Hercules, CA) and a scaled version of the manufacturer’s protocol. For buffy coat, a 1:4 dilution in phosphate buffered saline was used. Anucleate red blood cells were lysed to facilitate centrifugal separation from nucleated white blood cells that contain DNA. Cellular and nuclear membranes from pelleted white blood cells were lysed with a DNA lysis solution containing detergent and chaotrope followed by removal of RNA with Rnase for 30 min at 37°C. Proteins were precipitated from the solution and removed by centrifugation. Following sequential precipitation and washes with isopropanol and 70% ethanol, the DNA was dried at 65°C for 10 min, dissolved in a hydration solution at 65°C for 10 min, and stored at –30°C to be used at a later time.

**DNA Quantitation**

Comparison of DNA samples between subjects requires precisely equivalent DNA concentration and purity. Therefore, a fluorescent DNA stain was used for these determinations rather than employing the common method of measuring the absorbance at 260 and 280 nm. Picogreen dsDNA Quantitation Kit (Molecular Probes; Eugene, OR)
was used for all DNA quantitation in order to detect quantities as little as 25 pg/ml of double-stranded DNA (dsDNA) for which it is specific. Briefly, DNA samples and a 2 µg/ml standard of lambda DNA were serially diluted in TE buffer in a 96-well microtiter plate. Picogreen reagent was added, and the plate was read on an FMAX fluorescent microplate reader (Molecular Devices; Sunnyvale, CA) at excitation ~480 nm and emission ~520 nm. Sample DNA concentrations were determined from a lambda DNA standard curve. In order to check DNA purity defined as a 260/280 nm absorbance ratio of > 1.8, random samples were evaluated spectrophotometrically following Picogreen analysis and found to be pure.

**MTHFR Genotype Determination**

The restriction fragment length polymorphism polymerase chain reaction protocol of Frosst et al. (3) was used to detect MTHFR 677C→T polymorphisms. Briefly, using appropriate forward and reverse primer pairs bracketing the mutation, DNA fragments were amplified by PCR from template DNA purified as described above. Amplified fragments from the gene that codes for the MTHFR enzyme were separated on an agarose gel. DNA fragments with two C alleles (CC genotype for the MTHFR 677C→T polymorphism) form a single band that is 198 base pairs long. DNA fragments containing two T alleles are 175 and 23 base pairs long and run further down the gel due to their lighter molecular weight. When the MTHFR enzyme contains both C and T alleles, as is the case in individuals who are heterozygous for the MTHFR 677C→T polymorphism, three bands appear, one 198 base pairs long, one 175 base pairs long, and one 23 base pairs long. A sample gel and more detailed description are presented in Appendix C.
Methyl Acceptance Assay

The extent of *in vivo* DNA methylation was determined using a modified method of Balaghi and Wagner (213), in which the bacterial DNA methylase SssI is used to incorporate methyl groups into unmethylated CpG residues with tritium labeled ([^3]H)methyl) SAM. The bacterial methylase SssI is isolated from the wall-less prokaryote *Spiroplasma* strain MQ1 (314). Although most prokaryotic methylases recognize and methylate sites composed of 4-6 bases, SssI is similar to mammalian methylases that recognize and methylate CpG sequences exclusively (314), making it an ideal enzyme for this assay. The incorporation of[^3]H)methyl groups *in vitro* is inversely proportional to DNA methylation *in vivo*.

In order to optimize and determine the reproducibility of the methyl acceptance assay, a series of controls were tested. These controls included a hypermethylated, pooled human DNA control (negative control), an unmethylated, pooled human DNA control, and an unmethylated poly dG-dC (Sigma; St. Louis, MO) control (positive control), the latter of which is a synthetic substrate for CpG methylation. In order to prepare the negative control, ~40 µg pooled human DNA was hypermethylated by incubation with 60 U SssI methylase (NEB; Beverly, MA) and 15 µl unlabeled SAM at 37°C for 1.5 h. To prepare the positive control, 10 µg of dG-dC was diluted with 1 ml of deionozed water. Triplicate 250 ng samples were assayed for every subject and control. The assay was performed using the following reaction mixture in a 0.5 ml conical screw cap tube: 250 ng DNA or control, 1 µl of[^3]H]SAM (Perkin Elmer, 0.55 mCi/ml, 15 Ci/mmol; Boston, MA), 1.5 U (0.375 µl) SssI methylase (NEB; Beverly, MA), 1.5 µl 10X NEB buffer (NEB; Beverly, MA), and sterile-filtered, deionized water to a 15 µl total reaction volume and incubated at 30°C for 1 h. Samples were placed immediately in
ice to stop the enzymatic reaction. The entire reaction mixture was loaded on a 2.5-cm round DE81 ion exchange paper filter (Whatman; Maidstone, England) and filtered through a 25mm/200 ml filter funnel unit (Gelman Sciences; Ann Arbor, MI) attached to a vacuum source. After successive washes 3 times each with 15 ml of 0.5 M sodium phosphate buffer, twice with 1 ml 70% ethanol, and twice with 1 ml 100% ethanol, filters were allowed to dry for 15 min. Once dried, filters were added to 10 ml liquid scintillation fluid (Fisher Scientific ScintiSafe; Fair Lawn, NJ) and counts per min (cpm) determined using a Beckman LS 2800SC (Beckman Instruments; Fullerton, CA) liquid scintillation counter. Samples were recounted after 24 h at room temperature to allow the counts to stabilize, and these triplicate cpm values for each sample were averaged to determine DNA methyl acceptance. Each subject’s samples at baseline, post-depletion, and post-repletion were processed in the same run to ensure consistency.

In order to determine residual labeled methyl groups remaining on the filters after washing, a background mixture containing all ingredients except DNA was run with each assay and subtracted from the sample counts. The unmethylated, pooled human DNA control processed with each assay was used to determine an inter-assay CV of 7%. Intra-assay CVs among triplicate samples were less than 15%. Counts were converted to disintegrations per min (dpm) by dividing cpm by 0.6 to account for counting efficiency. Subject counts were multiplied by two and reported as dpm/0.5 µg DNA.

**Liquid Chromatography/Mass Spectrometry/Mass Spectrometry Assay**

Deoxymethylcytidine (mCyt) and deoxycytidine (Cyt) were quantified to produce mCyt/total Cyt (tCyt) ratios in enzymatically hydrolyzed DNA samples by liquid chromatography/mass spectrometry/mass spectrometry (LC-MS/MS) (Quinlivan et al. unpublished). Before analysis, DNA was hydrolyzed enzymatically using a modified
method of Crain (315). In order to prepare the DNA samples for hydrolysis, 1 µg of DNA (50 µl) for each subject at baseline, post-depletion, and post-repletion was added to a 0.5 ml conical screw cap tube, placed in a boiling water bath for 4 min to denature the DNA, and then immediately placed in an ice bath. Samples were mixed with 5 µl of 0.1 M ammonium acetate (pH 5.3) and incubated with 6 U (3 µl) P1 nuclease (Sigma; St. Louis, MO) at 50ºC for 2 h to nick various phosphate bonds of the DNA. The second hydrolysis involved the addition of 6 µl 1.0 M ammonium bicarbonate (pH 7.75) and incubation with 3.25 mU (2.5 µl) phosphodiesterase (Sigma; St. Louis, MO) at 37ºC for 2 h to complete the digestion of the phosphate backbone. Finally, samples were incubated with 0.5 U (2.5 µl) alkaline phosphatase (Sigma; St. Louis, MO) at 37ºC for 1 h to cleave the sugar-phosphate bonds leaving only the nucleosides (sugar + base). Samples were then chromatographed on a 5-micron Discovery C18 column (100 x 4.6 mm; Supelco; Bellefonte, PA) and eluted with a 50 mM ammonium formate (Solvent A): methanol (Solvent B) gradient. The analysis was started using an eluent of 95% A and 5% B for 4 min, followed by a gradient from 5% B to 65% B over 4 min at a flow rate of 0.6 ml/min, then maintained at 65% solvent B for 3 min, reversed to the original composition (95% solvent A, 5% solvent B) over 1 min, and reequilibrated at that composition for 2 min. Mass spectrometry was performed in the selective reaction monitoring mode using a Finnigan TSQ 7000 (Thermo Finnigan; San Jose, CA) (Table 3-2). The result of the LC-MS/MS analysis of a standard sample is given in Figure 3-2 and that of the DNA hydrolysis products is shown in Figure 3-3. The top panel of each figure (Fig. 3-2 and 3-3) illustrates the sum of all the ions monitored, the middle panel the deoxymethylcytidine peak, and the bottom panel the deoxycytidine peak. In order to quantify the mCyt and
Table 3-2. Selective reaction monitoring fragmentation table.

<table>
<thead>
<tr>
<th>Nucleoside</th>
<th>Retention time (min)</th>
<th>Parent ion</th>
<th>Daughter ion</th>
</tr>
</thead>
<tbody>
<tr>
<td>deoxymethylcytidine</td>
<td>7.0</td>
<td>241.6</td>
<td>125.8</td>
</tr>
<tr>
<td>deoxycytidine</td>
<td>3.9</td>
<td>227.6</td>
<td>111.8</td>
</tr>
</tbody>
</table>

Collision energy for MS/MS was 20V.

Figure 3-2. LC-MS/MS analysis of a standard. Top panel: sum of all ions monitored; middle panel: deoxymethylcytidine peak; bottom panel: deoxycytidine peak.

Cyt in the samples, an external standard consisting of 6% mCyt was prepared by adding 23.5 µl deoxymethylcytidine (Sigma; St. Louis, MO) at a concentration of 10 ng/ml and 15 µl deoxycytidine (Acros Organics, Fisher Scientific; Pittsburg, PA) at a concentration of 100 ng/mL to 961.5 µl sterile- filtered, deionized water. Sample mCyt and Cyt concentrations were quantified by comparing their peak areas to standard curves calculated from the peak areas of deoxymethylcytidine (0.29 to 2.35 ng/ml; r = 0.99) and deoxycytidine (18.75 to 150 ng/ml; r = 0.99). Ratios were calculated by dividing the...
mCyt concentration by the total Cyt concentration (mCyt + Cyt = tCyt).

**Statistical Analysis**

At baseline, one-way analysis of variance (ANOVA) was used to test for differences in mean serum and red blood cell folate concentrations, plasma homocysteine concentration, and DNA methylation ([³H]methyl group acceptance and mCyt/tCyt ratio) between genotype groups. Analysis of covariance (ANCOVA) assesses changes between genotype groups by accounting for initial values in order to correct for any variance between values due to differences at baseline. ANCOVA was used as the primary analysis to evaluate potential differences in serum and red blood cell folate concentrations, plasma homocysteine concentration, and DNA methylation between genotype groups at wk 7 (adjusting for wk 0 values) and at wk 14 (adjusting for wk 7 and wk 0 values). The least squares (LS) means, which are the means adjusted for initial
values by using an ANCOVA model, were used to describe the magnitude of the
differences between each genotype group. As a secondary analysis, ANOVA was
performed on the raw and percent change values from wk 0 to wk 7, wk 7 to wk 14, and
wk 0 to wk 14 for serum and red blood cell folate and plasma homocysteine
concentrations and DNA methylation.

In addition to assessing differences between genotype groups, it also was of interest
to evaluate differences by serum folate status. Two methods for categorizing serum
folate status were employed. Method one involved classifying subjects based on whether
their serum folate values were equal to or above or below the overall median serum folate
values at wk 0, 7, and 14. The second method involved categorizing subjects based on
whether their serum folate values were equal to or above or below 13.6 nmol/L at wk 0,
7, and 14, a concentration that is considered to represent a moderate folate deficiency
(312). For clarity, method one and two will be referred to as "serum folate median
status" and "serum folate depletion status", respectively. A 2x2 contingency table was
constructed and Fischer’s Exact test used to evaluate serum folate depletion status by
subject genotype (CC vs TT); comparisons of other dependent measure means by folate
status and genotype were performed via ANOVA.

Pearson’s correlation analysis was used to evaluate the strength of the relationships
between the dependent variables at each point in time (wk 0, 7, and 14). Specifically, at
each time point the following were correlated: serum folate vs red blood cell folate,
plasma homocysteine, $[^3]$Hmethyl group acceptance, and mCyt/tCyt ratio; red blood cell
folate vs serum folate, plasma homocysteine, $[^3]$Hmethyl group acceptance, and
mCyt/tCyt ratio; plasma homocysteine vs serum and red blood cell folate, $[^3]$Hmethyl
group acceptance, and mCyt/tCyt ratio; $[^3]$Hmethyl group acceptance vs serum and red blood cell folate, plasma homocysteine, and mCyt/tCyt ratio; and mCyt/tCyt ratio vs serum and red blood cell folate, plasma homocysteine, and $[^3]$Hmethyl group acceptance. Correlation coefficients less than 0.35 were considered a low correlation, while correlation coefficients between 0.35 and 0.70, and greater than 0.70 were used to indicate moderate or high correlation, respectively.

A sign test for proportion of trends analysis (316) was used to compare the expected and observed combination of trends for DNA methylation indicators and plasma homocysteine, serum folate, and red blood cell folate concentrations over the depletion and repletion periods. Specifically, regression analysis was used to determine the slope of each individual’s response over the specified time period. The signs of the regression slope values (positive or negative) were tallied and the observed proportion tested against the proportion expected by chance alone (i.e., there are 4 possible trends: ++, +−, −+, and −−, such that by chance alone the proportion of any possible combination is 25% or 0.25). To evaluate the strength of the relationship between each status indicator over the depletion and repletion phases, regression and Pearson correlation techniques were used. Specifically, linear regression was used to determine the slope of each individual’s response over the specified time period. Correlations of combinations of these coefficients (e.g., plasma homocysteine and serum folate concentrations) were determined to assess the magnitude of the relationship.

Exploratory statistical methods employing ANOVA models with main factors for genotype, serum folate status (median status and depletion status separately), as well as the interaction term were used to evaluate potential differences in $[^3]$Hmethyl group
acceptance, mCyt/tCyt ratio, and plasma homocysteine concentration at each time point (wk 0, wk 7, and wk 14). In addition, one-way ANOVA was used as a secondary exploratory analysis to evaluate potential differences in $[^3H]$methyl group acceptance, mCyt/tCyt ratio, and plasma homocysteine concentration by serum folate status (median status and depletion status separately) within each genotype group (CC and TT separately) at each point in time (wk 0, wk 7, and wk 14). For these analyses, alpha was adjusted as alpha/n where n = the number of comparisons. The adjusted alpha was therefore $0.05/12 = 0.004$. For all other comparisons, the alpha level was set apriori to 0.05. All statistics were computed using SAS 8.00 (Cary, NC).
Folate Content of Menus

The folate content of each menu in the 5-d cycle is presented in Table 4-1.

Table 4-1. Folate content of meals with snacks and total daily intake (µg DFE).

<table>
<thead>
<tr>
<th>Day</th>
<th>Breakfast &amp; Snack</th>
<th>Lunch &amp; Snack</th>
<th>Dinner &amp; Snack</th>
<th>Average Daily Total (µg DFE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>40.8 ± 6.8</td>
<td>43.4 ± 5.6</td>
<td>55.0 ± 7.1</td>
<td>139.2</td>
</tr>
<tr>
<td>B</td>
<td>21.7 ± 3.7</td>
<td>32.8 ± 2.6</td>
<td>35.3 ± 4.3</td>
<td>89.8</td>
</tr>
<tr>
<td>C</td>
<td>24.0 ± 3.9</td>
<td>29.9 ± 2.8</td>
<td>40.8 ± 3.1</td>
<td>94.7</td>
</tr>
<tr>
<td>D</td>
<td>19.3 ± 3.2</td>
<td>59.4 ± 6.3</td>
<td>41.6 ± 3.8</td>
<td>120.3</td>
</tr>
<tr>
<td>E</td>
<td>34.6 ± 5.5</td>
<td>42.8 ± 5.6</td>
<td>40.3 ± 5.1</td>
<td>117.7</td>
</tr>
</tbody>
</table>

The 5-d cycle menu provided an average of 115 ± 20 µg DFE/d. All meals and corresponding snacks were prepared, extracted, and measured in duplicate at three different time points during the study to verify that folate content remained constant.

Serum Folate Concentration

Serum folate concentrations (mean ± SD) throughout the study are illustrated in Figure 4-1 for subjects by genotype. The mean (± SD) serum folate concentration (nmol/L) at baseline, post-depletion, and post-repletion and changes in serum folate status are presented in Table 4-2. Although no significant (P = 0.12) difference in serum folate concentration existed at baseline between subjects with the TT genotype compared
Figure 4-1. Weekly unadjusted mean (± SD) serum folate concentrations (nmol/L) by genotype groups throughout the study. *Significant difference between genotype groups at wk 7 (P = 0.03).

to the CC genotype, values for subjects with the TT genotype were lower than those with the CC genotype (41.5 ± 19.7 vs 52.2 ± 22.5 nmol/L, respectively) (Fig. 4-2). Overall serum folate concentration decreased (P < 0.0001) during the depletion phase by 59 ± 15%. Serum folate for subjects with the TT genotype decreased (P < 0.0001) during depletion by 61 ± 16% compared to a 57 ± 15% decrease (P < 0.0001) in subjects with the CC genotype. No significant (P = 0.42) difference in serum folate percent change during depletion was detected between genotypes. The raw change (nmol/L) in serum folate concentration throughout depletion was significant overall (P < 0.0001) and by genotype (P < 0.0001) (Table 4-2). Post-depletion, there was a significantly lower (P = 0.03) serum folate concentration in subjects with the TT compared to the CC genotype (LS mean ± SEM: 19.5 ± 1.2 vs 15.3 ± 1.3 nmol/L, respectively) (Fig. 4-2). Post-
Table 4-2. Serum folate concentration (mean ± SD) at baseline, post-depletion and post-repletion and mean changes in serum folate concentration during depletion and repletion by genotype

<table>
<thead>
<tr>
<th>MTHFR Genotype</th>
<th>Mean serum folate concentration nmol/L ± SD (range)</th>
<th>Percent change % ± SD</th>
<th>Raw change nmol/L ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Post-depletion^2</td>
<td>Post-repletion^3</td>
</tr>
<tr>
<td>CC</td>
<td>52.2 ± 22.4 (25.9 – 94.7) n = 22</td>
<td>20.2 ± 6.3^4 (8.9 – 31.0) n = 20</td>
<td>26.8 ± 4.8 (17.8 – 32.4) n = 10</td>
</tr>
<tr>
<td>TT</td>
<td>41.5 ± 19.7 (12.7 – 88.3) n = 19</td>
<td>14.5 ± 5.9^4 (7.8 – 24.2) n = 17</td>
<td>21.9 ± 6.9 (13.7 – 35.5) n = 10</td>
</tr>
<tr>
<td>Overall Mean</td>
<td>47.2 ± 21.7 (12.7 – 94.8) n = 41</td>
<td>17.6 ± 6.7 (7.8 – 30.9) n = 37</td>
<td>24.4 ± 6.3 (13.7 – 35.5) n = 20</td>
</tr>
</tbody>
</table>

1 All means listed in table are unadjusted means.
2 Four (2 CC; 2 TT) subjects were only on the depletion diet for 5 wk so they were not included in post-depletion calculations.
3 The current study was performed in conjunction with another study whose subjects did not continue through repletion.
4 Means significantly different between genotypes post-depletion: LS Mean CC: 19.5; TT: 15.3; P=0.0304.
5 Significant change during depletion: CC (P<0.0001); TT (P<0.0001), overall (P<0.0001).
6 Significant change during repletion: CC (P<0.01); TT (P<0.006); overall (P<0.0001).
depletion, a greater proportion of subjects with the TT genotype (59%) had low folate status (serum folate < 13.6 nmol/L) compared to the CC genotype (15%) (P = 0.008).

Overall serum folate increased over the repletion phase of the study by 48 ± 45%, representing a significant (P < 0.0001) change from wk 7 to wk 14. Mean serum folate concentration for subjects with the TT genotype increased (P = 0.006) by 57 ± 50% during repletion compared to the significant (P = 0.013) increase in serum folate (38 ± 39%) of subjects with the CC genotype. No difference (P = 0.36) in the percent change in serum folate between genotypes was detected during repletion. The differences detected for raw change paralleled the percent change results, with significant increases overall in serum folate and by genotype (Table 4-2). No significant differences were detected (P = 0.60) in the mean serum folate concentration of subjects with the TT vs CC genotype (21.9 ± 6.9 vs 26.8 ± 4.9 nmol/L, respectively) post-repletion (Table 4-2).
Red Blood Cell Folate Concentration

Weekly red blood cell folate concentrations throughout the study for subjects by genotype are illustrated in Figure 4-3. Mean (± SD) red blood cell folate concentration at baseline, post-depletion, and post-repletion are presented in Table 4-3. Subjects with the TT genotype tended (P = 0.06) to have a lower red blood cell folate concentration at baseline compared to subjects with the CC genotype (1486 ± 564 vs 1806 ± 497 nmol/L, respectively) (Fig. 4-4). Mean red blood cell folate concentration for all subjects decreased (P < 0.0001) by 18 ± 15% during the depletion phase of the study. The red blood cell folate concentration of subjects with the TT and CC genotypes decreased significantly (P = 0.0004 and P < 0.0001, respectively) during depletion by 17 ± 15% vs 19 ± 16%, respectively, but these decreases in percent change were not different between...
Table 4-3. Red blood cell folate concentration (mean ± SD) at baseline, post-depletion and post-repletion and mean changes in red blood cell folate concentration during depletion and repletion by genotype.  

<table>
<thead>
<tr>
<th>MTHFR Genotype</th>
<th>Mean red blood cell folate concentration nmol/L ± SD (range)</th>
<th>Percent change % ± SD</th>
<th>Raw change nmol/L ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Post-depletion$^2$</td>
<td>Post-repletion$^3$</td>
</tr>
<tr>
<td>CC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1806 ± 497 (1021 – 2883)</td>
<td>1456 ± 273 (969 – 1952)</td>
<td>1205 ± 150$^4$ (1028 – 1428)</td>
</tr>
<tr>
<td></td>
<td>n = 22</td>
<td>n = 20</td>
<td>n = 10</td>
</tr>
<tr>
<td>TT</td>
<td>1486 ± 564 (637 – 2505)</td>
<td>1194 ± 392 (749 – 1885)</td>
<td>1033 ± 284$^4$ (589 – 1434)</td>
</tr>
<tr>
<td></td>
<td>n = 19</td>
<td>n = 17</td>
<td>n = 10</td>
</tr>
<tr>
<td>Overall Mean</td>
<td>1658 ± 547 (637 – 2883)</td>
<td>1336 ± 354 (749 – 1952)</td>
<td>1120 ± 238 (589 – 1434)</td>
</tr>
<tr>
<td></td>
<td>n = 41</td>
<td>n = 37</td>
<td>n = 20</td>
</tr>
</tbody>
</table>

$^1$ All means listed in table are unadjusted means.

$^2$ Four (2 CC; 2 TT) subjects were only on the depletion diet for 5 wk so they were not included in post-depletion calculations.

$^3$ The current study was performed in conjunction with another study whose subjects did not continue through repletion.

$^4$ Means significantly different between genotypes post-repletion: LS Mean CC: 1203; TT: 1036; P=0.0395.

$^5$ Significant change during depletion: CC (P<0.0001); TT (P≤0.0004), overall (P<0.0001).

$^6$ Significant change during repletion: TT (P≤ 0.02); overall (P<0.02).
Figure 4-4. Mean red blood cell folate concentration (nmol/L ± SEM) by genotype at baseline (wk 0; unadjusted mean), post-depletion (wk 7; adjusted mean), and post-repletion (wk 14; adjusted mean). *Significant difference between genotype groups at wk 14 (P < 0.05).

The raw change analysis for red blood cell folate concentration during depletion produced analogous results, with significant overall (P < 0.0001) and by genotype (TT: P < 0.0003; CC: P < 0.0001) decreases during depletion (Table 4-3). No significant differences were detected (P = 0.33) in red blood cell folate concentration between genotypes post-depletion (TT: 1194 ± 392; CC: 1456 ± 273 nmol/L, respectively) (Table 4-3).

The overall red blood cell folate concentration continued to decrease (P = 0.02) during the repletion phase by 11 ± 19%. Only the red blood cell folate concentration of subjects with the TT genotype continued to decrease significantly (−17 ± 19%; P = 0.02) during repletion. This is in contrast to the red blood cell folate concentration response of subjects with the CC genotype, which did not decrease significantly (−6 ± 18%; P = 0.36). The percent change in red blood cell folate concentration during repletion was not
different between genotypes ($P = 0.19$). The raw change analysis for red blood cell folate concentration paralleled the percent change analysis, with significant decreases detected for all subjects ($P = 0.02$) and subjects with the TT genotype ($P = 0.02$) during repletion (Table 4-3). Post-repletion, the mean red blood cell folate concentration of subjects with the TT genotype was significantly lower ($P = 0.04$) than that of subjects with the CC genotype (LS mean $\pm$ SEM: $1036 \pm 53$ vs $1203 \pm 53$ nmol/L, respectively) (Fig. 4-4).

**Homocysteine Concentration**

Weekly mean plasma homocysteine concentrations for subjects by genotype are illustrated in Figure 4-5. Mean ($\pm$ SD) plasma homocysteine concentrations at baseline, post-depletion, and post-repletion are presented in Table 4-4. No significant difference was detected ($P = 0.18$) in plasma homocysteine concentration at baseline between subjects with the TT compared to the CC genotype ($7.1 \pm 2.1$ vs $6.3 \pm 1.4$ $\mu$mol/L,
respectively) (Fig. 4-6). The overall plasma homocysteine concentration increased by 52 ± 42% (P < 0.0001) during the depletion phase. Plasma homocysteine concentration increased (P < 0.0001) by 62 ± 52% for subjects with the TT genotype and 43 ± 32% (P < 0.0001) for subjects with the CC genotype during depletion. The plasma homocysteine percent change during depletion was not different between genotypes (P = 0.19). The plasma homocysteine raw change also was significantly increased overall (P < 0.0001) and for both genotype groups (P < 0.0001) during depletion (Table 4-4). Although the plasma homocysteine percent change during depletion was not significantly (P = 0.19) different between genotype groups, there was a trend (P = 0.09) for subjects with the TT genotype to have a greater raw change in homocysteine concentration during depletion than subjects with the CC genotype (3.9 ± 3.0 vs 2.5 ± 1.7, respectively). These increases paralleled significant decreases in serum folate during depletion (Fig. 4-7). Throughout the study, subjects with the TT genotype had numerically higher plasma homocysteine concentrations and lower serum folate concentrations than subjects with the CC genotype (Fig. 4-7). Women with the TT genotype tended (P = 0.09) to have higher mean homocysteine concentration post-depletion than women with the CC genotype (10.5 ± 3.3 vs 8.9 ± 1.9 µmol/L, respectively) post-depletion (Table 4-4). A low but inverse correlation was detected (r = −0.32; P = 0.05) between serum folate and homocysteine concentrations for all subjects post-depletion. In addition, a moderate inverse correlation was detected (r = −0.46; P = 0.004) between the slopes for serum folate and plasma homocysteine concentrations post-depletion.

In response to folate repletion, overall plasma homocysteine concentration decreased (P = 0.02) by 10 ± 18%. The mean homocysteine concentration for subjects
Table 4-4. Homocysteine concentration (mean ± SD) at baseline, post-depletion and post-repletion and mean changes in plasma homocysteine concentration during depletion and repletion by genotype

<table>
<thead>
<tr>
<th>MTHFR Genotype</th>
<th>Mean homocysteine concentration µmol/L ± SD (range)</th>
<th>Percent change % ± SD</th>
<th>Raw change µmol/L ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Post-depletion</td>
<td>Post-repletion</td>
</tr>
<tr>
<td>CC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.3 ± 1.4 (4.2 – 9.4) n = 22</td>
<td>8.9 ± 1.9 (5.8 – 14.1) n = 20</td>
<td>7.2 ± 1.9 (4.4 – 9.9) n = 10</td>
</tr>
<tr>
<td>TT</td>
<td>7.1 ± 2.1 (4.3 – 14.1) n = 19</td>
<td>10.5 ± 3.3 (5.9 – 17.4) n = 17</td>
<td>8.9 ± 1.6 (6.2 – 11.1) n = 10</td>
</tr>
<tr>
<td>Overall Mean</td>
<td>6.6 ± 1.8 (4.2 – 14.1) n = 41</td>
<td>9.6 ± 2.7 (5.8 – 17.4) n = 37</td>
<td>8.1 ± 1.9 (4.4 – 11.1) n = 20</td>
</tr>
</tbody>
</table>

1 All means listed in table are unadjusted means.
2 Four (2 CC; 2 TT) subjects were only on the depletion diet for 5 wk so they were not included in post-depletion calculations.
3 The current study was performed in conjunction with another study whose subjects did not continue through repletion.
4 Significant change during depletion: CC (P<0.0001); TT (P<0.0001), overall (P<0.0001).
5 Significant change during repletion: CC (P<0.03); overall (P<0.02).
with the CC genotype decreased (P = 0.02) during repletion (−15 ± 17%). This decrease in plasma homocysteine concentration paralleled an increase in serum folate concentration during repletion (Fig. 4-7). This is in contrast to the homocysteine concentration in subjects with the TT genotype, which did not change (P = 0.47) during repletion and tended (P = 0.08) to be higher than that of subjects with the CC genotype (8.9 ± 1.6 vs 7.2 ± 1.9 µmol/L, respectively) post-repletion (Table 4-4). Although mean homocysteine concentrations were not significantly different between genotypes post-repletion, homocysteine by genotype did not behave the same way over the course of the study. Specifically, based on raw and percent changes from wk 0 to wk 14, the mean homocysteine concentration returned to baseline levels (P = 0.1327 and 0.1088, respectively) for subjects with the CC genotype but not the TT genotype (P = 0.0016 and
0.0039, respectively), whose levels increased on average 2.3 µmol/L (36%) throughout the study. Although plasma homocysteine concentration for subjects with the TT genotype did not decrease during repletion, an increase in serum folate concentration during repletion was detected (Fig. 4-7).

**DNA Methylation**

DNA [³H]methyl group acceptance and percentage of methylated cytosine (LC-MS/MS method) are presented in Tables 4-5 and 4-6. No significant difference in DNA [³H]methyl group acceptance (P = 0.20) or mCyt/tCyt ratio (P = 0.53) was detected at baseline between genotype groups. A significant (P = 0.03) interaction was detected for mCyt/tCyt ratio and serum folate status at baseline for all subjects below the median. Specifically, subjects with a serum folate concentration below the median (40 nmol/L) for serum folate tended (P = 0.01; adjusted alpha P = 0.0004) to have a lower mCyt/tCyt
Table 4-5. DNA [$^3$H]methyl group acceptance at baseline, post-depletion, and post-repletion for all subjects and by MTHFR genotype

<table>
<thead>
<tr>
<th>Genotype</th>
<th>CC</th>
<th>TT</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[$^3$H]methyl group acceptance (mean dpm X 10^2/0.5 µg DNA ± SD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>470 ± 80 (n=22)</td>
<td>500 ± 70 (n=19)</td>
<td>490 ± 70 (n=41)</td>
</tr>
<tr>
<td>Post-depletion</td>
<td>490 ± 80 (n=22)</td>
<td>520 ± 70 (n=19)</td>
<td>500 ± 80 (n=41)</td>
</tr>
<tr>
<td>Post-repletion</td>
<td>550 ± 150 (n=10)</td>
<td>470 ± 70 (n=10)</td>
<td>510 ± 120 (n=20)</td>
</tr>
</tbody>
</table>

Table 4-6. Percentage (%) of methylated cytosine at baseline post-depletion, and post-repletion for all subjects and by MTHFR genotype

<table>
<thead>
<tr>
<th>Genotype</th>
<th>CC</th>
<th>TT</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of methylated cytosine, mean ± SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>4.7 ± 0.5 (n=22)</td>
<td>4.8 ± 1.0 (n=19)</td>
<td>4.7 ± 0.8 (n=41)</td>
</tr>
<tr>
<td>Post-depletion</td>
<td>4.6 ± 0.7 (n=22)</td>
<td>4.6 ± 0.7 (n=19)</td>
<td>4.6 ± 0.7 (n=41)</td>
</tr>
<tr>
<td>Post-repletion</td>
<td>4.6 ± 0.4 (n=10)</td>
<td>4.5 ± 0.5 (n=10)</td>
<td>4.6 ± 0.5 (n=20)</td>
</tr>
</tbody>
</table>

ratio (i.e., less DNA methylation) than subjects with a serum folate concentration above the median. During depletion, there was a trend (P = 0.08) for all subjects to have an increase in percent change of DNA [$^3$H]methyl group acceptance (Table 4-7), suggestive

Table 4-7. Percent (%) change in DNA [$^3$H]methyl group acceptance during folate depletion and repletion for all subjects and by MTHFR genotype

<table>
<thead>
<tr>
<th>Genotype</th>
<th>CC</th>
<th>TT</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% change, mean ± SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Depletion (wk 0–7)</td>
<td>5 ± 20 (n=22)</td>
<td>6 ± 18 (n=19)</td>
<td>5 ± 19 (n=41)</td>
</tr>
<tr>
<td>Repletion (wk 7–14)</td>
<td>5 ± 32 (n=10)</td>
<td>−8 ± 21 (n=10)</td>
<td>−1 ± 27 (n=20)</td>
</tr>
</tbody>
</table>

* Trend (P=0.08) for significant increase in % change during depletion for all subjects.
of a decrease in global DNA methylation for all subjects. A nonsignificant (P = 0.12) decrease in mean raw change of mCyt/tCyt ratio during depletion as determined by LC-MS/MS during depletion was observed only in subjects with the TT genotype (raw change = 0.003), suggestive of a slight decrease in methylated cytosine (i.e., less DNA methylation) during folate depletion in these subjects (Table 4-8). In addition, a

Table 4-8. Raw change in mCyt/tCyt ratio during folate depletion and repletion for all subjects and by MTHFR genotype

<table>
<thead>
<tr>
<th>Genotype</th>
<th>CC</th>
<th>TT</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depletion (wk 0–7)</td>
<td>−0.0003 ± 0.006 (n=22)</td>
<td>−0.003 ± 0.007 (n=19)</td>
<td>−0.001 ± 0.006 (n=41)</td>
</tr>
<tr>
<td>Repletion (wk 7–14)</td>
<td>0.0002 ± 0.003 (n=10)</td>
<td>0.002 ± 0.002 (^1) (n=10)</td>
<td>0.001 ± 0.002 (n=20)</td>
</tr>
</tbody>
</table>

\(^1\) Significant (P = 0.03) raw change during repletion for subjects with the TT genotype.

nonsignificant (P = 0.17) decrease in percent change was observed for subjects with the TT genotype. During depletion and repletion an inverse relationship between serum folate or red blood cell folate concentration and \(^{3}H\)methyl group acceptance was expected (i.e., it was expected that serum folate concentrations would decrease and \(^{3}H\)methyl group acceptance would increase with folate depletion). Conversely, a direct relationship between plasma homocysteine concentration and \(^{3}H\)methyl group acceptance was expected in response to folate depletion. Based on the sign test for trends analysis, an inverse relationship between serum folate and \(^{3}H\)methyl group acceptance was observed in 61% of all subjects (P < 0.0001), 55% of subjects with the CC genotype (P = 0.0064), and 68% of subjects with the TT genotype (P = 0.0004). An inverse relationship also was observed between red blood cell folate and \(^{3}H\)methyl group
acceptance in 59%, 50%, and 68% of all (P < 0.0001), CC (P = 0.0162), and TT (P = 0.0005) subjects, respectively. A direct relationship was observed during depletion for plasma homocysteine and $[^3\text{H}]$methyl group acceptance in 61% of all subjects (P < 0.0001), 55% of subjects with the CC genotype (P = 0.0064), and 68% of subjects with the TT genotype (P = 0.0005). During depletion and repletion, a direct relationship between serum folate or red blood cell folate and mCyt/tCyt ratio was expected and an inverse relationship between homocysteine and mCyt/tCyt ratio was expected. During depletion a direct relationship between serum folate and mCyt/tCyt ratio was observed in 42% and 50% of all (P = 0.02) and CC (P = 0.0162) subjects, respectively. No significant relationship was observed for subjects with the TT genotype. Only subjects with the CC genotype had a direct relationship between red blood cell folate and mCyt/tCyt ratio (46%; P = 0.0369). There was a trend for all subjects (37%; P = 0.0680) and no significant relationship for subjects with the TT genotype. In addition, an inverse correlation was observed between homocysteine and mCyt/tCyt ratio in 42% and 50% of all (P = 0.02) and CC (P = 0.0162) subjects, respectively. There was no significant relationship for subjects with the TT genotype. No significant difference in $[^3\text{H}]$methyl group acceptance (P = 0.30) or mCyt/tCyt ratio (P = 0.39) between genotypes was detected post-depletion. A moderate inverse relationship was found between red blood cell folate concentration and DNA $[^3\text{H}]$methyl group acceptance for all subjects (r = −0.37; P = 0.02) and for subjects with the CC genotype (r = −0.48; P = 0.03) post-depletion.

In response to repletion, although not significant (P = 0.62) a small positive increase in percent change in $[^3\text{H}]$methyl group acceptance was observed in subjects with
the CC genotype (Fig. 4-8), suggestive of a slight decrease in DNA methylation. This is in contrast to the nonsignificant negative trend in percent change in $[^3]$Hmethyl group acceptance in subjects with the TT genotype ($P = 0.24$) and for all subjects ($P = 0.81$).

Figure 4-8. Percent (%) change in $[^3]$Hmethyl group acceptance for all subjects and by MTHFR genotype during depletion and repletion.

(Fig. 4-8). These nonsignificant trends suggested by DNA $[^3]$Hmethyl group acceptance data were more clearly defined by the significant ($P = 0.04$) increase in raw change (Table 4-8) and percent change (Fig. 4-9) in mCyt/tCyt ratio, which was detected for subjects with the TT genotype during repletion and indicated a positive response to folate repletion in this group. The overall raw change and percent change in mCyt/tCyt ratio tended ($P = 0.07$ and $P = 0.06$, respectively) to increase, but no increase was noted for subjects with the CC genotype ($P = 0.52$) (Table 4-8 and Fig. 4-9).

During repletion, an inverse relationship was observed between serum folate and $[^3]$Hmethyl group acceptance in 60% of all ($P = 0.0029$), CC ($P = 0.0303$), and TT ($P = 0.0303$) subjects ($P < 0.03$). A direct relationship also was observed between
homocysteine and $[^3]H$ methyl group acceptance in 50% of all subjects ($P = 0.02$). A trend towards a significant direct relationship was observed for 50% of CC and TT subjects ($P = 0.08$). No significant difference in $[^3]H$ methyl group acceptance ($P = 0.17$) or mCyt/tCyt ratio ($P = 0.56$) between genotypes was detected post-repletion.
CHAPTER 5
DISCUSSION AND CONCLUSIONS

The first objective of this study was to use a low-folate diet as a tool to evaluate the response to suboptimal folate intake (115 µg DFE/d) in women of reproductive age by assessing indicators of folate status and global DNA methylation between women with the CC genotype and the TT genotype for the MTHFR 677 C→T polymorphism. The second objective was to evaluate differences in folate status and DNA methylation indicators between the CC and TT genotype groups in response to repletion with the current Recommended Dietary Allowance (RDA) for folate (400 µg DFE/d). There is a large body of observational data (82,86) indicating that individuals with the TT genotype have lower blood folate and higher plasma homocysteine concentrations (65,67,82) than individuals with the CC genotype. Interpretation of data from these epidemiological cross-sectional studies is complicated by the fact that confounding dietary and environmental variables that may affect folate status and plasma homocysteine concentration are not controlled (e.g., folate intake, caffeine, alcohol and tobacco use). The strength of the current metabolic study is that a controlled folate intake was used to assess folate status and DNA methylation response to moderate folate depletion and repletion with the current RDA in young women by MTHFR genotype while controlling for other dietary and environmental factors.

Women with the TT genotype responded more negatively to folate depletion and less positively to folate repletion than women with the CC genotype. This was demonstrated by the significantly lower serum folate concentration and the trend for a
higher plasma homocysteine concentration post-depletion. During repletion, subjects with the TT genotype did not have a significant decrease in plasma homocysteine concentration, resulting in a trend for a higher plasma homocysteine concentration compared to the CC genotype post-repletion. In addition, women with the TT genotype had a significantly lower red blood cell folate concentration post-repletion. During repletion, women with the TT genotype also had a significant increase in global DNA methylation based on percent change in mCyt/tCyt ratio.

Although there are many observational studies available that provide data on comparisons between the TT and CC genotypes for the MTHFR 677C→T polymorphism, this discussion focuses on a very recently published metabolic study (87) in young women and a previously published metabolic study (307) in elderly women with research designs and protocols similar to the present study.

A similar controlled folate depletion-repletion feeding study in young women was recently published by Guinotte et al. (87), but an important difference in the current study is the ethnicity of the population group. Guinotte et al. (87) restricted their population to Hispanic women who were all of Mexican American origin, whereas the current study consisted of 90% non-Hispanic white, 7% non-Hispanic Black, and 3% Hispanic (Puerto Rican) women. The Mexican American women in the study by Guinotte et al. (87) had a lower overall baseline mean serum folate concentration (30.8 nmol/L) and a lower overall baseline mean plasma homocysteine concentration (5.5 µmol/L) than that observed in the women in the current study (47.2 nmol/L and 6.6 µmol/L, respectively). These data agree with previously published data from the third National Health and Nutrition Examination Survey, which indicated that serum and red blood cell folate and plasma
homocysteine concentrations were significantly lower in a Mexican American population compared to non-Hispanic whites and non-Hispanic blacks (281). Serum and red blood cell folate concentrations were still lower in Mexican American women than non-Hispanic white women after controlling for dietary intake assessed by food frequency questionnaires. Additionally, Caudill et al. (317) reported lower serum folate concentrations in a combined group of socioeconomically advantaged (SEA) and disadvantaged (LSES) Hispanic women compared to a combined group of Caucasian women. They also reported a lower plasma homocysteine concentration in the LSES Hispanic women compared to LSES Caucasian women. Although the ethnicity of the Hispanic women was not reported, it is likely that these women were predominantly Mexican American based on other reports published by that group (87,303). The influence of ethnicity on folate status at baseline in the women of the current study and that of Guinotte et al. (87) may explain the differences in results between the two studies, which are discussed below.

The magnitude of the effect of the low-folate diet on serum folate as evidenced by the 59% reduction in values is similar to that observed previously by our research group (307) in elderly women following a similar 7 wk folate depletion protocol and that observed by Guinotte et al. (87) in the young Mexican American women (65% and 58%, respectively). After consuming the low-folate diet, approximately one-third of the young women in the current study had a serum folate concentration between 7 and 13.6 nmol/L, suggestive of moderate folate depletion, and none of these subjects was severely deficient (< 7 nmol/L) (312). In contrast, 21% of the elderly women in our previous study were severely deficient by wk 7 (< 7 nmol/L) despite having comparable baseline values and
consuming comparable low folate diets to that of the young women in the current study (46 vs 47 nmol/L, respectively, and 118 vs 115 µg DFE/d, respectively) (307). The mean serum folate concentration of the young women at wk 7 was 17.6 nmol/L versus 11.3 nmol/L in the elderly women, suggestive of a possible influence of age on the decline of folate status in response to an inadequate intake (307). A negative effect of age on folate status has been observed in rat studies. Choi et al. (318) reported significantly lower plasma folate concentrations in aged rats compared to young rats fed a folate deficient diet for 8 or 20 wk. The overall post-depletion serum folate concentration in the present study differs from that reported in Mexican American women post-depletion (12.9 nmol/L) (87), which are comparable to the values of the elderly women in our previous study (307). During the 7 wk depletion phase, the young women in the present study decreased 29.6 nmol/L or 4.2 nmol/L/wk. The women in the Guinotte et al. (87) study decreased 19.8 nmol/L or 2.6 nmol/L/wk. The mechanism behind this difference in depletion is not yet understood. These data are consistent with observational data that indicate lower mean blood folate values in Mexican American compared to non-Hispanic women (274,281).

Genotypic differences were evident in the present study. The young women with the TT genotype for the MTHFR 677C→T polymorphism responded more negatively to the folate deficient diet compared to those with the CC genotype based on the significantly lower serum folate concentration post-depletion. In addition, more women in the current study with the TT genotype had low serum folate concentrations (< 13.6 nmol/L) post-depletion than subjects with the CC genotype (59% vs 15%, respectively). Ethnic differences by genotype also were evident between the populations of the Guinotte
et al. (87) study and the women of the current study. Serum folate concentrations were
~10 and ~20 nmol/L lower in Mexican women with the TT and CC genotypes,
respectively, at baseline compared to the predominantly non-Hispanic women in the
current study (87). In addition, Guinotte et al. (87) observed that Mexican American
women with the TT genotype had significantly lower serum folate concentrations than
their subjects with the CC genotype throughout depletion. Increased age coupled with
the TT genotype may have a compounding negative effect on folate status. The elderly
women in our previous study with the TT genotype had a significantly (P = 0.04) greater
decrease in serum folate concentration during depletion (−72 ± 21.6%) compared to the
decrease in a subset (n = 22) of the young women in the current study with either
genotype (CC = −64.6 ± 10.8%; TT = −69.9 ±12.1%).

After repletion with the RDA for folate (400 µg DFE/d) for 7 wk, overall mean
serum folate increased significantly (P < 0.0001) by 48%. In contrast, Guinotte et al. (87)
observed a much smaller yet significant overall increase in serum folate (23%) in
response to repletion with 400 µg DFE/d in Mexican American women (87). The
Mexican American women in the Guinotte et al. (87) study appeared to be less
responsive than the women in the present study to folate repletion with 400 µg DFE/d
based on changes in serum folate concentration. During the 7 wk repletion phase, serum
folate concentration increased 6.8 nmol/L or ~1 nmol/L/wk in the present study
compared to only 3.2 nmol/L or 0.45 nmol/L/wk in the Guinotte et al. (87). This lower
rate of repletion in the Guinotte et al. (87) study is consistent with the lower rate of
depletion described earlier. It is likely that ethnicity-based differences, including genetic
differences, exist between these groups. In the current study, the serum folate
concentration of subjects with the CC genotype increased 38% in contrast to a 57% increase in subjects with the TT genotype. Although all subjects had normal serum folate concentrations (> 13.6 nmol/L) at wk 14, two subjects with the TT genotype had borderline normal values (15.6 and 13.7 nmol/L), suggesting a more impaired response to folate depletion in subjects with the TT genotype compared to subjects with the CC genotype. This is in contrast to the study by Guinotte et al. (87) in which 50% of the Mexican American women with the TT genotype and 14% of those with the CC genotype were still moderately folate deficient (7 to 13.6 nmol/L) after repletion with the current RDA for folate. It is likely that the lower mean serum folate concentration reported by Guinotte et al. (87) post-depletion explains the greater number of Mexican American women still moderately folate deficient post-repletion compared to the women in the present study.

During repletion with the current RDA for folate, red blood cell folate concentrations continued to decrease for all women, culminating with a significantly lower mean red blood cell folate concentration in women with the TT genotype compared to the CC genotype. Due to the long half-life of red blood cells and the fact that folate is only taken up by the developing reticulocyte, it takes longer to detect changes in red blood cell folate concentration as a result of folate depletion or repletion. Only a study with a longer repletion phase would determine how long it would take to restore normal red blood cell folate concentration.

Overall baseline homocysteine concentration was approximately 3.4 µmol/L lower in the women in the current study compared to that observed in the elderly women studied previously (307). It is a well-established fact that homocysteine concentration
increases with age (27). Herrmann et al. (319) found significantly higher plasma homocysteine concentrations in elderly individuals aged 65 to 75 compared to a younger control group (19 to 60 y). They attributed these higher concentrations to an age-related decline in cystathionine-β-synthase or a decrease in intracellular vitamin B12. In addition, a reduced vitamin intake (320), changes in renal function (321) and impaired renal homocysteine metabolism (322) also may account for age-related decreases in homocysteine concentration.

The negative influence of the MTHFR 677 TT genotype on plasma homocysteine concentration was evident in the trend for women with the TT genotype to have higher mean homocysteine concentration than the women with the CC genotype post-depletion. It also is reflected in the trend for subjects with the TT genotype to have a greater raw change in plasma homocysteine concentration in response to folate depletion than subjects with the CC genotype. The fact that the mean homocysteine concentration was not significantly different between genotype groups post-depletion differs from our previous study with elderly women in which individuals with the TT genotype had a significantly (P = 0.001) greater mean homocysteine concentration post-depletion than women with the CC genotype (LS Mean 13.7 ± 0.7 vs 10.4 ± 0.5 µmol/L, respectively) (84). It is likely that age and the TT genotype may also be having a negative synergistic effect on homocysteine status during depletion. Results from the study in elderly women were compared to the results from the present study. At baseline, elderly women with the TT genotype had a plasma homocysteine concentration that was 1.5 µmol/L higher than elderly women with the CC genotype (10.0 vs 8.5 µmol/L, respectively). Elderly women with the TT genotype had a 2.9 µmol/L higher homocysteine concentration post-
depletion than elderly women with the CC genotype. This is almost twice the difference in homocysteine concentration noted for the young women with the TT compared to the CC genotype (1.6 µmol/L) post-depletion.

Baseline homocysteine concentrations in women with the TT and CC genotype in the present study are higher than values reported by Guinotte et al. (87) in Mexican American women with the TT and CC genotype (7.1 vs 5.4 and 6.3 vs 5.3 µmol/L, respectively). Although the mean plasma homocysteine concentration increased significantly in both populations during depletion, the Mexican American women had an overall mean plasma homocysteine concentration of 7.2 µmol/L post-depletion compared to 9.6 µmol/L in the women of the present study. Thus, the Mexican American women in the Guinotte et al. (87) study had a 1.7 µmol/L increase in homocysteine concentration compared to 3 µmol/L increase in the women in the present study during depletion. Guinotte et al. (87) attributed this low post-depletion value to the low initial overall mean homocysteine concentration of 5.5 µmol/L. Their low baseline values are consistent with pre-fortification reports that Mexican American women have lower plasma homocysteine concentrations than non-Hispanic white and non-Hispanic black women (281). There are currently no plasma homocysteine concentration data in these women post-fortification. Lifestyle factors or genetic differences in Mexican American women may be associated with the lower homocysteine concentration in spite of the lower blood folate concentrations observed in these women compared to predominantly non-Hispanic women.

Another key difference between the two studies is that in the study by Guinotte et al. (87) choline was supplemented to provide the Adequate Intake (AI) of 425 mg/d
(306), unlike the current study in which the dietary choline content was 285 mg/d (67% of AI). Choline is a methyl-rich compound that can be oxidized to betaine in order to supply methyl groups to remethylate homocysteine to methionine (323). This reaction takes place via the betaine:homocysteine methyltransferase enzyme, which is only found in the liver and kidneys (306). Choline supplementation in the Guinotte et al. (87) study may have contributed to the smaller increase in plasma homocysteine concentration reported during depletion in their women compared to that observed in the present study.

Rat studies have demonstrated that choline is utilized to remethylate homocysteine when folate is not available in the diet (232,324). After performing an experimental choline depletion-repletion study in healthy adult men, Zeisel et al. (325) concluded that choline is essential for humans when methionine and folate are limited in the diet. Steenge et al. (326) supplemented healthy men and women with 6 g of betaine, the oxidized form of choline, daily for 6 wk and observed a significant decrease in plasma homocysteine concentration. In addition, Schwahn et al. (327) supplemented mice with all three genotypes for the MTHFR 677C→T polymorphism (CC, CT, TT) with betaine (25 mmol/kg) and detected significant decreases in homocysteine concentration for all three genotype groups. They also reported results from a human observational study in which a weak but highly significant inverse correlation ($r = -0.2543; P = 0.0049$) between plasma homocysteine concentration and plasma betaine concentration was observed (327). The results of the aforementioned studies support the conclusion that choline supplementation may have affected the plasma homocysteine response in the Guinotte et al. (87) study.
Another explanation is that the lower initial homocysteine values contributed to the smaller overall increase in homocysteine. It is well established that pretreatment homocysteine concentrations affect the extent to which folic acid supplementation decreases homocysteine concentrations. The Homocysteine Lowering Trialists’ Collaboration (328) reported that individuals with higher pretreatment homocysteine or lower pretreatment folate concentrations benefited the most from folic acid supplementation. Based on these data, it is likely that baseline homocysteine concentration affects homocysteine response in either direction.

Jacob et al. (323) retrospectively determined the choline status of men and women participating in previously published folate depletion studies (237,286). The men participated in a 108 d study that included three folate intake periods: a 9-d baseline period consisting of 440 µg/d followed by two low folate periods in which subjects consumed 25 µ/d for 30 d and 99 µg/d for 15 d. The three periods were then repeated. The choline content (238 mg) of this low folate diet was comparable to that in the present study (285 mg/d). Jacob et al. (323) observed that the men consuming the low folate, low choline diet for the first trial (48 d), a comparable period of time to the depletion phase in the current study, did not have a decrease in plasma choline concentrations. They concluded that hepatic choline stores must have been adequate to maintain choline status during the study. When the trial was repeated, plasma choline concentrations decreased significantly. Based on the data from this study in men, they concluded that more than 250 mg of choline/d is required to maintain adequate choline status when folate intake is low. Although the choline content of the diet consumed during the 49 d folate depletion phase of the current study was slightly lower (67%) than the current AI for choline, the
choline status indicators in our subjects are not likely to have been affected based on the
data from the study by Jacob et al. (323). The women also participated in a feeding study
consisting of a low folate diet of 56 µg/d for 35 d and 111 µg/d for another 30 d. The
choline content of this diet was approximately half of the content in the diet consumed by
the men (147 mg/d). Plasma choline concentrations decreased significantly after 35 d of
the lower folate intake. Weekly plasma choline concentrations would need to be
analyzed in order to definitively conclude that choline status remained adequate in the
present study. It is likely that all diets low in folate, including those consumed
chronically in countries that do not practice fortification, also may be low in choline,
which may compound the negative effect on homocysteine concentrations.

There also was a significant difference in homocysteine response to folate repletion
between genotypes in the current study. Repletion with the current RDA was sufficient
to significantly decrease mean plasma homocysteine concentrations for women in the
current study with the CC genotype. In contrast, there was no significant change in mean
plasma homocysteine concentration during repletion for women with the TT genotype.
This differs from the response in the Guinotte et al. (87) study in which 400 µg DFE/d
was sufficient to significantly decrease plasma homocysteine concentrations in both
genotype groups (87).

Jacques et al. (7) reported that plasma homocysteine concentrations were elevated
only in individuals with the TT genotype who had plasma folate concentrations below the
median for the group. In the present study, 59% and 15% of subjects with the TT and CC
genotypes, respectively, had low folate status (serum folate < 13.6 nmol/L) post-
depletion. The mean plasma homocysteine concentrations of these women with low
folate status were 11.1 µmol/L and 8.2 µmol/L in subjects with the TT and CC genotypes, respectively. This difference in homocysteine concentration (~3 µmol/L), although not significantly different in this relatively small group of subjects, may be physiologically significant. This point is illustrated by a prospective study of plasma homocysteine concentration and risk for myocardial infarction in US physicians (329). These researchers reported a 3-fold increase in risk in physicians in the highest 5% (> 15.8 µmol/L) vs the lowest 90% (< 14.1 µmol/L) of plasma homocysteine, which is a difference of 1.7 µmol/L between the two groups. The 3 µmol/L difference between genotype groups in the present study is within a normal range that is not associated with vascular disease (< 14 µmol/L) (27). An increase of 3 µmol/L in an individual with a baseline homocysteine concentration of 12 µmol/L would increase their homocysteine concentration into a range that is associated with cardiovascular disease. This is especially important in European countries where homocysteine concentrations tend to be higher especially in individuals with the TT genotype (82) and an increased risk for cardiovascular disease would be likely. In addition, Boushey et al. (330) conducted a meta-analysis of observational studies investigating the relationship between homocysteine concentrations and vascular disease risk. They reported that throughout a range of homocysteine concentrations from 10 to 15 µmol/L, a decrease in homocysteine concentrations by ~1 µmol/L was associated with a ~10% reduction in risk. Therefore, a difference of 3 µmol/L between genotype groups in the current study may confer a difference in risk for vascular disease.

Differences in the means for indicators of global DNA methylation were not detected at any time point during this study in women with either genotype. The first
method used to assess global DNA methylation was the $[^3]H$ methyl group acceptance assay. There was a trend for all subjects to have an increase in the percent change in $[^3]H$ methyl group acceptance during depletion, which is suggestive of a decrease in global DNA methylation. In our previous study of elderly women (238), a significant increase in labeled methyl group acceptance was observed in response to consumption of a comparable low folate diet. The young women in the current study had been consuming fortified foods for approximately 5 y and had very high mean folate concentration at baseline. The folate depletion protocol used in this study may not have been of sufficient duration to reduce their folate status to detect a change in DNA methylation. In contrast, 21% of elderly women studied previously (307) were severely folate deficient post-depletion despite having baseline values comparable to the young women. Another explanation could be that DNA methylation in young women may not be affected as negatively by folate depletion as DNA methylation in elderly women, suggesting an effect of age on DNA methylation response. Richardson (331) reviewed studies that support the theory that DNA methylation decreases with age. Some mechanisms that may contribute to this decrease are endogenous changes such as altered expression of DNA methyltransferases, and exogenous changes such as dietary factors, drugs, and UV light (332). Stern et al. (12) reported significantly higher labeled methyl group incorporation in individuals with the TT compared to the CC genotype in an observational study that included older individuals (25 to 75 y). The mean ages for the TT and CC genotype groups were 52 and 49 y, respectively. Also, the mean plasma folate values of these individuals (CC: 23.8 nmol/L vs TT: 21.3 nmol/L) were very similar to the post-depletion values in the present study. Perhaps increased age and the
chronically low folate values in these subjects may have affected the DNA methylation of these individuals. Although both genotype groups experienced an increase in the mean percent change of [$^3$H]methyl group acceptance during depletion in the present study, only subjects with the CC genotype had an increase in [$^3$H]methyl group acceptance during repletion. This is in contrast to the subjects with the TT genotype who had a significant decrease in percent change in [$^3$H]methyl group acceptance during repletion.

Another method available to indirectly assess global DNA methylation is the cytosine-extension assay developed by Pogribny et al. (295). This method is based on the selective use of methylation-sensitive restriction enzymes that leave a 5’ guanine overhang after DNA cleavage followed by single nucleotide primer extension with [$^3$H]dCTP (295). It has advantages over the methyl acceptance assay in that it has the ability to simultaneously detect both global and CpG island methylation in one assay; requires less DNA; and is independent of DNA integrity (295). This analytical method was evaluated in the present study to assess global DNA methylation but was not used on study samples due to the large intra-and interassay variations.

In addition to the indirect methods available to determine DNA methylation (e.g., [$^3$H]methyl group acceptance assay and cytosine-extension assay), newer methods have been developed to measure methylcytosine and cytosine directly using liquid chromatography and mass spectrometry (LC/MS) (13). Friso et al. (13) first developed an LC/MS method to directly measure methylcytosine in subjects with the TT and CC genotypes for the MTHFR 677C→T polymorphism in an observational study of an Italian population. Plasma folate and quantities of methylcytosine were significantly lower in subjects with the TT genotype compared to the CC genotype. An important
limitation to this study is that it was observational data and folate intake was not controlled. Overall folate concentrations (serum and red blood cell folate) were much lower than that reported in the present study, which likely reflects the consumption of unfortified foods by the Italian population.

A different LC/MS analytical procedure was developed by our research group for determination of methylcytosine (mCyt) and cytosine (Cyt). Our method differed from the method used by Friso et al. (13) in that we used MS/MS detection in order to detect the bases. Another difference between the analytical procedures was the fact that our method did not involve the use of internal standards. In contrast, external standards were used to quantify the mCyt and Cyt and calculate the ratio of mCyt/total Cyt (tCyt).

Although a significant difference in mCyt/tCyt ratios between genotype groups was not detected at any time point in the present study of young women, there was a trend for subjects with serum folate concentrations below the baseline median (40 nmol/L) to have a lower mCyt/tCyt ratio than subjects with serum folate concentrations above the median. The inability to detect differences between genotype groups may have been a result of the relatively small sample size and high folate intake of our population compared to the Italian population (n = 292) with chronically low folate intakes evaluated in the observational study reported by Friso et al. (13).

In response to folate repletion, a significant increase in the percent change in mCyt/tCyt ratio in subjects with the TT genotype was detected. This is consistent with the results by Friso et al. (13) who found significantly more methylcytosine in subjects with the TT genotype with plasma folate concentrations in the highest tertile compared to the lowest tertile. This indicates that as folate status is improved in these individuals,
DNA methylation is restored. A change in mCyt/tCyt ratio during repletion in subjects with the CC genotype was not detected, which is also consistent with data by Friso et al. (13) who did not find a significant difference in DNA methylation among tertiles of plasma folate in subjects with the CC genotype. Based on all of the global DNA methylation indicators, subjects with the TT genotype seemed to have been more negatively affected by folate depletion and more positively affected by folate repletion.

A large percentage of women of reproductive age in countries other than the US and Canada consume unfortified diets that provide a mean folate intake of ~200 µg/d (82,333,334) which has been demonstrated to be inadequate to maintain normal folate status (287). Chronic consumption of these low folate diets by women of reproductive age with the TT genotype for the MTHFR 677C→T polymorphism may increase their risk of impaired pregnancy outcome should pregnancy occur (304). Since initiation of the folic acid fortification program in the United States and Canada, typical folic acid intakes have increased by ~200 µg/d (275,278), which probably accounts for the overall higher mean serum folate concentration at baseline (47 nmol/L) in the present study. The pre-depletion serum folate and plasma homocysteine concentrations of subjects in this study agree with other reports of folate status of young women in the US during the post-fortification period (303,317). In addition, 41% of the women in this study (9 TT, 8 CC) were taking a folic acid containing supplement prior to the start of the study. This may have contributed to the higher overall folate values at baseline. These high values differ with the folate status in countries that do not practice folic acid fortification, where blood folate concentrations are much lower than those in the United States. For example, in a recent population-based study in the Netherlands (82), the mean serum folate and plasma
homocysteine concentrations were 7.5 nmol/L and 13.6 µmol/L, respectively, compared to the overall means of 47 nmol/L and 6.6 µmol/L observed in the present study. In addition, subjects with the TT genotype for the MTHFR 677C→T polymorphism in the Netherlands study had a mean plasma homocysteine concentration of 17.1 µmol/L (82) compared to 7.1 µmol/L in the present study. Individuals with chronically elevated homocysteine concentrations may be at an increased risk for cardiovascular disease (335), NTDs (138), or pregnancy complications (139).

In conclusion, subjects with the TT genotype responded more negatively to folate depletion (115 µg DFE/d) than subjects with the CC genotype as indicated by the lower serum folate concentration post depletion and the trend for an increased plasma homocysteine concentration post-depletion. In addition, subjects with the TT genotype responded less positively to repletion with the current RDA for folate (400 µg DFE/d) than subjects with the CC genotype as evidenced by the significantly lower red blood cell folate concentration post-repletion and the lack of a significant decrease in plasma homocysteine concentration post-repletion. Plasma homocysteine concentration also tended to be higher in subjects with the TT genotype post-repletion. Therefore, subjects with the TT genotype may require more daily folate to maintain status than subjects with the CC genotype. In addition, the data suggest that changes in global DNA methylation of subjects with the TT genotype responded more favorably to repletion than subjects with the CC genotype.
A 14 wk metabolic study was conducted to determine differences in the folate status and DNA methylation response of women of childbearing age with the CC and TT genotypes for the MTHFR 677C→T polymorphism to folate depletion (115 µg DFE/d) and repletion with the current RDA for folate (400 µg DFE/d). Serum and red blood cell folate and plasma homocysteine concentration, [³H]methyl group incorporation, and mCyt/tCyt ratio were evaluated in response to folate depletion and repletion. The findings of the current study indicate that women of reproductive age with the TT genotype for the MTHFR 677C→T polymorphism who consume low-folate diets are at greater risk for impaired folate status than women with the CC genotype. Individuals with the TT genotype for the MTHFR 677C→T polymorphism who are folate deficient are more likely to develop an increased homocysteine concentration, a risk factor for NTDs and pregnancy complications, than individuals with the CC genotype (7,139). These data suggest that women of childbearing age with the TT genotype may require higher folate intakes to maintain normal folate status and prevent an elevation in plasma homocysteine concentration than women with the CC genotype. These data also suggest that DNA methylation of women with the TT genotype may respond more positively to increased folate intake compared to women with the CC genotype. The results of this study provide important new data that can be considered in future revisions of the folate DRI in which genotype differences are considered.
## APPENDIX A
### VITAMIN-MINERAL SUPPLEMENT COMPOSITION

Table A-1. Vitamin/mineral RDA, five-day diet average, and supplement calculations.

<table>
<thead>
<tr>
<th>Vitamin/mineral</th>
<th>RDA for young women</th>
<th>5 d average from diet</th>
<th>Amount in supplement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin A (RE)</td>
<td>800</td>
<td>799</td>
<td>0.8</td>
</tr>
<tr>
<td>Vitamin D (µg)</td>
<td>5</td>
<td>5.7</td>
<td>0</td>
</tr>
<tr>
<td>Vitamin E (µg)</td>
<td>15</td>
<td>12.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Vitamin K (µg)</td>
<td>65</td>
<td>64.8</td>
<td>0.2</td>
</tr>
<tr>
<td>Vitamin C (mg)</td>
<td>75</td>
<td>80.5</td>
<td>0</td>
</tr>
<tr>
<td>Thiamin (mg)</td>
<td>1.1</td>
<td>0.8</td>
<td>0.3</td>
</tr>
<tr>
<td>Riboflavin (mg)</td>
<td>1.1</td>
<td>1.2</td>
<td>0</td>
</tr>
<tr>
<td>Niacin (mg)</td>
<td>14</td>
<td>15.2</td>
<td>0</td>
</tr>
<tr>
<td>Pantothenic Acid (mg)</td>
<td>5</td>
<td>3.4</td>
<td>1.6</td>
</tr>
<tr>
<td>Vitamin B₆ (mg)</td>
<td>1.3</td>
<td>1.3</td>
<td>0.04</td>
</tr>
<tr>
<td>Folate (µg DFE)</td>
<td>400</td>
<td>115</td>
<td>0</td>
</tr>
<tr>
<td>Vitamin B₁₂ (µg)</td>
<td>2.4</td>
<td>2.4</td>
<td>0</td>
</tr>
<tr>
<td>Calcium (mg)</td>
<td>1000</td>
<td>830</td>
<td>170</td>
</tr>
<tr>
<td>Phosphorus (mg)</td>
<td>700</td>
<td>1138</td>
<td>0</td>
</tr>
<tr>
<td>Magnesium (mg)</td>
<td>310</td>
<td>232</td>
<td>77.8</td>
</tr>
<tr>
<td>Iron (mg)</td>
<td>15</td>
<td>9.1</td>
<td>5.9</td>
</tr>
<tr>
<td>Zinc (mg)</td>
<td>12</td>
<td>7.5</td>
<td>4.5</td>
</tr>
<tr>
<td>Copper (µg)</td>
<td>3</td>
<td>1.1</td>
<td>1.9</td>
</tr>
<tr>
<td>Selenium (µg)</td>
<td>55</td>
<td>84.9</td>
<td>0</td>
</tr>
<tr>
<td>Sodium (mg)</td>
<td>2400</td>
<td>3064</td>
<td>0</td>
</tr>
<tr>
<td>Potassium (mg)</td>
<td>2000</td>
<td>1810</td>
<td>190</td>
</tr>
</tbody>
</table>

RE = retinol equivalents; µg = micrograms; mg = milligrams
# APPENDIX B
## CHOLINE CONTENT OF DIET

Table B-1. First analysis of dietary choline content.

<table>
<thead>
<tr>
<th>Day</th>
<th>Breakfast &amp; Snack</th>
<th>Lunch &amp; Snack</th>
<th>Dinner &amp; Snack</th>
<th>Average Daily Total (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>62</td>
<td>111</td>
<td>187</td>
<td>359</td>
</tr>
<tr>
<td>B</td>
<td>35</td>
<td>84</td>
<td>111</td>
<td>229</td>
</tr>
<tr>
<td>C</td>
<td>39</td>
<td>106</td>
<td>135</td>
<td>280</td>
</tr>
<tr>
<td>D</td>
<td>34</td>
<td>99</td>
<td>103</td>
<td>235</td>
</tr>
<tr>
<td>E</td>
<td>58</td>
<td>60</td>
<td>209</td>
<td>327</td>
</tr>
</tbody>
</table>

Daily Average: 286 ± 57 mg

Table B-2. Second analysis of dietary choline content.

<table>
<thead>
<tr>
<th>Day</th>
<th>Breakfast &amp; Snack</th>
<th>Lunch &amp; Snack</th>
<th>Dinner &amp; Snack</th>
<th>Average Daily Total (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>66</td>
<td>103</td>
<td>150</td>
<td>320</td>
</tr>
<tr>
<td>B</td>
<td>82</td>
<td>86</td>
<td>98</td>
<td>266</td>
</tr>
<tr>
<td>C</td>
<td>43</td>
<td>77</td>
<td>105</td>
<td>225</td>
</tr>
<tr>
<td>D</td>
<td>66</td>
<td>107</td>
<td>121</td>
<td>294</td>
</tr>
<tr>
<td>E</td>
<td>75</td>
<td>74</td>
<td>162</td>
<td>310</td>
</tr>
</tbody>
</table>

Daily Average: 283 ± 38 mg
Figure C-1. Sample gel with amplified DNA fragments.

Amplified DNA fragments were separated on an agarose gel and visualized by the use of ethidium bromide. Individuals with the MTHFR 677C→T CC genotype have DNA fragments that are 198 base pairs long. This forms a single band as seen in Lane 2. Individuals with the MTHFR 677C→T TT genotype (Lane 3) have DNA fragments that are 175 and 23 base pairs long (the 23 base pair band is not shown above). When the gene contains both C and T alleles, as seen in individuals heterozygous for the MTHFR 677C→T polymorphism (Lane 4), three bands appear: one 198 base pairs long, one 175 base pairs long, and one 23 base pairs long (the 23 base pair band is not shown above).
LIST OF REFERENCES


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BIOGRAPHICAL SKETCH

Karla Pagán Shelnutt was born in Rio Piedras, Puerto Rico on August 6, 1975. She grew up in Coral Springs, Florida where she graduated from Marjory Stoneman Douglas High School in 1993. She graduated with high honors from the University of Florida in 1997 with a Bachelor of Science degree in Food Science and Human Nutrition (with a specialization in dietetics). In June of 1998, she completed her dietetic internship at University of Alabama at Birmingham and passed her dietetic registration examination later that year. In April 2000, she was awarded a Master of Science degree in Clinical Nutrition also from the University of Alabama at Birmingham. After working as a clinical dietitian for four months at West Boca Medical Center in Boca Raton, Florida, she entered the doctoral program in the Food Science and Human Nutrition Department at the University of Florida in August of 2000. She graduated with a doctoral degree in August 2003 and plans to work as a Postdoctoral Research Associate with Dr. Gail Kauwell at the University of Florida.