ACKNOWLEDGEMENTS

I would like to sincerely thank my supervisory committee members Lynn B. Bailey, PhD, Gail P. A. Kauwell, PhD, RD, LDN, and Linda Young, PhD for the unique contributions that each of them made to my research project. In particular, I would like to thank my committee co-chairs, Drs. Lynn Bailey and Gail Kauwell, for guiding and encouraging me through this challenging experience. They are both remarkable mentors and role models and have been an inspiration from the very beginning. Without their expertise and support, I could not have accomplished this endeavor. I have learned more in the past few years than I ever could have anticipated, and I know that the knowledge and wisdom I have gained will take me great places in the future.

I also would like to thank Dave Maneval for his assistance in the laboratory, James Colee for his assistance with the statistical analyses of my data, and Karla Shelnutt, PhD, RD, Kristina von-Castel-Roberts, PhD, and Amanda Wittmann, MS, RD, LDN for being great listeners. Each of them played an important role in guiding me through this long journey.

My family and friends have been an amazing support system to me and have been a constant source of encouragement and sage advice for which I am very grateful. Finally, I would like to thank Dominick Savoca. He reminded me everyday how proud he was of my hard work and accomplishments. His love and encouragement gave me the strength to complete this program.
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<tr>
<td>$^{125}$I</td>
<td>iodine-125</td>
</tr>
<tr>
<td>1-C</td>
<td>one-carbon</td>
</tr>
<tr>
<td>AI</td>
<td>Adequate Intake</td>
</tr>
<tr>
<td>ASCII</td>
<td>American Standard Code for Information Interchange</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>C</td>
<td>cytosine</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celcius</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>CH2</td>
<td>methylene</td>
</tr>
<tr>
<td>CH3</td>
<td>methyl</td>
</tr>
<tr>
<td>CHD</td>
<td>coronary heart disease</td>
</tr>
<tr>
<td>CRC</td>
<td>colorectal cancer</td>
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<tr>
<td>CSFII</td>
<td>Continuing Survey of Food Intakes by Individuals</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>d</td>
<td>day</td>
</tr>
<tr>
<td>DASH™</td>
<td>dynamic allele-specific hybridization</td>
</tr>
<tr>
<td>DFE</td>
<td>dietary folate equivalents</td>
</tr>
<tr>
<td>DHF</td>
<td>dihydrofolic acid</td>
</tr>
<tr>
<td>DHQ</td>
<td>Dietary History Questionnaire</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DRI</td>
<td>Dietary Reference Intakes</td>
</tr>
<tr>
<td>dTMP</td>
<td>thymidylate</td>
</tr>
<tr>
<td>dUMP</td>
<td>deoxyuridylate</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>RDA</td>
<td>Recommended Dietary Allowance</td>
</tr>
<tr>
<td>RFC</td>
<td>reduced folate carrier</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RTE</td>
<td>ready-to-eat</td>
</tr>
<tr>
<td>SAH</td>
<td>S-adenosylhomocysteine</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosylmethionine</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SE</td>
<td>standard error</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>T</td>
<td>thymine</td>
</tr>
<tr>
<td>TF</td>
<td>total folate</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydofolic acid</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>UL</td>
<td>Tolerable Upper Intake Level</td>
</tr>
<tr>
<td>μmol</td>
<td>micromole</td>
</tr>
<tr>
<td>UNC</td>
<td>University of North Carolina</td>
</tr>
<tr>
<td>US</td>
<td>United States</td>
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<tr>
<td>USDA</td>
<td>United States Department of Agriculture</td>
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Folate, a water-soluble vitamin essential for one-carbon metabolism, is available in the diet as naturally occurring food folate or synthetic folic acid (1). Since 1998, the United States (US) Food and Drug Administration has mandated folic acid fortification of all enriched cereal-grain products with the intent of increasing folic acid intake of women of reproductive potential to help reduce neural tube defect (NTD) risk (2). With the introduction of folic acid fortification, researchers have been interested in determining the impact of fortification on folate status and intake of the population (3-6). The aim of this study was to assess folate/folic acid intake and folate status of non-supplement consuming healthy young men and women, and to examine the relative contribution of different food categories to folate/folic acid intake. Furthermore, the relationship between folate status and methylenetetrahydrofolate reductase (MTHFR) 677 C→T genotype was examined because of previous reports suggesting a genotype effect on folate status.

Folate status was determined for men (n = 140) and women (n = 162) (ages 18 to 49 years). Mean serum and red blood cell folate (RBC) concentrations for males (39.9 nmol/L; 810 nmol/L, respectively) did not differ (P>0.2) from that of females (41.7 nmol/L; 767 nmol/L). The mean plasma homocysteine concentration was significantly higher for males (8.0 µmol) than
females (6.6 µmol/L) (P<0.0001). Comparisons of folate status by MTHFR 677 C→T genotype for all subjects revealed that individuals with the CC genotype had higher RBC folate and lower plasma homocysteine concentrations than individuals with the CT or TT genotypes.

To estimate usual folate/folic acid intake, subjects completed a modified Dietary History Questionnaire. Mean total intake expressed as dietary folate equivalents (DFE) for males and females (652 µg DFE/d; 512 µg DFE/d, respectively) exceeded the Recommended Dietary Allowance of 400 µg DFE/d. Average intake of folic acid for females was 128 µg/d, with only 3% of females consuming at least 400 µg/d of folic acid, the amount recommended by the Institute of Medicine to reduce the risk of an NTD-affected pregnancy (7). The largest contributors to folic acid intake for males and females included enriched cereal-grain products (41.1%; 41.9%, respectively), fortified ready-to-eat (RTE) cereals and bars (29.3%; 36.0%), and combination foods that included “enriched” ingredients (17.5%; 14.8%). The food categories that provided the most naturally occurring food folate in the diets of males and females, respectively, were vegetables (31.6%; 38.4%) and legumes and nuts (16.2%; 14.4%).

The present study is one of the first to use the US Department of Agriculture National Nutrient Database for Standard Reference (Release 17) to assess dietary folate intake expressed as food folate, folic acid, total folate, and DFEs (8). Dietary folic acid from enriched cereal-grain products and fortified RTE cereals positively affected total folate intake and status of males and females; however, folic acid intake for females was less than the level recommended for NTD risk reduction (i.e., 400 µg/d). Daily consumption of fortified RTE cereals may be an effective intervention strategy for increasing folic acid intake in women of reproductive potential.
CHAPTER 1
INTRODUCTION

Folate is a water-soluble B-complex vitamin that enables enzymes to transfer one-carbon groups to a variety of target molecules that are essential to the regulation of one-carbon (1-C) reactions involved in pyrimidine metabolism and methylation. Folate is an important nutrient that is found in the diet as naturally occurring food folate or synthetic folic acid. Foods naturally rich in food folate include dark green leafy vegetables, legumes, and orange juice.

In 1998, the United States (US) Food and Drug Administration (FDA) mandated folic acid fortification of all enriched cereal-grain products (e.g., bread, pasta, flour, rice, corn meal, and cereals, including ready-to-eat (RTE) breakfast cereals) with the intent of reducing neural tube defect (NTD) risk by increasing daily folic acid intake of women of reproductive potential (2). To decrease the risk of having a NTD affected pregnancy, it is currently recommended that women of reproductive age consume 400 µg/d of folic acid from supplements or fortified foods, in addition to dietary food folate (9). For the general population, it is recommended that both males and females 19 years and older consume the Recommended Dietary Allowance (RDA) of 400 µg DFE/d. The RDA was based on controlled studies on the adequacy of folate intake to maintain normal blood concentrations of folate status indicators, including serum and RBC folate, and plasma homocysteine concentrations (10). A continuous public health challenge is to ensure that adults, especially women capable of becoming pregnant, consume the recommended amount of folic acid intake on a daily basis.

Since the FDA mandated folic acid fortification, there has been public interest in determining the folate status and intake of adult men and women. Dietary intake of folate has been assessed in adult men and women through a variety of methods, including food frequency questionnaires. Research has consistently shown that folic acid fortification has improved the
folate status of the population compared to pre-fortification and dramatically decreased the incidence of NTDs (11-16). However, more recent studies have suggested that despite folate status improvement, women of reproductive age still fail to consume the recommended amount of folic acid daily (13). Furthermore, public health educational programs have focused attention on the recommendation that folic acid supplements be taken daily by women of reproductive age; however, recent surveys indicated that only 33% of women reported taking a daily supplement containing folic acid (17).

The data from this study provide valuable information regarding the relative and total contributions of dietary sources of folic acid to daily folic acid intake, which may help determine improved strategies for helping women meet the recommended daily level of intake for folic acid. In addition, this study adds valuable information concerning the folate status of healthy young men and women. To evaluate folate status, this study was also designed to evaluate plasma homocysteine concentration, which is a known folate status indicator. Blood folate and homocysteine concentrations are inversely correlated indicating homocysteine concentration is elevated in response to reduced folate status (18).

With regard to other factors that may influence folate status, this study provides validating evidence of a genetic single-nucleotide polymorphism that has been extensively studied and demonstrated to influence the folate-dependent metabolic pathway of 1-C metabolism. This polymorphism is a cytosine (C) to thymine (T) substitution at base pair 677 in the gene that encodes for 5,10-methylenetetrahydrofolate reductase (MTHFR) (19). The MTHFR 677 C→T variant can be characterized as homozygous (TT) or heterozygous (CT) and is prevalent in approximately 12% and 50%, respectively, of the overall population (20). In individuals who are homozygous (TT) for the MTHFR 677 C→T polymorphism, the impaired
enzyme activity is associated with reduced blood folate and elevated plasma homocysteine concentrations, both of which can be significantly improved in response to improved folate status (21).

Overall Goal

The goal of the present study was to assess folate status of non-supplement consuming healthy young men and women and to estimate the relative contribution of folate/folic acid from different food categories to overall intake.

Specific Objectives

1. To assess folate/folic acid intake and folate status of young male and female non-supplement users.

2. To evaluate the adequacy of folate intake relative to the RDA for men and women, and to evaluate the adequacy of folic acid intake for women relative to the recommendation for NTD risk reduction.

3. To compare the actual and relative contribution of specific food categories to folate/folic acid intake in young male and female non-supplement users.

4. To compare folate status of young adults by folate intake and by MTHFR 677C→T genotype.
CHAPTER 2
BACKGROUND AND LITERATURE REVIEW

Folate

Chemistry

Folate is a water-soluble B-complex vitamin that is consumed in the diet as either naturally occurring food folate or synthetic folic acid. Folic acid is the synthetic, monoglutamate form of folate used in fortified foods and supplements (1, 22). The chemical structure of folic acid, which has a molecular weight of 441.4, is formed through the combination of three structural features that are required for its functional activity: a para-aminobenzoic acid molecule is linked at one end to a pteridine ring by a methylene bridge (C-9–N-10), and at the other end joined by peptide linkage to a glutamic acid molecule (1, 23) (Figure 2-1). Naturally occurring food folates exist in various chemical forms, containing a side-chain composed of two to ten additional glutamate residues joined to the first glutamate residue (1, 9) (Figure 2-1).

The structure of the folate molecule can change through the reduction of the pteridine ring to dihydrofolate acid (DHF) and tetrahydofolic acid (THF), elongation of the chain of glutamate residues, and 1-C substitutions at the N-5, N-10, or both positions of THF (23). The polyglutamyl form of the THF molecule can be converted to various forms of folate through the transfer of methyl (CH3), methylene (–CH2–), methenyl (–CH=), formyl (–CH=O), or formimino (–CH=NH) groups (1) (Figure 2-1). These one carbon entities can be transferred in diverse pathways including those required for the synthesis of deoxyribonucleic acid (DNA), amino acid interconversions (e.g., homocysteine to methionine), and DNA methylation.

Dietary Sources

Eukaryotic cells are unable to synthesize the folate molecule in the body. Thus, folate must be consumed through dietary sources, which include naturally occurring food folate and
foods fortified with synthetic folic acid. Endogenous food folate is found in a variety of foods including orange juice, dark green leafy vegetables, asparagus, strawberries, peanuts, and legumes such as kidney beans, black beans and lima beans (9). Meat is not considered a good source of food folate, except for liver. Additionally, folate concentrations are higher in raw foods than cooked foods, due to folate losses that occur through cooking processes, with losses dependent on oxygen exposure, ascorbic acid content, and the quantity of water used. Folate in plant foods can lose up to 40% of their folate content during cooking (24).

Synthetic folic acid is another major source of folate in the diet and is found in enriched cereal-grain products and fortified RTE breakfast cereals. Since January 1, 1998, the FDA has mandated folic acid fortification of all enriched cereal-grain products (e.g., bread, pasta, flour, rice, corn meal, and cereals, including RTE breakfast cereals) and mixed food products containing these grains (9). The target folic acid concentration of cereal-grain products is 140 µg folic acid per 100 g cereal-grain, with a range in recommended amount to be added to various foods (95 to 309 µg per 100 g of product) (2). The majority of RTE breakfast cereals provide 25% of the Daily Value of folic acid (~100 µg/serving), with some brands providing up to 100% (400 µg/serving) (9, 25). Naturally occurring food sources of folate are considered “excellent” sources of folate if they contain 100 to 200 µg DFE/serving, “good” sources if they contain 50 to 99 µg DFE/serving, “moderate” sources if they contain 25 to 49 µg DFE/serving, and “fair to poor” sources if they contain <25 µg DFE/serving (9).

Total folate is the term that refers to the combination of endogenous food folate and/or folic acid from fortification. Total folate intake per day is expressed as µg/d. Total folate does not take into account the difference in bioavailability between naturally occurring food folate and
folic acid, whereas the expression of folate intake in terms of DFE accounts for the higher bioavailability of folic acid compared to food folate.

**Bioavailability**

The bioavailability of folate refers to the degree to which folate is absorbed and utilized, including differences in folate absorption, transport, metabolism, catabolism, enterohepatic circulation and urinary excretion (25). Physiological conditions, variability of food constituents, and pharmaceutical drugs can possibly affect the efficiency of intestinal absorption of food folate through a variety of ways: entrapment of food folate within the food matrix; the presence of enzyme inhibitors required for the deconjugation of the polyglutamate form of food folate to the monoglutamate form required for absorption; the instability of THF in the acidic pH of the stomach before absorption; and the presence of food constituents that may inhibit folate deconjugation (1, 25).

A great deal of ambiguity exists among food folate research with regard to potential factors affecting bioavailability, particularly the difference in bioavailability between the monoglutamate and polyglutamate folate forms, and the degree of bioavailability of food folates. Research findings reported by McKillop and colleagues suggest that the extent of conjugation is not a factor affecting the efficiency of absorption of food folate in the intestines (25). Variation in bioavailability reported in previous research could be due to differences in folate digestion, absorption and metabolism, as well as inconsistencies between protocols and analytical methods used to measure and report bioavailability. Alcohol and pharmaceutical drugs also can inhibit the absorption and metabolism of folate (26).

Estimates of the bioavailability of food folate relative to folic acid have varied in the measured percentage of absorption, ranging from 10 to 98% (25, 27-32); however, the best
estimate is provided by data from Sauberlich and colleagues indicating that food folate is approximately 50% bioavailable relative to folic acid (9, 30). When folic acid is consumed as a supplement in the fasting state or without food, it is 100% bioavailable (33). Folic acid absorption is slightly reduced and estimated at 85% bioavailable when folic acid is consumed with food, which is the case with enriched cereal-grain products or fortified foods (10, 34, 35). The physiological requirement for folate represents the amount the body requires post-folate absorption. Considering the differences in folate bioavailability, a larger quantity of food folate compared to folic acid must be ingested to meet the physiological requirement (9). These data coupled with the estimate that food folate is approximately 50% bioavailable were used by the Dietary Reference Intake (DRI) Committee of the Institute of Medicine (IOM) to develop the concept of DFE, a unit of measure that acknowledges differences in bioavailability and absorption among the various chemical forms of the vitamin (9). The DFE values for folic acid in enriched cereal-grain products and vitamin supplements are calculated using conversion factors that account for differences in bioavailability relative to naturally occurring food folate (9). Folic acid in supplemental form taken while fasting is estimated to be twice as bioavailable relative to food folate (i.e., 100:50). Folic acid from supplements taken with food or folic acid from enriched or fortified food sources is estimated to be 1.7 times more bioavailable than naturally occurring food folate (i.e., 85:50) (9). The total folate content of any food product in DFE units can be calculated by multiplying the micrograms of synthetic folic acid by 1.7 then adding this value to the micrograms of food folate present (9). No calculation or adjustment is needed when solely determining food folate intake.
Absorption, Transport and Storage

Folate must be in the monoglutamate form in order for absorption to occur. Synthetic folic acid is already a monoglutamate, and thus readily absorbed. However, since food folates are polyglutamates, they must first be hydrolyzed by the enzymatic action of folypoly-γ-glutamate carboxypeptidase II, also known as pteroylpolyglutamate hydrolase or folate conjugase, before absorption can occur (36). Following complete deconjugation of the polyglutamate molecule, folates are transported across the brush border membrane in the jejunum via an acidic pH-dependent (pH of 6.5 to 7.0) carrier-mediated mechanism (37, 38). Drugs or diseases that impair jejunal pH may impair folate absorption (1).

Prior to entry into the portal system, the majority of folate is reduced by dihydrofolate reductase to DHF and THF (36). Once folate is absorbed, it travels to the liver via portal circulation, primarily in the form of 5-methylTHF. To allow for folate storage and retention in the liver, folates are reduced and conjugated. A small fraction of 5-methylTHF in the plasma can be present as free folate or bound with high affinity to a folate binding protein; however, the majority of plasma folate is bound with low affinity to albumin (23, 39, 40).

Two mechanisms exist for transport of folate into the cells: a folate transporter and a folate receptor (41, 42). These transport mechanisms are required for absorption of folate in the small intestine, reabsorption of endogenous folate by the kidneys, and uptake of folate through the plasma membrane into a developing embryo (41). The reduced folate carrier (RFC) is the folate transporter in the intestinal epithelial cells and is a classic facilitated transport protein that carries reduced folate (5-methylTHF) across the membrane; it is encoded by the RFC gene (RFC1), which is expressed in most tissues and is capable of mediating bi-directional flux of folate. The second transport mechanism is a folate receptor-mediated process by which folate
binds with high-affinity to a folate-receptor at the membrane surface and is transported into the cells via a unidirectional system. The efficiency of cellular uptake of the carrier-mediated process is much greater than that of the receptor–mediated process (41, 42). Pharmacological quantities of folic acid are absorbed via diffusion as absorption by the RFC is a saturable process (38).

Once cellular uptake of 5-methylTHF is complete, it is demethylated by methionine synthase and converted to the polyglutamyl form through the action of folylpolyglutamate synthetase. This mechanism allows for intracellular folate retention because polyglutamates are unable to cross the cell membrane (43). Previous measurements of folate concentrations in liver tissue indicate that the liver specifically stores approximately 6 to 14 mg of folate. Based on the assumption that the liver stores 50% of total body folate, it is estimated that the body stores 15 to 30 mg of folate (44).

**Metabolism and Excretion**

When folate polyglutamates are released from tissues into circulation, the polyglutamate form of folate molecules is reconverted to the monoglutamyl form by γ-glutamylhydrolase. The mechanisms of polyglutamation and monoglutamation are necessary for the function of 1-C metabolism. The majority of circulating folate is reabsorbed in the proximal tubules of the kidney primarily via a folate receptor (45-47). Folate may also be packaged as part of bile (~100 µg/d), concentrated and stored in the gall bladder, and subsequently secreted into the intestinal tract. The majority of folate in bile is recycled during enterohepatic circulation, although some may be excreted in the feces along with unabsorbed dietary folate, folate from endogenous secretions, and folate synthesized by bacteria in the gut (48). Research findings have determined that folate losses via fecal and urinary excretion are comparable and represent approximately 0.5
to 1% of folate (48, 49). These loses must be replaced daily through dietary folate or supplemental folic acid to maintain normal folate metabolism.

**Dietary Reference Intakes**

Dietary Reference Intakes represent the most current recommendations for each vitamin and mineral and include for each nutrient the Estimated Average Requirements (EAR), RDA, Adequate Intake (AI), and Tolerable Upper Limit (UL). See Table 1-1 for definitions of each DRI (10).

The most recent DRI recommendations were published by the National Academy of Sciences IOM in 1998 (10). The previous recommendations for nutrient intake were established to prevent clinical deficiencies. In establishing the 1998 DRI recommendations, the IOM shifted the focus of recommendations from nutrient quantities needed to prevent clinical deficiencies to intakes that ensure optimum health (10). Dietary Reference Intake recommendations for folate are presented in Table 1-2. The RDA for men and non-pregnant women 19 years and older is 400 µg DFE/d. The recommendations are increased to 600 µg DFE/d and 500 µg DFE/d during pregnancy and lactation, respectively. Since folate is not associated with toxic side-effects, the UL of 1,000 µg/d of synthetic folic acid is based solely on the concern that folic acid supplementation can mask the diagnosis of a vitamin B12 deficiency, which is prevalent in approximately 10 to 15% of the elderly population over 60 years of age (10, 50).

The IOM also recommends that all women of childbearing age consume 400 µg/d of synthetic folic acid from fortified foods and/or supplements in addition to consuming food folate from a varied diet to reduce the risk of having an NTD-affected pregnancy (9). However, the achievement of the latter is problematic as compliance with the supplementation recommendation is often suboptimal. It is estimated that approximately 33% of women of
reproductive age take a daily folic acid-containing supplement (17). Evidence for an association between folic acid intake and NTD risk reduction provided the basis for the mandated folic acid fortification of cereal-grain products.

**Biochemical Functions**

Interconnected reactions requiring specific forms of folate to accept and transfer one-carbon units, together referred to as 1-C metabolism, include amino acid metabolism, homocysteine remethylation, purine and pyrimidine synthesis, and the generation of S-adenosylmethionine (SAM) (1). One-carbon metabolism is a series of folate-dependent pathways that requires the donation of one-carbon units from individual folate coenzymes resulting in the regeneration of THF. An illustration of the 1-C metabolism pathway is presented in Figure 2-2. In 1-C metabolism, THF is the primary acceptor molecule and is required for continuation of the cycle allowing for nucleotide biosynthesis and methylation reactions (1).

**Key-Folate Dependent Reactions in One-Carbon Metabolism**

**Nucleotide biosynthesis.** Through a reversible reaction, THF is converted to 5,10-methyleneTHF in association with the conversion of serine to glycine, both of which are non-essential amino acids, by the vitamin B6-dependent enzyme serine hydroxymethyltransferase (SHMT). DNA synthesis is dependent upon the availability of 5,10-methyleneTHF, which can donate a one-carbon unit to deoxyuridylate (dUMP) through the action of thymidylate synthase to irreversibly synthesize thymidylate (dTMP). This rate-limiting step is essential for DNA synthesis to proceed. During the process of donating a one-carbon group, 5,10-methyleneTHF is oxidized to form DHF and reduced back to THF by dihydrofolate reductase. Additional nucleotide production requires 5,10-methyleneTHF for the formation of 10-formylTHF by 10-
formylTHF synthetase, which can be used to donate multiple one-carbon units for purine synthesis and the regeneration of THF for recirculation (1, 51).

**Methylation reactions.** The folate-dependent reactions of 1-C metabolism hinge on the production of 5,10-methyleneTHF, which is also required for several other functional outcomes. In order for the remethylation of methionine from homocysteine to occur, 5,10-methyleneTHF is first reduced by MTHFR to 5-methylTHF. The 5-methylTHF form of folate, in conjunction with methionine synthetase and cobalamin (vitamin B12), irreversibly donates a one-carbon group to cobalamin to become methyl cobalamin. The methyl group from methyl cobalamin is transferred to homocysteine to form methionine, and 5-methylTHF is ultimately converted to THF. Regeneration of methionine and THF is required for the 1-C metabolism cycle to continue. During a cobalamin deficiency, a secondary folate deficiency often occurs. The “methyl-trap hypothesis” suggests the latter emerges because there is not enough cobalamin to accept and donate a one-carbon unit to homocysteine (52). Thus, folate is “trapped” within 5-methylTHF, and THF is not regenerated to continue the cycle through the synthesis of dTMP. Consequently, DNA replication and cellular mitosis are interrupted.

Methionine is essential for the formation of S-adenosylmethionine (SAM) by the enzyme SAM synthase and activation by adenosine 5’-triphosphate (ATP). Methionine, an essential amino acid, can be derived from dietary protein intake or by generation from homocysteine. SAM is the primary methylating agent that donates a methyl group, originally accepted from 5-methylTHF, that is used in more than 100 methyltransferase reactions, including the methylation of DNA, ribonucleic acid (RNA), protein, phospholipids and neurotransmitters (53). Once SAM donates its methyl group, it is converted to S-adenosylhomocysteine (SAH) by various cellular methyltransferases. SAH is hydrolyzed by SAH hydrolase to homocysteine and adenosine
through a reversible reaction in which SAH is favored in the balance. SAM also regulates the production of 5-methylTHF. When dietary methionine is adequate, SAM will inhibit MTHFR from producing 5-methylTHF and thus methionine; whereas, when the availability of methionine is low, the concentration of SAM will be decreased (54). This releases the inhibition of MTHFR to allow for greater production of 5-methylTHF and regeneration of methionine (54).

SAM also regulates the pyridoxal phosphate-dependent reaction in which homocysteine is catabolized to cystathionine in combination with serine and the enzyme cystathionine β-synthase. The transsulfuration pathway catabolizes excess homocysteine not required for methyl transfer reactions. This transsulfuration pathway ultimately leads to the synthesis of cysteine (non-essential amino acid) and glutathione (antioxidant) (1).

**Methylenetetrahydrofolate Reductase (MTHFR) Polymorphism**

Methylenetetrahydrofolate reductase, the enzyme required to reduce 5,10-methyleneTHF to 5-methylTHF, is the main circulating form of folate and the major methyl donor for the remethylation of homocysteine to methionine. The most extensively studied polymorphisms related to folate metabolism are MTHFR polymorphisms. Frosst and colleagues discovered that individuals homozygous for the 677C→T polymorphism (TT genotype) have decreased MTHFR enzyme activity resulting in reduced levels of 5-methylTHF available to donate a methyl group for the remethylation of homocysteine to methionine (19). This substitution renders the enzyme “thermolabile” and may cause elevated plasma levels of the amino acid homocysteine. The homozygous TT genotype is termed thermolabile because the enzymatic activity is decreased compared to the CC genotype (55). Kang and colleagues discovered the TT genotype had a specific enzymatic activity of approximately 50% of the normal levels seen with the CC genotype (55). Decreases in MTHFR function have been associated with mild
hyperhomocysteinemia and may therefore be a risk factor for the development of cardiovascular disease (CVD) (56). Approximately 12% of the population is homozygous for the 677C→T variant (i.e., TT genotype), whereas approximately 50% is heterozygous (i.e., CT genotype) (20). 

Matthews and colleagues characterized the decrease in MTHFR enzyme activity through an increased dissociation of flavin adenine dinucleotide (FAD) from thermolabile MTHFR (56). Furthermore, these researchers found that folic acid supplementation provided protection by increasing the affinity of the enzyme for FAD. These results agreed with the findings by Guenther and colleagues (57) who also found that dissociation of FAD resulted in a loss in MTHFR enzyme activity. These investigators monitored enzyme activity and found the TT genotype loses activity at a rate ten times faster compared to the enzyme activity associated with the CC genotype (57).

**Folate Status Assessment**

**Serum and Red Blood Cell Folate Concentrations**

Serum folate concentration best reflects current and recent intakes of folate and is an early and sensitive measure of short-term folate status (58). Controlled, metabolic studies have shown that serum folate concentrations decrease quickly within a period of one to three weeks when folate intake is limited (21, 52, 59). These metabolic changes precede decreases in RBC folate concentrations. The lower limit of normal used to define inadequate serum folate status using the microbiological assay is $\leq 13.6 \text{ nmol/L}$ (60). An alternative method for assessing folate status is the radiobinding assay, which has been shown to yield lower blood folate values relative to the microbiological assay (61). The lower limit of normal for the radiobinding assay is $<7 \text{ nmol/L}$. 
Red blood cell folate is considered a better indicator of long-term folate status than serum folate concentration as this marker reflects tissue folate storage. A previous study using liver tissue biopsies found that RBC folate concentrations correspond to liver folate concentrations (62). Uptake of folate into erythrocytes only occurs in the bone marrow during the early stages of erythropoiesis. Since folate cannot permeate the membrane of a mature RBC during its 120-day lifespan, RBC folate reflects folate status over the preceding three months. The lower limit of normal used to define inadequate RBC folate status using the radiobinding assay is \( \leq 317 \) nmol/L (10, 52).

**Plasma Homocysteine Concentration**

In addition to measuring blood folate concentrations, other “functional” indicators should be used to evaluate folate status as these indices may reflect abnormalities in metabolic function that may or may not be reflected in altered blood folate concentrations. The most notable “functional” indicator is total plasma homocysteine concentration (63). An elevated homocysteine concentration reflects not only a reduction in blood folate concentration secondary to inadequate folate intake, but also an insufficient concentration of 5-methylTHF required to convert homocysteine to methionine in the 1-C metabolism pathway (10, 18). Plasma homocysteine concentration has been shown to be directly and inversely correlated with folate status; however, it is not specific to folate deficiency because other nutrient deficiencies (i.e., vitamins B12 and B6), genetic abnormalities, renal insufficiency, and dietary and lifestyle factors may influence homocysteine concentration (64, 65). Although discrepancies exist when defining acceptable homocysteine concentrations, individuals with a plasma homocysteine concentration \( \geq 12 \) µmol/L are often considered to be at higher risk for adverse health effects, including vascular disease (4).
To assess homocysteine status in the US, Ganji and colleagues (66) presented the distribution of plasma homocysteine concentrations of adults in the US by using data from the National Health and Nutrition Examination Surveys (NHANES) 1999-2000 and 2001-2002. These researchers studied the homocysteine distribution by age, gender and race-ethnicity in 9,196 subjects. Results showed that plasma homocysteine concentrations were higher in men than in women (aged 19-70 years) and in older persons compared to younger persons (66). In a preceding study, these researchers also reported that mean circulating homocysteine concentrations in the period since folic acid fortification were 7.6 µmol/L in 1999-2000 and 7.9 µmol/L in 2001-2002, as compared with 9.5 µmol/L during the period prior to fortification (1994 to 1998) (67).

**Folate Deficiency**

**Etiology**

In addition to inadequate folate in the diet, a folate deficiency can develop through other causes, such as drug-nutrient interactions, genetic variations, impaired intestinal folate absorption or folate metabolism secondary to high alcohol consumption, and increased renal folate excretion. High doses of nonsteroidal anti-inflammatory drugs (i.e., aspirin, ibuprofen, and acetaminophen) have been shown to exert antifolate activity (68-71). However, impaired folate status has not been reported with low doses of these drugs. Folate deficiency associated with genetic variations, alcohol consumption and dietary deficiency are discussed in the following sections.

**Megaloblastic Anemia**

The development of a folate deficiency is associated with a progression of events that can eventually lead to megaloblastic anemia seen with chronic severe folate deficiency. The
sequence of events begins with a reduction in serum folate concentration, which can rapidly decrease within one to three weeks. Following a long phase of folate deficiency, RBC folate concentration will also decline (58).

The most common etiology of megaloblastic anemia is impaired DNA synthesis, linked to abnormalities in the folate pathway (1-C metabolism). A decrease in the availability of folate from circulation and tissues will lead to a decline in DNA synthesis, reducing cell division, and ultimately resulting in the formation of large, immature RBCs characteristic of megaloblastic anemia. Abnormalities in RBC formation will lead to decreases in hemoglobin, hematocrit, and RBC number.

Clinical manifestations of megaloblastic anemia can develop after a period of compromised folate status. Folate status can be compromised and a folate deficiency can develop through various factors including: inadequate folate intake; malabsorption induced by abnormalities of the small intestine, drugs or alcohol; altered metabolism; increased requirement (i.e., pregnancy, diseases); increased losses (i.e., dialysis, some skin diseases); abnormalities of folate metabolism; and inherited abnormalities of folate absorption and metabolism (72).

Pregnancy is a common cause of megaloblastic anemia in women. Folate requirements increase five to ten fold for pregnant women relative to non-pregnant women. The higher folate requirement during pregnancy is related to increases in cellular proliferation secondary to the growth and development of the fetus, placenta, and maternal tissues (73, 74), rather than an increase in folate catabolism as substantiated by Caudill and colleagues (75). This elevated demand for folate must be met by adequate dietary intake.
Neural Tube Defects

Neural tube defects are congenital malformations that occur in the brain and spinal column that are caused primarily when closure of the neural tube fails during embryonic development (76). The development and closure of the neural tube of an embryo occurs within 28 days after conception, often before women are aware they are pregnant. The extent of an NTD varies with different degrees of tissue protrusion and neural involvement. The two most common forms of NTDs are anencephaly (failure of the brain to develop) and spina bifida (exposure of the spinal cord due to defective closure of the neural tube). In some instances, defects in the orofacial cleft can develop from an abnormal increase in cerebrospinal fluid pressure on the closed neural tube during the first trimester of embryonic development (76). The incidence of NTDs (anencephaly or spina bifida) is 0.75 cases per 10,000 births in the US, with roughly 400,000 cases per year worldwide (77).

It has long been suspected that a cause-and-effect relationship exists between diet and NTDs. Hibbard was one of the first researchers to address the importance of folate during pregnancy. He proposed that the incidence of megaloblastic anemia in mid to late pregnancy may have resulted from a defect in folate metabolism (78). Shortly after, Hibbard hypothesized that fetal spontaneous abortions and placental abruption were associated with folate deficiencies during pregnancy (79). In 1976, Smithells and colleagues further suggested that folate deficiency was linked to NTDs because women who gave birth to NTD-affected infants had low blood folate concentrations (80). These investigators reported that supplementation with folic acid consumed periconceptionally reduced the recurrence of NTD pregnancies in high-risk mothers. These results spawned further research studies that have continued to explore the benefits of folic acid on reducing the incidence of NTDs. The powerful driving force in folate
research was the report from the Medical Research Council (MRC) Vitamin Study Research Group (81). In 1983, the MRC launched a large-scale, randomized, double-blind, prevention trial evaluating the effect of multivitamin supplementation with and without folic acid on the recurrence of NTD-affected pregnancies. The study was ended early because 72% of NTDs were prevented with 4 mg/d of supplemental folic acid (81). Moreover, in 1992, Czeizel and Dudas found that periconceptional folic acid supplementation (800 µg/d) decreased first time occurrence of NTDs in Hungarian women compared to subjects receiving no folic acid (82). Further research led to the conclusion that folic acid supplementation can prevent up to 70% of NTDs (83).

A subsequent intervention study conducted by Berry et al. (84) supported the work of earlier investigators who observed a relationship between folic acid and NTDs. Berry and his team investigated the effect of periconceptional use of folic acid in Chinese women living in northern regions of China where NTDs rates were high and southern regions of China where NTDs rates were low (84). Among the fetuses or infants of women who did not take folic acid, NTDs rates were 4.8 and 1.0 per 1,000 pregnancies in the northern and southern regions of China, respectively; whereas, 400 µg/d of folic acid reduced the occurrence of NTDs in the northern and southern regions of China to 1.0 and 0.6 per 1,000 pregnancies, respectively. These NTD rate decreases are equivalent to a respective 79% and 40% reduction in the incidence of NTDs in the northern and southern regions.

In a study of 56,000 Irish women, Daly and colleagues (85) examined the relationship between blood folate concentration and number of NTDs. The investigators observed that as RBC folate concentration increased, the risk for NTDs decreased. They also reported an eight-fold difference in the incidence of NTDs between women with RBC concentrations <341 nmol/L
and women with concentrations ≥908 nmol/L (85). It was estimated that a RBC folate concentration of 908 nmol/L could be achieved with 400 µg/d of folic acid.

In a case-control study by Moore and colleagues, investigators reported an NTD risk of 3.4 cases per 1,000 pregnancies for women who consumed <150 µg DFE/d. Risk for NTDs significantly decreased by 77% to 0.8 cases per 1,000 pregnancies for women who consumed ≥1,200 µg DFE/d. These researchers concluded that the prevalence of NTDs decreased by 0.78 cases per 1,000 pregnancies with folate intake increments of 500 µg DFE/d (86). These data suggest that NTD risk declined significantly with modest increases of total folate intake during early pregnancy. Overall, an overwhelming amount of scientific evidence supports an association between adequate folate status/folic acid intake and NTD risk reduction (31).

**MTHFR polymorphism and neural tube defects.** The MTHFR 677C→T polymorphism was identified as the first genetic risk factor for NTDs. The MTHFR 677C→T mutation, as previously reviewed, causes reduced activity of the enzyme. The homozygous mutation (TT genotype) is associated with an elevated plasma homocysteine concentration and reduced RBC and plasma folate concentrations. It has also been suggested that individuals with the TT genotype have higher folate requirements (87).

Proposed mechanisms for folate-responsive NTDs include decreased rates of DNA synthesis and cell division due to impaired dTMP synthesis, and a reduction in DNA methylation (83). Embryonic tissues grow very rapidly, with high requirements for DNA synthesis and methyl groups from SAM (88). Individuals homozygous for the MTHFR genotype may need more folate in their diets to provide adequate amounts of methyl groups and to provide substrate for the DNA methylation and synthesis via 1-C metabolism. During early pregnancy, a mild deficiency can impair DNA synthesis and methylation, the latter of which can affect gene
expression. Disordered gene expression may explain how a relatively mild deficiency can
initiate the development of severe neural tube deformities in an embryo (88).

van der Put and colleagues (89) examined the frequency of the MTHFR 677C→T
polymorphism in patients with spina bifida (n = 55) and their parents (n = 130) and controls who
did not have spina bifida or offspring with spina bifida (n = 207). Only 5% of the control
subjects were homozygous for the TT genotype, whereas 13% of patients with spina bifida had
the TT genotype, as well as 16% and 10% of their mothers and fathers, respectively. The
researchers found that all subjects and controls with the TT genotype had decreased MTHFR
activity, low plasma folate concentrations, and high plasma homocysteine and RBC folate
concentrations (89). These researchers later reported that the risk of having offspring born with
spina bifida was strongest when both the mother and her child carried the homozygous variant
genotype (TT) (90). van der Put and colleagues concluded that the 677C→T polymorphism
should be regarded as a genetic risk factor for spina bifida.

Vascular Disease and Stroke

Several epidemiological studies have confirmed an inverse association between folate
and homocysteine concentrations based on the metabolic role of folate as a coenzyme in the
regulation and methylation of homocysteine in 1-C metabolism. Furthermore, studies have
indicated the role of an elevated plasma homocysteine concentration, also known as
hyperhomocysteinemia, as a significant risk factor for atherosclerotic vascular and coronary
heart disease. Homocysteine may increase vascular and coronary heart disease risk and
atherogenesis through direct toxicity to endothelial cells via lipid peroxidation, increased
coagulation and platelet aggregation, decreased endothelial cell reactivity, and stimulation of
smooth muscle cell proliferation (91-94).
To establish if there is a dose-dependent effect of folic acid supplementation on plasma homocysteine concentration, meta-analyses of randomized controlled trials have been conducted (95). Specifically, the Homocysteine Lowering Trialists’ Collaboration reported results from randomized controlled trials in two different meta-analyses (1998 and 2005) to determine the size of reduction in homocysteine concentration achieved by different daily supplemental doses of folic acid, with or without vitamins B12 or B6 (96, 97). The first meta-analysis revealed that the effect of folic acid on blood homocysteine concentration seemed to depend on the pretreatment blood homocysteine and folate concentrations (97). Participants with lower blood folate concentration or higher baseline plasma homocysteine concentration experienced the greatest improvement from supplements containing folic acid. After adjusting for pretreatment blood homocysteine and folate concentrations, results indicated that daily folic acid doses of <1 mg/d (mean = 0.5 mg/d), 1–3 mg/d, and >3 mg/d lowered homocysteine concentration by approximately 25%.

The second meta-analysis provided new data regarding a folic acid dose-dependent lowering effect on homocysteine concentrations (96). Baseline plasma folate and homocysteine concentrations before treatment with folic acid were standardized at 12 nmol/L and 12 µmol/L, respectively. Results suggested that doses ≥800 µg/d of folic acid are typically required to achieve maximum reductions in plasma homocysteine concentrations produced by folic acid supplementation. Folic acid doses of 200 and 400 µg/d were associated with homocysteine reductions of 60% and 90%, respectively, from baseline levels. The results revealed that 400 µg/d of folic acid produced near-maximum lowering effects in plasma homocysteine concentration, which may be significant to public health (96).
Considering the role of supplemental folic acid in lowering homocysteine, the main unanswered question is whether folic acid supplementation will reduce the overall rate of vascular disease. Cardiovascular disease is the leading cause of death in the US, accounting for approximately 37% of all deaths (98). Between 1980 and 1994, Rimm and colleagues (99) examined the intakes of folate and vitamin B6 of 80,082 women in the Nurses’ Health Study Cohort in relation to the incidence of nonfatal myocardial infarction (MI) and fatal coronary heart disease (CHD). The results revealed an approximate 30% lower relative risk of CHD for women with higher folate intake (~700 µg/d) compared to intakes at or below the RDA at that time (~160 µg/d), after controlling for vascular disease risk factors (i.e., smoking, hypertension, alcohol consumption, fiber, vitamin E, and saturated, polyunsaturated, and trans fat) (99).

Because this study was conducted before implementation of folic acid fortification, the largest contributor to the overall intake of folate was multivitamins, followed by RTE cereals, orange juice, lettuce, eggs, broccoli, and spinach (99). The researchers noted that their findings were also consistent with previous data linking higher intakes of folate with lower homocysteine concentrations. However, one limitation to this study was that it was an observational study, and the individuals who fell into the highest range of folate intake might have had overall “healthier diets” and were taking supplements that might have contained other nutrients that could have affected CHD risk.

In addition, Refsum and colleagues (100) conducted a meta-analysis in 1998 including data from 80 clinical and epidemiological studies (~10,000 patients). The results of this analysis provided unequivocal evidence that hyperhomocysteinemia is a common, independent CVD risk factor in the general population, and may also enhance the effect of other conventional risk factors (100). Because supplementation with B vitamins, in particular folic acid, is an efficient,
safe, and inexpensive means to reduce an elevated homocysteine level, studies have tried to establish whether such therapy will reduce CVD risk. A meta-analysis conducted by the Homocysteine Studies Collaboration assessed the relationship of homocysteine concentrations with primary prevention of vascular disease and determined that an estimated 25% lower usual homocysteine concentration was associated with approximately 11% lower ischemic heart disease risk and 19% lower stroke risk (101).

Moreover, a meta-analysis by Bazzano and colleagues (102) summarized the results of twelve randomized, controlled clinical trials on dietary supplementation with folic acid on CVD risk reduction. These studies included data from 16,958 participants with preexisting vascular disease. Folic acid supplementation was not shown to reduce recurrence risk of CVD or all-cause mortality among subjects with a prior history of vascular disease (102). It is possible that folic acid supplementation may have a protective effect on risk reduction and prevention of a primary rather than secondary occurrence of a vascular event. Several ongoing randomized, controlled, clinical trials in the US, Canada, Europe and Australia with large sample sizes as part of the B-Vitamin Treatment Trialists’ Collaboration might provide a more definitive answer to the role of folic acid on primary risk reduction (103). Although the results of these studies are uncertain, subjects enrolled in trials conducted in the US and Canada are expected to have lower baseline homocysteine concentrations because folic acid fortification is mandatory in these regions and has previously been associated with significant reductions in plasma homocysteine concentrations. These ongoing intervention trials in the US and Canada may not observe the same decreases in homocysteine that might appear in populations not exposed to fortification (11). Furthermore, it is difficult to detect a cause-and-effect relationship between folic acid supplementation and vascular disease risk secondary to difficulty in controlling for other
nutrients in the diet (i.e., vitamins B12 and B6) that may significantly lower homocysteine, as well as the fact that the underlying causes of vascular disease are multifactorial (99, 100, 102).

Research studies have also been conducted to investigate the efficacy of folate therapy on stroke prevention. Wang and colleagues conducted a meta-analysis of eight randomized trials of folic acid that reported stroke as an endpoint (104). The data indicated that folic acid supplementation significantly reduced the risk of stroke by 18%, and an even greater beneficial effect was seen in those trials in which homocysteine concentration was reduced by more than 20%, treatment duration was more than 36 months, no fortification program or partial fortification of grains had been introduced, and there was no history of stroke. Investigators concluded that the inverse relationship detected between the duration of folic acid supplementation and the risk of stroke suggests that folic acid supplementation can effectively reduce the risk of stroke in primary prevention (104).

**MTHFR polymorphism and vascular disease.** Studies have also indicated that individuals with the homozygous variant (TT) of the MTHFR 677 C→T polymorphism have elevated plasma homocysteine concentrations compared to individuals who express either the heterozygous (CT) or normal (CC) genotypes when folate status is low. Jacques and colleagues (105) assessed the potential interaction between the MTHFR 677 C→T polymorphism and folate status in homocysteine metabolism. This study included 365 individuals from the National Heart, Lung, and Blood Institute Family Heart Study. The investigators found that among individuals with lower plasma folate concentrations (<15.4 nmol/L), those with the TT genotype had a mean total fasting homocysteine concentration (12.1 µmol/L) that was 24% greater than individuals with the CC genotype (9.8 µmol/L). A difference in homocysteine concentrations between the TT and CC genotypes (7.9 and 7.8 µmol/L, respectively) was not seen for
individuals with a folate concentration $\geq 15.4$ nmol/L (105). A meta-analysis by Brattstrom and colleagues (106) combined the results of studies that have documented plasma homocysteine concentrations in relation to the MTHFR genotypes (CC, CT and TT). Similar to previous findings, the meta-analysis revealed that individuals with the TT genotype had higher mean homocysteine concentration (25% or 2.6 $\mu$mol/L increase) than subjects with the CC genotype when both populations had suboptimal folate status. A difference in homocysteine concentrations among the MTHFR genotypes was not detected when folate status was high. Ultimately, these studies concluded that the MTHFR $677C \rightarrow T$ mutation is a major cause of mild hyperhomocysteinemia and that individuals are at greater risk for hyperhomocysteinemia when they have the MTHFR $677$ TT genotype coupled with low serum folate concentrations. However, there appears to be little evidence that individuals with the TT genotype are at greater risk for vascular disease.

Similarly, Kauwell and colleagues reported data suggesting that older women with the TT genotype may be at greater risk for elevated plasma homocysteine in response to low dietary intake of folate in comparison to older women with the CC or CT genotypes (59). Studies investigating the effects of the MTHFR $677C \rightarrow T$ polymorphism on homocysteine metabolism suggest that individuals with the TT genotype may have higher folate requirements to maintain adequate folate status and prevent hyperhomocysteinemia.

Cancer

Compelling evidence from epidemiologic, animal and human studies suggests that folate status is associated with risk reduction for developing several types of cancers, including breast, cervical, pancreatic, brain, lung, and colorectal cancer (CRC) (107), although more recent evidence is contradictory to the latter. Research also suggests that folate depletion enhances
carcinogenesis, whereas large doses of supplemental folic acid, above what is presently recommended for the general population, appear to have a protective effect. Although the exact mechanism is still uncertain, the beneficial effect of folate on carcinogenesis is speculated to come from the metabolic role of folate in DNA methylation and stability (107). When there is a deficiency of folate in circulation, dTMP synthesis is impaired and results in nucleotide errors that incorporate uracil into DNA strands. This may lead to the instability of DNA strands that are associated with increased cancer risk. Moreover, folate can affect cellular SAM levels, which regulate DNA methylation and gene transcription by methylating specific cytosines in DNA (108). As a consequence of folate deficiency, SAM is depleted, which in turn induces DNA hypomethylation and potentially induces expression of cancer-prone genes (109). Research has shown that folate administration can repair DNA synthesis by reversing excessive DNA uracil misincorporation thereby reducing the number of chromosomal breaks, and supplying adequate methyl groups for DNA methylation and reducing expression of cancer-related genes (110).

Significant evidence from population cohorts from the Nurses’ Health Study (15,984 women) and Health Professional Follow-Up Study (9,490 men) supports the association between folate and CRC risk (111). These epidemiological studies revealed a 30 to 40% reduction in the risk for CRC and adenocarcinomas in participants with the highest folate intake (>700 µg/d), the majority of who were supplement users, compared to those with the lowest intake of folate (166 and 241 µg/d for women and men, respectively) (111).

In the Nurses’ Health Study, a reduction in the risk for CRC was not associated with folate intake from food sources alone (111). However, when total folate intake exceeded 400 µg/d, a 30% lower risk was observed compared to the group of women who consumed ≤200 µg/d. It was noted that of the group of women who consumed ≥400 µg/d of folate, 86%
consumed multivitamins. An apparent association between length of supplement use and risk for cancer was also noted. Women who had been consuming supplements containing folic acid for at least 15 years had a 75% decreased risk for CRC compared to non-supplement users; whereas no significant reductions in risk were observed in women who had been taking multivitamins for shorter lengths of time (111).

In contrast, more recent population-based observations from two data sets have suggested that the incidence of CRC in North America increased as a result of folic acid fortification. Mason and colleagues (112) noted a surge in absolute rates of CRC in 1996 to 1998 (US) and 1998 to 2000 (Canada), despite a downward trend in CRC incidence in both countries during the decade prior to fortification. These investigators attributed this incidence to the role of folate in nucleotide synthesis, including the vitamin’s role as a potential growth factor for neoplastic cells. Evidence indicating that high doses of folic acid can accelerate the growth of established neoplasms has been documented in previous research (113, 114). Subsequent research also noted that supplemental folic acid is only protective before neoplastic cells appear in the intestine. Once these proliferative cells are established, microscopic and macroscopic tumors develop faster as more folic acid is administered (115, 116). The observations by Mason and colleagues that rates in CRC spiked as a result of fortification suggest that fortification “unveiled” a significant number of suppressed neoplasms that would have otherwise never transformed into cancers or would have gradually evolved into cancer over a longer period of time (112).

In a double-blind, placebo-controlled, randomized clinical trial (the Aspirin/Folate Polyp Prevention Study) conducted by Cole and colleagues (117), 1,021 participants with recently removed colorectal adenomas were randomized to receive either 1 mg of folic acid or a placebo
over a period of three to five years. Analysis of the final follow-up colonoscopy did not reveal a protective effect of folic acid supplementation; rather, incidence of at least 1 recurrent advanced lesion was significantly higher for folic acid (11.6%) compared to the placebo (6.9%) \( (P = 0.05) \) (117). Further research is needed to investigate the potential of folic acid supplementation to accelerate the growth of existing neoplasms and increase risk of CRC.

In contrast to the studies by Mason et al. (112) and Cole et al. (117), the American Cancer Society, the Centers for Disease Control and Prevention (CDC), the National Cancer Institute, and the North American Association of Central Cancer Registries found that for all races/ethnicities combined in the US, favorable trends in lower incidence and mortality were noted for lung and CRC in men and women and for breast cancer in women (118). Cancer incidence data were available for up to 82% of the US population, and cancer deaths were available for the entire US population. Overall cancer death rates decreased by 2.1% per year from 2002 through 2004, nearly twice the annual decrease of 1.1% per year from 1993 through 2002. Among men and women, death rates declined for most cancers. Breast cancer incidence rates in women decreased 3.5% per year from 2001 to 2004, the first decrease observed in 20 years. Colorectal cancer incidence and death rates and prostate cancer death rates declined, with CRC death rates dropping more sharply from 2002 through 2004.

The role of folic acid supplementation in risk reduction for other cancers also is supported by evidence of the role of adequate folate intake in the reduction of breast cancer in relation to alcohol consumption. Folate status may be compromised in chronic alcohol abusers secondary to inadequate folate intake. Ethanol may also aggravate folate deficiency by impairing intestinal folate absorption, interfering with folate metabolism, or increasing renal folate excretion (119). Excessive alcohol consumption is defined as \( \geq 14 \) to 15 g/d, which is
equivalent to 5 to 6 ounces of wine and 13 to 14 ounces of beer. Data from the Nurses’ Health Study revealed that for women consuming $\geq 15$ g/d of alcohol, risk for breast cancer was lowest in those women consuming $\geq 600$ µg/d of folate from both food sources and supplements compared to women who consumed 150 to 200 µg/d of folate (61). Furthermore, a 26% reduction in risk for breast cancer was observed in women consuming alcohol and taking multivitamins compared to similar women who were not taking supplements. Additional data from the Canadian National Breast Screening Study (56,837 women) showed a 43% reduction in breast cancer risk associated with folate consumption (>300 µg/d) in women consuming $\geq 14$ g/d of alcohol compared to women with equivalent alcohol intake who consumed <225 µg/d of folate (120). These results suggest that a diet high in folate may compensate for the negative effects on folate metabolism caused by alcohol intake and may consequently reduce breast cancer risks (11). Ongoing research is still being conducted with the hope of providing better insight into the potential role of folate in cancer prevention.

**Folic Acid Fortification**

Following reports from clinical intervention studies identifying a direct association between periconceptional folic acid intake and NTD risk reduction, the US Public Health Service issued a recommendation in September 1992 that women of childbearing age (i.e., 15 to 44 years) capable of becoming pregnant should consume 400 µg of the folic acid daily to reduce the number of cases of NTD. Since then, an ongoing national public health campaign has encouraged women to consume dietary supplements containing folic acid. In addition, the FDA mandated in 1996 that all “enriched” cereal-grain products be fortified with folic acid by 1998 (2). Although the effective date was January 1, 1998, the majority of food manufacturers had implemented folic acid fortification by mid-1997. The predicted increase of daily folic acid
consumption attributable to fortification of enriched cereal-grain products was estimated to be approximately 100 µg/d.

Originally, the FDA considered several options that included fortification of enriched cereal-grain products, RTE breakfast cereals, dairy products, and fruit juices. However, analysis of folic acid intake by consumers in some non-target groups (i.e., men and elderly) showed that when fruit juices and dairy products were fortified with folic acid, in addition to cereal-grain products, RTE breakfast cereals and dietary supplements, folic acid intake exceeded the UL of 1,000 µg/d even at the lowest level of fortification. As a result of FDA’s analysis, the FDA determined that fortification should be limited to cereal-grain products, RTE breakfast cereals and dietary supplements (2).

The FDA reviewed information showing that cereal-grain products and RTE breakfast cereals are consumed on a daily routine basis by 90% of the target population (i.e., women of childbearing age). Furthermore, representatives of manufacturers in the cereal-grain industry stated that these products could be easily fortified with folic acid and that a fortification mandate would not be unfair to the industry. Thus, the FDA determined that mandatory folic acid fortification of cereal-grain products with 140 µg per 100 g, along with voluntary fortification of RTE breakfast cereals up to 400 µg per serving and dietary supplements up to 400 µg per unit or per serving, would provide increased intakes of folic acid for women in reproductive years while keeping intakes for the non-target population below the UL (2). In addition to the US, other countries that have implemented a mandated folic acid fortification program include Canada (121), Chile (122), and some Latin American countries (123).
Effect of Fortification on Folate Status of Women of Reproductive Potential

The initiation of fortification of the US food supply with folic acid has had a positive effect on the folate status of the entire population and has decreased the incidence of NTD-affected pregnancies in the US by approximately 26% (13). Dietrich and colleagues have assessed the benefit of fortification by comparing serum and RBC folate concentrations for women of reproductive age from two separate NHANES data sets. NHANES III, conducted during 1988 to 1994, reflects the time prior to folic acid fortification, and NHANES 1999-2000, reflects the time period post-fortification. Compared to pre-fortification concentrations, both serum and RBC folate concentrations increased significantly post-fortification. For women between the ages of 20 to 39 years and 40 to 59 years who did not use supplements, the mean serum folate concentration increased more than two-fold from 10.3 to 26.0 nmol/L and 11.4 to 27.1 nmol/L, respectively. These increases represent a respective 153% and 137% increase in serum folate concentrations (13).

The mean RBC folate concentration increased for women 20 to 39 years old and 40 to 59 years old from 341 to 556 nmol/L and 386 to 629 nmol/L, respectively, between NHANES III to NHANES 1999-2000. A 63% increase in RBC folate concentration was observed for both age groups of women. These increases appear to be associated with the consumption of folic acid in enriched and fortified foods (i.e., enriched cereal-grain products and fortified RTE breakfast cereals) because data for supplement users were evaluated separately (11, 17).

Jacques and colleagues (4) evaluated the effect of fortification on indices of folate status in a cross-sectional study with participants in the Framingham Offspring Cohort (men and women, ages 30 to 59). These researchers found that among nonusers of folic acid supplements, mean plasma folate concentration among individuals examined after fortification increased 46%
(10.4 to 22.7 nmol/L; P <0.001) relative to individuals examined before fortification. Furthermore, the prevalence of low folate concentrations (<7 nmol/L) significantly decreased from 22.0 to 1.7% (P <0.001). Choumenkovitch and colleagues also evaluated folate status of participants in the Framingham Offspring Cohort and observed a 38% increase in mean RBC folate concentration of subjects comparing pre- to post-fortification (3).

In addition, Lawrence and colleagues reviewed pre-existing data on serum folate concentrations in more than 98,000 blood samples submitted to Kaiser Permanente’s Southern California Endocrinology Laboratory between 1994 and 1998 to evaluate changes in serum folate concentrations since implementation of folic acid fortification of cereal-grain products (5). These researchers observed a median pre-fortification serum folate value of 28.6 nmol/L in 1994, which gradually increased by 48% during the transition period between initiation of fortification in 1996 and full implementation in 1998 to 42.4 nmol/L.

Recently the CDC assessed the trends in folate status of women and reported that blood folate concentrations among non-pregnant US women of childbearing age declined from NHANES 1999-2000 through NHANES 2003-2004 (124). Based on data from NHANES 1999-2000, 2001-2002, and 2003-2004, the median serum folate concentrations among women 15 to 44 years old were 28.6 nmol/L, 25.8 nmol/L and 24.0 nmol/L, respectively. This reduction in medium serum folate concentrations reported from 1999-2000 through 2003-2004 represented a statistically significant reduction of 16% (P <0.001). Similarly, RBC folate concentration decreased 8% (P = 0.03) from 578 nmol/L reported during NHANES 1999-2000 to 533 nmol/L reported from NHANES 2003-2004 data. Although a decline has been noted, the majority of the women participating in NHANES 2003-2004, particularly non-Hispanic Caucasian women, achieved the 2010 national health objective of a median RBC folate concentration of 500 nmol/L.
Nevertheless, this recent decline in folate status emphasizes the need for enhanced folic acid awareness for women of reproductive age.

**Effect of Fortification on Neural Tube Defects**

Folic acid fortification has a direct positive and significant effect on the reduction of NTD birth prevalence. Honein and colleagues (14) estimated reductions in NTDs in the US by evaluating data from birth certificates from 45 states and Washington, DC and reporting the number of infant birth certificates reporting births affected by either spina bifida or anencephaly. Data post-fortification revealed a decline in the prevalence of spina bifida and anencephaly by 23% and 11%, respectively. However, the data included only live births, since NTD-affected infants who were either miscarried or stillborn were not reported (14). The CDC reported that rates of NTDs have decreased 26% in the US since folic acid fortification was mandated. The prevalence of anencephaly reported on birth certificates declined from 18.38 cases per 100,000 live births in 1991 to 9.40 in 2001. The prevalence of spina bifida reported on birth certificates declined from 24.88 cases per 100,000 live births in 1991 to 20.09 in 2001 (15). Although the level NTD risk reduction observed in the US was not as high as originally expected, research has shown that the apparent positive reduction in NTD incidence has been associated with improved folate status post-fortification and supports food fortification as an effective intervention strategy for individuals (11).

In 2006, Botto and colleagues (12) assessed two crucial issues relative to the benefits and impact of folic acid in the prevention of birth defects: whether folic acid supplementation alone, without fortification, is effective in reducing the population-wide rates of NTDs, and whether the current policies can reduce the occurrence of other birth defects. These investigators used data from 15 birth registries from areas with either official supplementation recommendations of 400
µg/d of folic acid or a folic acid fortification program to assess the effectiveness of both on the rates and trends of major birth defects, including NTDs. The results revealed significant changes in the incidence of NTDs in areas with folic acid fortification, but not in areas with folic acid supplementation recommendations and no fortification program. The investigators concluded that recommending folic acid supplementation alone remains an ineffective approach in translating the known protective effect of folic acid in reducing NTD rates. In contrast, fortification appears to be effective in reducing NTDs (12).

When folic acid fortification was first implemented, the estimated increase of folic acid intake in the US food supply that resulted from consumption of folic acid enriched and fortified products was approximately 200 µg/d or two times the quantity originally predicted at 100 µg/d (16). Actual measurements of total folate content in enriched cereal-grain products have shown that many of these products contained total folate levels that were higher than the amounts required by federal regulation (125-127). Although there is a lack of consistent research evaluating the folic acid content of enriched cereal-grain products, recent research groups have reported data suggesting a decline in the amount of folic acid added to foods since the initiation of fortification. Johnston and Tamura (128) measured the folate content of white sandwich breads containing enriched flour during 2001 to 2003 and found that the mean folate content in enriched white bread significantly declined from 2001 to 2002 or 2003. In addition, Póo-Prieto and colleagues (129) analyzed the folic acid content of numerous enriched foods and found no evidence of folic acid overages in enriched products contrary to previous reports. In fact, many of the fortified foods tested tended to contain less folic acid than required by law (129).
Folic Acid Recommendations and Awareness

Despite significant reductions in the incidence of NTDs observed since folic acid fortification, the majority of women capable of becoming pregnant have not achieved the level of intake associated with NTD risk reduction (13). Therefore, increasing the number of women who consume 400 µg of folic acid daily from dietary supplements or folic acid fortified foods remains an important public health goal for NTD prevention.

Numerous public health policies have been implemented worldwide based on the strength of the evidence relating periconceptional folic acid supplementation to NTD risk reduction. In 2005, the CDC and the March of Dimes sponsored the first National Summit on Preconception Care and launched the Preconception Health and Health Care Initiative, the goal of which is to improve both the health of mothers before pregnancy and maternal and infant health outcomes (130). The recommendations from this summit were published on April 21, 2006 (131). The recommendations noted that the time has come to ensure that efforts promoting adequate folic acid intake and improved pregnancy outcomes, including NTD risk reduction, are not limited to prenatal care but should be expanded to include preconception health and health care. The panel, which included an array of health care providers, public health practitioners, and researchers, developed strategies to implement the recommendations across three areas: clinical practice, consumer roles, and public health practice (130). Current and future plans to promote folic acid include developing and distributing clinical guidelines and tools, educating consumers, integrating preconception care activities into clinical and public health programs, educating and training clinical and public health care providers, developing a research agenda, identifying, documenting, and promoting best practices, and supporting state and local initiatives (130).
However, despite these recommendations and other folic acid awareness campaigns, there does not appear to be a significant increase in the number of women taking folic acid supplements. A survey conducted by the March of Dimes recently provided evidence that only 33% of women of reproductive age (18 to 45 years) take a folic acid containing supplement daily, compared to 40% in 2004 and 28% in 1995 (17). Furthermore, reports have indicated that women not contemplating pregnancy, women between the ages of 18 to 24 years, non-white women, those less educated, and those of lower socioeconomic status have both lower folic acid knowledge and lower folic acid supplement use (132-134). The March of Dimes has also reported that only 24% of women aged 18 to 24 years took a supplement with folic acid daily, yet this population accounts for at least one third of all US births (135). In addition, although 84% of women reported awareness of folic acid, up from 78% in 2004, only 25% of women reported knowing that folic acid prevents birth defects, and only 7% of women reported knowing that folic acid should be taken preconception (17). Comparable data were reported in a survey conducted in Puerto Rico, where nationwide campaigns had been promoting folic acid for four years prior to the study. Despite the majority of pregnant women surveyed who understood the importance of folic acid (88%), regardless of whether or not they planned their pregnancy, only 32% actually took a folic acid supplement prior to conception (136).

**Analytical Methodology for Assessing Folate Status**

There are several analytical methods used to assess blood folate status. Not all researchers have used the same methodology for detecting folate concentrations, and even when research groups have used the same method, there may be differences in protocols between labs. This section reviews the two most commonly used methods.
Blood Folate Analysis

Serum and RBC folate concentrations are most often measured using the radiobinding or the microbiological assays. The microbiological assay is considered the gold standard method for determining folate concentrations in blood, urine, tissue, and food samples (137, 138). The microbiological assay uses the test organism *Lactobacillus rhamnosus* as it metabolizes the highest number of folate derivatives, including 5-methylTHF, the predominant folate form in plasma and RBC (22). Ascorbic acid and a phosphate buffer (pH 6.1) are added to the test sample and micro-organism to offer better stability and prevent the oxidative loss of labile reduced folates. Each 96-well flat bottom microtiter plate contains serial dilutions of a control sample, samples of folic acid standard, and subject samples. Samples are inoculated with *L. rhamnosus* and incubated at approximately 37°C to allow for growth of the organism. Growth of the folate-dependent microorganism is assessed by comparing the degree of turbidity of the sample compared to the turbidity of known concentrations of folic acid standard. Cell growth is determined by absorbance at 650 nanometers using a computer-interfaced microtiter plate reader and data reduction software (137). A standard curve is generated by plotting the log-linear absorbance against the folic acid standard concentration to interpolate unknown folate concentrations of samples.

The radiobinding assay also is used for blood folate analysis (139). The radiobinding assay uses a competitive folate binding protein attached to microbeads and iodine-125 [¹²⁵I]-labeled folic acid, which are used to quantify serum or RBC folate concentrations (140). In the assay, the folate binding protein has an equal affinity for the standard and the folate present in the serum or plasma. The unlabeled folate competes with the labeled folate for the limited
number of available binding sites on the folate binding protein. Thus, the level of radioactivity bound is inversely related to the concentration in the sample or standard.

The radiobinding assay is conducted by adding dithiothreitol solution to the folate tracer ($^{125}$I, borate buffer with human serum albumin, dextran, potassium cyanide, dye and preservative). This mixture is added to serum or RBC samples and heated in a water bath at 100°C for 15 minutes. Once cooled, the folate binder is added to the mixture, which is protected from exposure to light, and incubated for one hour. During incubation, endogenous folate and $^{125}$I compete for binding sites to the folate binder. Samples are centrifuged and bound folates and microbeads precipitated. The bound folate (labeled and unlabeled) accumulates in a pellet, while unbound folate is in the supernatant, which is gently discarded. The radioactivity of the pellet is measured using a scintillation gamma counter.

The microbiological assay has been the preferred method for measuring folate concentrations. However, the method is tedious and time consuming, requires microbiological expertise, and growth of the test organism may be inhibited by non-folate substances, such as prescription medications (e.g., antibiotics or methotrexate) taken by subjects. The advantages of using the radiobinding assay include the fact that it is relatively inexpensive, easy to perform, and has a high specificity towards folate isomers. One limitation of the radiobinding assay is that it gives lower folate values than the microbiological assay since this method underestimates certain folate forms as evidenced by recovery analysis (61).

Another method used to assess folate concentrations in biological samples uses liquid chromatography-tandem mass spectrometry (LC-MS/MS). Fazili and colleagues (61) compared serum folate species analyzed using LC-MS/MS with total folate measured by the microbiological and Bio-Rad radiobinding assays. Advantages of the LC-MS/MS method
compared to the microbiological assay are that it provides information on the different folate species in addition to total folate and is less prone to interferences such as antibiotics. Mean and median total folate concentrations measured by LC-MS/MS and microbiological assays were generally in agreement, but the radiobinding assay values were much lower (-29% relative to LC-MS/MS values). Fazili and colleagues concluded that the radiobinding assay produces much lower results, on average, probably due to underrecovery of 5-methylTHF, which is the main circulating form of folate (61). The Bio-Rad QuantaPhase II radiobinding assay has been used to measure blood folate in NHANES for 25 years. Due to the fact that the radiobinding assay is being discontinued in 2007, NHANES is switching to the microbiological assay for all samples, and will use the LC-MS/MS method for a subset of the population.

**Plasma Homocysteine Analysis**

Determination of plasma homocysteine concentration is generally measured as total homocysteine concentration. The most commonly used approach for quantifying homocysteine concentration is capillary gas chromatography-mass spectrometry with selected ion monitoring (141). The term “total homocysteine” as applied to biological samples (i.e., serum or plasma) refers to the sum of homocysteine “that is linked via disulfide bond formation in a variety of compounds that include homocysteine, homocysteine-cysteine mixed disulfide, proteins via their cysteine moieties, and peptides such as glutathione via their cysteine moieties (141).” The assay is conducted by adding 2-mercaptoethanol to the sample in order to chemically reduce and release endogenous homocysteine from proteins and other disulfides. The samples are analyzed using a gas chromatograph-mass spectrometer equipped with a falling needle injector. Quantitation is based on the ratio of the areas of the base peak ion for homocysteine to the areas of the base peak ion for the derivative of the respective stable isotope internal standards (141).
MTHFR Genotype Determination

Since the discovery of single nucleotide polymorphisms (SNP), technological advances have been made in methods for determining subject genotypes for these SNPs. A common method for genotype determination involves polymerase chain reaction (PCR) to amplify the desired region or target sequence of the DNA strand. Once the preferred region is magnified, specific restriction enzymes are added depending on the SNP being studied. The SNP of greatest interest with regard to folate metabolism is the MTHFR 677 C→T that results in the substitution of the amino acid alanine with valine in the gene product (19).

In order to achieve unambiguous determination of all SNP variations, Howell and colleagues (142) created a new SNP scoring method known as dynamic allele-specific hybridization (DASH™). Using this approach, the target sequence is amplified by PCR with one biotinylated primer. The biotinylated product strand is bound to a streptavidin-coated microtiter plate well, and the non-biotinylated, unbound strand is removed with alkali. An oligonucleotide probe, specific for the desired allele, is hybridized to the bound DNA at low temperature. This forms a probe-target duplex that interacts with a double strand-specific intercalating dye. The temperature is increased and the denaturation temperature and fluorescence emitted from the dye, which is proportional to the amount of double-stranded DNA present, are recorded. For analysis, the negative derivatives of the melting curves are plotted to show a lower temperature peak for a homozygous allele mismatch, a higher temperature peak for a homozygous match, or both peaks for a heterozygous sample (142).
Dietary Intake Assessment

USDA National Nutrient Database

The USDA National Nutrient Database for Standard Reference is the major source of food composition data in the US and provides the foundation for most public and private sector databases. The 2007 release (Release 20) contains nutrient data for 7,517 food items for up to 140 food components including vitamins, minerals, amino acids, and fatty acids (143). Major applications of the USDA National Nutrient Database include the development of the Food and Nutrient Database for Dietary Surveys by the Food Surveys Research Group, which is used to process dietary data records from the survey “What We Eat in America”, the dietary intake component of NHANES. The version of the USDA National Nutrient Database used to estimate nutrient intakes for subjects in the present study was Release 17 (2004), which contains nutrient data for 6,839 food items for up to 128 food components (8). In contrast to older database releases, a unique component of the Release 17 nutrient composition database is that it provides data for four categories of dietary folate including folic acid (µg/d), natural food folate (µg/d), total folate (µg/d), and DFE (µg DFE/d). Until recently, researchers reporting folate intake from NHANES data used an older version of the nutrient database that only provided nutrient composition data for total folate (µg/d).

Food Frequency Questionnaire

A food frequency questionnaire (FFQ) is often used as one method for determining usual dietary intake. The FFQ is the most practical and economical method for collection of comprehensive dietary data and is most commonly used in large epidemiological studies (144). Compared with other dietary assessment methods, such as 24-hour dietary recalls or multiple-day food records, the FFQ obtains less detailed information regarding food type or portion size.
Although alternative methods are more detailed, FFQ is self-administered and is designed to capture usual dietary intake, unlike records or recalls. The FFQ often generalizes food intake into food groupings, such as fortified cereal, whereas a 24-hour recall method or multiple-day food record provides more detailed information such as brand names of foods consumed. The FFQ has predetermined questions and portion sizes to which an individual responds. This type of questionnaire can be electronically scanned and the information uploaded by software for further analysis (144). The limitation with this dietary assessment method is that there is little opportunity for individuals to list dietary intake data not included as part of the questionnaire. Concern regarding measurement error has stimulated numerous validation studies comparing nutrient intakes estimated from FFQs with those estimated by other methods.

Validation of this method, which is the process of determining if estimated nutrient intake is true to actual intake, is essential to providing valid estimates of dietary intake for observational epidemiological studies and clinical trials. The National Cancer Institute (NCI) Diet History Questionnaire (DHQ) is an FFQ developed by staff at the Risk Factor Monitoring and Methods Branch (144). The DHQ captures data on frequency of consumption and estimated portion size from a list 124 individual food and beverage items over the past year. The 124 food items currently included in the DHQ were selected based on the work of Subar and colleagues (145) who originally categorized 5,261 individuals foods with 170 food groups. The DHQ has been refined over the years based on results from intensive cognitive interviewing. Subar and colleagues (146) conducted cognitive evaluation of various approaches to asking about usual dietary intake and identified ways to improve the FFQ so completing the FFQ would be easier while the accuracy of responses would be enhanced. Numerous cognitive issues in FFQs have been addressed in the DHQ, including comprehension, order of food items, intake of seasonal
foods (e.g., fruits and vegetables), specificity of low-fat or fat-free food items, intake averages from multiple food items, and format. Other improvements to the DHQ include modification of portion size categories based on analysis of data and responses from 10,019 adults in the CSFII (1994-1996) study (147). Rather than categorizing portion sizes as small, medium, or large, portion size choices were changed to provide more detail about specific intake of a food item, such as “less than 1 cup”, “1 to 2 cups”, and “more than 2 cups”. These changes have ultimately improved the validity and accuracy of nutrient intake estimated by the DHQ (146, 148).

In 2001, Subar and colleagues (144) evaluated the NCI DHQ against repeated 24-hour recalls and compared to the Block and Willett FFQ, and ultimately validated the DHQ as a suitable method for estimating nutrient intake. The Block FFQ is an eight-page questionnaire that inquires about 106 food items, and categorizes portion size choices as small, medium, or large (144). This questionnaire also has eight questions concerning use of added fats or low-fat foods, 13 dietary supplement questions, five summary questions, and six questions on eating at restaurants. The Willett FFQ asks about 126 foods over four pages, including ten questions each on supplement fat intake. This questionnaire does not include separate portion size questions, but rather categorizes responses into intake frequency of a reference portion size. This validation study consisted of 1,301 men and women who completed four 24-hour recalls during one session over the telephone. The subjects were then randomized to complete two FFQs, either the DHQ and Block FFQ or the DHQ and Willett FFQ. Researchers found that the DHQ was more accurate compared to the 24-hour recall method than the Block or Willett FFQ in determining nutrient intake (144).
Assessment of Folate/Folic Acid Intake in Women of Reproductive Potential

Assessment of folate/folic acid intake among men and women typically has been evaluated using data from NHANES or the Framingham Offspring Cohort Studies. However, there are few studies that have evaluated folate/folic acid intake for women of reproductive potential who do not consume supplements containing folic acid.

Choumenkovitch and colleagues (125) examined food and nutrient intake of 1,480 individuals who participated in the Framingham Offspring Cohort Studies to assess folic acid intake from fortification in the US. Participants completed a 126-item semi-quantitative FFQ developed by Willett et al. (149) that allowed for estimation of usual nutrient intakes during the previous year. Among the 186 women (ages 30 to 80 years) who did not use supplements, intake of folic acid after exposure to fortification increased by a mean of 192 µg/d (125). Furthermore, 9% of non-supplement consuming women post-fortification compared to 55% of women pre-fortification consumed less than the EAR (<320 µg DFE/d) (125). A study by Dietrich and colleagues (13) who also used data from NHANES 1999-2000 estimated mean total folate intake for women between the ages of 20 to 39 years and 40 to 59 years to be 294 µg/d and 302 µg/d, respectively.

A study by Yang and colleagues (6) used NHANES 2001-2002 data to compare differences in folic acid intake in women of childbearing age (ages 15 to 49 years) in the US after folic acid fortification. Information on folic acid intake from fortified foods and intake of food folate was obtained from a single 24-hour food recall questionnaire. Nutrient values were calculated using the USDA National Nutrient Database (Release 17). Yang and colleagues reported that estimated mean consumption of folic acid from fortified foods in women of childbearing age was 128 µg/d. Approximately 8% of non-supplement consuming women
reported consuming ≥400 µg/d of folic acid from fortified foods alone, and 26% of the women surveyed reported taking ≥400 µg/d of folic acid from supplements in the previous month. The mean food folate intake from dietary sources was 151 µg/d. Yang and colleagues concluded that at the present level of folic acid fortification, most women need to take a supplement containing folic acid to achieve the IOM recommendation for 400 µg/d of folic acid (6). However, a limitation to this study is that it did not characterize sources of folate/folic acid. Further research is needed to identify which components of the diet contribute to folic acid intake in women of reproductive potential who do not consume a folic acid supplement or supplements containing this nutrient.
Figure 2-1. Folate/folic acid structures adapted from Present Knowledge in Nutrition (1). Folic acid consists of a para-aminobenzoic acid molecule linked on one side by a methylene bridge to a pteridine ring, and joined by peptide linkage to a glutamic acid molecule on the other side. Naturally occurring food folates exist in various chemical forms, containing a side-chain composed of two to ten additional glutamate residues (n) joined to the first glutamic acid. The pteridine ring of the folate/folic acid structure can be reduced to form dihydrofolate acid and tetrahydrofolic acid (THF). Folate coenzymes are formed by substitution of one-carbon units at the N5, N10, or both positions (R) to the polyglutamyl form of THF.
Table 2-1. Definitions of Dietary Reference Intake (DRI) recommendations.

<table>
<thead>
<tr>
<th>DRI recommendation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estimated Average Requirement (EAR)</td>
<td>A daily nutrient intake value that is estimated to meet the requirement of half the healthy individuals in a group.</td>
</tr>
<tr>
<td>Recommended Dietary Allowance (RDA)</td>
<td>The average daily dietary intake level that is sufficient to meet the nutrient requirement of nearly all (97 to 98%) healthy individuals in a particular life stage and gender group. Individuals should aim for this intake level.</td>
</tr>
<tr>
<td>Adequate Intake (AI)</td>
<td>A recommended daily intake value based on observed or experimentally determined approximations of nutrient intake by a group (or groups) of healthy people that are assumed to be adequate—used when an RDA cannot be determined.</td>
</tr>
<tr>
<td>Tolerable Upper Intake Level (UL)</td>
<td>The highest level of daily nutrient intake that is likely to pose no risk of adverse health effects to almost all individuals in the general population. As intake increases above the UL, the risk for adverse health effects increases.</td>
</tr>
</tbody>
</table>

Adapted from the Institute of Medicine (10)

Table 2-2. Folate intake recommendations for men and non-pregnant women ≥19 years.

<table>
<thead>
<tr>
<th>DRI recommendation</th>
<th>Physiological folate requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estimated Average Requirement (EAR)</td>
<td>320 µg DFE/d</td>
</tr>
<tr>
<td>Recommended Dietary Allowance (RDA)</td>
<td>400 µg DFE/d</td>
</tr>
<tr>
<td>Adequate Intake (AI)</td>
<td>Not applicable</td>
</tr>
<tr>
<td>Tolerable Upper Intake Level (UL)</td>
<td>1,000 µg synthetic folic acid/d</td>
</tr>
</tbody>
</table>

Adapted from the Institute of Medicine (10)
Figure 2-2. One-carbon metabolism folate-dependent pathway adapted from Present Knowledge in Nutrition (1).
CHAPTER 3
MATERIALS AND METHODS

Study Design and Methods Overview

Approximately 1,000 healthy male and female adult volunteers were recruited through newspaper advertisements, flyers, and radio broadcasts. Prospective subjects were screened by phone using a screening questionnaire (Appendix A) to determine their eligibility. Individuals were included in the study if they met the following criteria: (a) age 18 to 49 years, (b) currently not using prescription medications (birth control medication was permitted), (c) low alcohol intake (< one drink per day), (d) non-smoker, (e) no history of chronic disease, (f) non-pregnant, (g) non-lactating, (h) no use of supplements within the last 6 months, and (i) no major dietary changes within the last 3 years. Individuals also were screened for consumption of highly fortified RTE cereals containing 400 µg/d of folic acid (e.g. Total®), and were not eligible to participate if they indicated they consumed these highly fortified RTE cereals. Eligible male and female volunteers (n = 388) were chosen to participate in the study.

Subjects were scheduled to have a fasting blood sample drawn by a certified phlebotomist and to receive comprehensive detailed verbal and written instructions lasting 15 to 20 minutes regarding the completion of the DHQ (Appendix B). Subjects took the DHQ and prepaid mailing envelopes home with them and were asked to mail the completed DHQ to the primary investigator within 2 weeks of receiving the in-person instructions.

Fasting blood samples were collected and processed for multiple analyses, including serum and RBC folate concentrations, plasma homocysteine concentration, and MTHFR genotype. Folate intake (total folate, DFE, folic acid, and food folate), in addition to other nutrients were assessed using the DHQ. If subjects completed all aspects of the study they were compensated
$50 for their participation. The University of Florida Institutional Review Board approved this protocol, and all subjects signed informed consent forms.

**Human Subject Procedures**

Eligible subjects enrolled in the study were scheduled for an appointment and were instructed to report to the Food Science and Human Nutrition building on the University of Florida campus following an overnight fast. Upon arrival, the purpose of the study was discussed with each subject who was then asked to sign the approved informed consent form. To ensure the privacy of personal health information, subjects who granted consent were assigned a subject identification number. As a follow up to questions asked during the phone screening, subjects were again asked about supplement use during the past 6 months. Subjects acknowledging that they had taken supplements were asked to provide more detailed information. This information was recorded on each subject’s data form.

Blood samples were collected by a phlebotomist for analysis of biochemical indices. Following the blood draw, subjects were given a snack and were instructed on how to complete the DHQ. During the 20 minute DHQ instruction session, each small group of participants received detailed verbal and written descriptions of how to complete the DHQ. Subjects were also taught how to report accurate portion sizes by reviewing how to read food labels and how to use measuring cups and spoons to visually interpret portion sizes correctly.

Additional handouts were reviewed and given to subjects to assist them in properly completing the DHQ. These handouts included a detailed drawing illustrating the proper method for marking or changing an answer (to ensure that the forms would scan correctly) and handouts on “Caffeinated versus Non-caffeinated Beverages,” “Fortified Cereals”, and “Seasonal Fruits
and Vegetables” (Appendix C). Subjects were given the opportunity to ask questions regarding the study and the DHQ.

**Dietary History Questionnaire**

This study was a collaborative effort between researchers at the University of Florida and the University of North Carolina (UNC), Chapel Hill. The original DHQ from NCI was modified with the addition of questions pertaining to beef consumption, beef-containing food products, and vitamin B12 fortified foods since the primary objective of this study was to assess vitamin B12 intake in the diet. The DHQ data also were used to evaluate the contributions of folate and folic acid containing foods to overall folate intake. The total intake of each nutrient was analyzed using the Diet*Calc Analysis program provided by NCI. This software program can be downloaded free of charge from the NCI website (www.riskfactor.cancer.gov). The nutrient database within the Diet*Calc software is from the USDA National Nutrient Database Standard Reference, Release 17 (8). The Diet*Calc Analysis program nutrient database had been updated in August 2004.

**Processing the Dietary History Questionnaire**

Subjects were instructed to mail the completed DHQ within 2 weeks of the initial instruction. Subjects who failed to return the DHQ within this time frame were contacted by phone or email. All returned DHQs were reviewed to ensure they were completed entirely and that no questions were missing a response. Marked responses were not reviewed at this time to prevent bias. Subjects who neglected to respond to one or more DHQ questions were contacted to obtain the missing information. The completed paper versions of the DHQ were mailed to Optimal Solutions Corporation, Lynbrook, New York, in groups of 100 where they were scanned electronically. After the scanning process was finished, Optimal Solutions Corporation mailed
the DHQs and electronic data in the form of an American Standard Code for Information Interchange (ASCII) text file to collaborators at UNC. Investigators at UNC uploaded the files into the Diet*Calc Analysis program to analyze the nutrient intake of each subject based on their DHQ responses.

Analysis of Nutrient Intakes

After the paper version of the DHQ was electronically scanned, each food item or question included in the DHQ was linked to a predetermined Food Identification Number (FIN). Each FIN had defined nutrients for gender and serving size. The FINs corresponding to the DHQ food items and questions were linked to the Diet*Calc Nutrient and Food Group database. The Diet*Calc Analysis program used the FIN associated with each response on the DHQ to evaluate the nutrient intake for each participant based on gender and serving size. When Diet*Calc identified a FIN from the ASCII text file, the software used the database to calculate an individual’s nutrient intake. The FINs and corresponding nutrient values estimated using each individual’s responses to questions on the DHQ were entered into a Microsoft Excel spreadsheet. The Diet*Calc software produced data files that were available for analysis: the details.txt file and the results.txt file. The details.txt file was an expanded version of the data calculated from the ASCII text file and allowed investigators to compare FINs to individual nutrient intake and daily frequency of intake. The results.txt file was a condensed version that provided combined daily nutrient intake values for each subject regardless of the food source.

Dietary History Questionnaire Instruction Pretest

To ensure that the instructions and handouts on how to complete the DHQ would be clear to the subjects, a pretest of the DHQ instructions was conducted with a group of 20 graduate students in the Food Science and Human Nutrition Department and five vegetarian and vegan
consumers prior to starting the study. This pretest group was instructed using the same script to be used during the study. The students were asked to follow the instructions and complete the DHQ as if they were participants in the study. They were also given an additional survey that asked questions regarding the length of time needed to complete the DHQ, if any regularly consumed foods were missing from the DHQ, and if they had any other comments to improve the instructions and/or the DHQ instrument. Feedback from this group was reviewed and incorporated into the DHQ and DHQ instructions.

**Blood Sample Collection and Processing**

Fasting blood samples were collected for each participant by a phlebotomist (Vacutainer® Blood Collection Set; Becton Dickinson, Vacutainer® Systems; Franklin Lakes, NJ). All blood samples were processed within one hour of collection. A total of 70 ml of blood were obtained from each subject.

Blood for serum samples was collected in 8.3 ml serum separator gel clot activator tubes (Vacutainer®, Becton Dickinson, Rutherford, NJ) and kept at room temperature for 30 to 60 minutes to allow time for clotting. Serum was obtained by centrifuging the tubes at 650 x g for 15 minutes 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 (Vacutainer®, Becton Dickinson, Rutherford, NJ). Blood for plasma homocysteine was kept on ice prior to processing. A small aliquot of whole blood held at room temperature was diluted 20-fold in 1 mg/ml ascorbic acid and aliquoted into 200 µl samples and frozen for measurement of RBC folate concentration. The iced blood was centrifuged at 2000 x g at 4°C for 30 minutes.
The plasma from these samples was frozen and used to measure the plasma homocysteine concentration. Following removal of the plasma, the samples were used to extract DNA to be used to determine genotype for the MTHFR 677 C→T polymorphism. Aliquots were frozen at -30°C for subsequent analysis of serum and RBC folate concentrations.

**Analytical Methods**

**Identification of Food Groups from the Dietary History Questionnaire**

All foods included in the DHQ were categorized into specific food groups: beef, pork/other meat, poultry, dairy, eggs, seafood, cereal, soy, meal replacements, mixed dishes with meat type unknown, non-dairy fats, beans, rice/pasta, soups/sauces, breads/crackers/cakes/pies, nuts/seeds, vegetables, fruit, syrup/honey/gelatin/candy, and beverages. A new set of food categories were derived including the following: (1) enriched cereal-grains, (2) fortified cereals and bars, (3) vegetables, (4) fruit, (5) juice (only orange and grapefruit), (6) legumes and nuts, (7) dairy, (8) eggs, (9) meat, (10) combination foods, (11) snacks, (12) miscellaneous, (13) other. Foods that were fortified with folic acid but consumed minimally were grouped together as (12) miscellaneous, and foods that were not fortified and contained minimal levels of food folate were grouped together in the (13) other category. Table 3-1 describes the types of food included in each food category. Although we attempted to screen out individuals who consumed highly fortified RTE cereals, individuals that later reported consumption of these cereals were included in the final analysis. Intake of highly fortified RTE cereals was included in the fortified cereals and bars food category. The nutrient composition database used to calculate nutrient estimates from the DHQ was the USDA National Nutrient Database Standard Reference Release 17 (Diet*Calc Nutrient and Food Group database). The nutrient database provided data for four
categories of dietary folate intake, including folic acid (µg/d), food folate (µg/d), total folate (µg/d) and DFE (µg DFE/d), from foods in each of these new food categories.

**Serum and RBC Folate Concentrations**

The serum and RBC folate concentrations of all subjects were determined using the MP Biomedicals, Inc. SimulTRAC-S® Radioassay Kit (Orangebury, New York), a competitive protein binding assay previously described in detail. Folate concentrations were inversely related to the measured radioactivity. Serum and RBC folate concentrations >12 nmol/L and >317 nmol/L, respectively, represented the lower limit of normal values for this study.

**Homocysteine Concentrations**

Samples to be analyzed for homocysteine were sent to Dr. Sally Stabler, University of Colorado Health Sciences Center, Division of Hematology (Denver, CO). Total homocysteine concentration was quantified using a gas chromatograph-mass spectrometer (141). A plasma homocysteine concentration ≥12 µmol/L was considered elevated when interpreting the results for this study.

**MTHFR Genotype Determination**

Samples were sent to DynaMetrix Limited, University of Leicester (United Kingdom, where primers and probes were designed for the MTHFR 677C→T polymorphism and analyses were performed using the DASH™ method with DynaScore Software v. 0.7.7 (http://www.dynametrix-ltd.com).

**Statistical Methods**

The initial statistical analysis determined basic means and standard deviations for demographic data, folate intake, and blood folate and homocysteine concentrations for the overall population. Another initial statistical analysis was conducted to determine if there was a
relationship among any of the covariates of interest, which included gender, genotype, age, and body mass index (BMI). For the continuous variables (i.e., BMI and age), a one-way analysis of variance was used to determine whether the mean BMI and mean age differed between males and females. A Chi-square test was used to determine whether the proportion of responses in each of the categories differed among groups when analyzing categorical response variables. All possible pairings were run, and only two pairs were found to be correlated. Significant differences in BMI were detected between males and females with males having a higher mean BMI compared to females. BMI also was found to be positively correlated with age. Since BMI and gender and BMI and age were both highly correlated, BMI was not used in the analysis as correlations could lead to confusing results. Therefore, only gender and age were used as covariates in the analysis. Linear regression analysis was used to test for relationships between folate/folic acid intake and folate and homocysteine concentrations. Generalized linear models (GLM) were used to conduct the rest of the analyses to determine correlations for given response variables.

The data used in the analyses were naturally bound at zero, because individuals cannot have either negative intake or blood concentrations. This resulted in data that were non-normal and positively skewed to the right. To correct for this, the data were transformed to the natural log scale. The GLM were used on the transformed data. The estimated least squares means obtained from the GLM were retransformed to original scale along with their appropriate confidence bounds. The estimated least squares means also are the means that are corrected for the covariates of interest (age and gender). Normalized data were used consistently in reporting the results of the analyses.
<table>
<thead>
<tr>
<th>Food group</th>
<th>Type of foods included</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enriched cereal-grains</td>
<td>Breads (enriched breads, rolls, bagels, muffins, croissants, dumplings, biscuits, dessert breads, pancakes, waffles, French toast)</td>
</tr>
<tr>
<td></td>
<td>Cakes, cookies, doughnuts, Danish, sweet rolls, pies, cobblers, crisps, enriched with cereal-grain</td>
</tr>
<tr>
<td></td>
<td>Enriched rice</td>
</tr>
<tr>
<td></td>
<td>Enriched pasta, noodles, pasta with meatless red sauce</td>
</tr>
<tr>
<td>Fortified cereals and bars</td>
<td>Fortified ready-to-eat cereals, hot breakfast cereals, cereal bars, granola bars, power bars, slim fast bars</td>
</tr>
<tr>
<td>Vegetable</td>
<td>Vegetables, vegetable juice, tomato juice, tomato sauce without meat, vegetable medley</td>
</tr>
<tr>
<td>Fruit</td>
<td>Fruit, fruit juice, fruit drinks (other than orange/grapefruit juice)</td>
</tr>
<tr>
<td>Juice (orange and grapefruit)</td>
<td>Orange and grapefruit juice</td>
</tr>
<tr>
<td>Legumes and nuts</td>
<td>Legumes, soup with legumes, chili without meat, nuts/seed, nut butters</td>
</tr>
<tr>
<td>Dairy</td>
<td>Dairy products (cheese, yogurt, milk, milkshakes, ice cream, frozen yogurt, cheesecake, sour cream, puddings/custards)</td>
</tr>
<tr>
<td>Eggs</td>
<td>Whole eggs, egg salad, egg whites, egg substitute</td>
</tr>
<tr>
<td>Meat</td>
<td>Meat, fish, poultry, meat/fish sauces, liver (all meats except products prepared with enriched cereal-grains [e.g., breaded, batter dipped, and meatloaf])</td>
</tr>
<tr>
<td>Combination</td>
<td>Combination foods (e.g., Mexican food, pizza, macaroni and cheese, chili with meat, lasagna with and without meat, pot pies, egg rolls, pasta salad, pasta with meat sauce)</td>
</tr>
<tr>
<td></td>
<td>Breaded meats (meats and fish, breaded, batter-fried or meatloaf with enriched flour)</td>
</tr>
<tr>
<td>Snacks</td>
<td>Snacks (chips, pretzels, crackers, popcorn)</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>Other fortified foods eaten minimally (beer, fortified vegetarian meals (e.g., Boca® entrée, burger, breakfast sausage, chicken, ground), tofu/soy products, soymilk, liquid meal replacements, fortified egg substitute)</td>
</tr>
<tr>
<td>Other</td>
<td>Other foods with negligible folate (fats/oils, alcohol [except beer], dressings, sugars, gelatins/jams/jelly, candy, soda, tea, coffee without cream, rice milk)</td>
</tr>
</tbody>
</table>
CHAPTER 4
RESULTS

Characteristics of Study Population

Three hundred eighty-eight subjects were enrolled in this study and 302 subjects (140 males, 162 females) were eligible for inclusion in the final analyses. Subjects excluded after enrollment included 62 subjects who reported supplement use within the past six months and six who failed to return their DHQ. Data from another 18 subjects were excluded because estimates of their total caloric intake derived from their DHQ data was less than 600 calories (n = 9 females) or greater than 5,000 calories (n = 9 males). These calorie levels are considered improbable, so these subjects were excluded from the study. Of the 302 eligible subjects, the mean daily caloric intake (mean ± SD) for males and females was 2,382 ± 958 and 1,744 ± 805, respectively; males consumed significantly more calories per day than females (P < 0.0001).

The demographic characteristics of males and females in this study population are presented in Table 4-1. The mean age (years) and BMI (kg/m²) (mean ± SD) for all study participants were 26.0 ± 7.7 and 23.5 ± 4.1, respectively. The education level of all subjects was 15.0 ± 2.4 years, which is equivalent to status as a junior at the university level. Body mass index was the only variable that was significantly different between genders with males having a higher BMI (P < 0.0001) than females.

All eligible participants were categorized by their genotype for the MTHFR 677C→T single nucleotide polymorphism. The number of subjects within each genotype group was as follows: CC genotype (n = 151), CT genotype (n = 118), and TT genotype (n = 31). Two subjects had unknown genotypes. The subjects with known genotype data included 140 men (68 CC, 56 CT, 16 TT) and 160 women (83 CC, 62 CT, 15 TT).
Dietary Folate Intake and Gender

In relation to objective 1, four categories of folate/folic acid intake were analyzed from the DHQ data. These categories included total folate, DFE, folic acid, and food folate. After adjusting for differences in age, the mean intake for each folate category (µg/d and µg/1,000 kcals/d) was evaluated for males and females. Mean daily folate/folic acid intake (µg/d) for males and females among the four folate categories is presented in Table 4-2 and illustrated in Figure 4-1. Unadjusted for calories, mean total folate, DFE, folic acid and food folate intake were significantly (P <0.01) higher for males than females.

Mean folate/folic acid intake for males and females was further analyzed after adjusting for calories (µg/1,000 kcals/d), the data for which are presented in Table 4-3 and illustrated in Figure 4-2. After adjusting for caloric intake, females had significantly higher mean intakes (µg/1,000 kcals/d) of total folate (P = 0.02), DFE (P = 0.05), and food folate (P <0.01) than males. There was no statistically significant difference between males and females for mean folic acid intake (µg/1,000 kcals/d) (P = 0.9).

Dietary Folate Intake and Genotype

Mean folate intake (µg/d) of subjects by MTHFR 677C→T genotype was compared for each folate category (Table 4-4) and adjusted for age and gender. As expected, no significant differences were detected among genotype groups for intakes of total folate (P = 0.7), DFE (P = 0.9), folic acid (P = 0.9), or food folate (P = 0.8). Similarly, when intake was expressed as µg/1,000 kcals/d significant differences among genotype groups were not detected for total folate (P = 0.6), DFE (P = 0.9), folic acid (P = 0.9), or food folate (P = 0.9) intake (Table 4-5).
Dietary Folate Equivalents, Folic Acid and Recommended Intakes

Relative to objective 2, total daily folate intake of males and females expressed as DFE was compared to the Recommended Dietary Allowance (RDA) of 400 µg DFE/d. A significantly higher proportion of males (81.4%) consumed the RDA for folate compared to females (67.9%) (P <0.01) (Figure 4.3). In addition, female subjects’ intake of diet-derived folic acid, including folic acid from fortified ready-to-eat (RTE) cereals, was compared to the recommended folic acid intake for women of reproductive potential (i.e., 400 µg/d). Only 3.1% of females (n = 5) met this recommendation (Figure 4-4). Of the remaining female subjects, 24.7% (n = 40) consumed 200 to 399 µg/d folic acid, and 72.2% (n = 117) consumed <200 µg/d folic acid. Consumption of folic acid did not exceed the UL (≥1,000 µg/d) for any of the male or female participants. The highest estimated intake of folic acid for any male or female subject was 947 µg/d and 665 µg/d, respectively.

Contribution of Folate from Food Groups

In relation to objective 3, to analyze the contribution of various foods to dietary folate intake, foods included in the DHQ were categorized into thirteen categories. A comprehensive descriptive list of foods within each food category is presented in Table 3-1. Foods were categorized based on type, folic acid enrichment/fortification status, and the amount of naturally occurring food folate. Mean daily total folate intake (µg/d) contributed by various food sources is presented in Table 4-6. Both genders consumed the greatest amount of total folate from enriched grain products, and fortified cereals and bars, including fortified RTE breakfast cereals, vegetables, and legumes and nuts. Mean daily DFE intake (µg DFE/d) contributed from various food categories is presented in Table 4-7. The largest contributors of DFE for both genders included fortified cereals and bars, enriched grain products, vegetables, and combination foods.
Mean daily folic acid intake (µg/d) contributed from various food categories is presented in Table 4-8. Both males and females primarily consumed folic acid in their diet from enriched grain products, fortified cereals and bars, and combination foods. Lastly, mean daily food folate intake (µg/d) contributed from various food categories is presented in Table 4-9. Vegetables were the largest contributor of food folate, two-fold higher than any other food source. However, other good sources of food folate for both genders included legumes and nuts, and fruits.

Within each folate category, no significant differences between males and females were detected in the folate/folic acid contribution from various food categories except for the snack category. The folate/folic acid contribution from snack foods was significantly higher (P <0.02) for females than males; however, snack foods were not a large contributor to the overall intake of folic acid or food folate.

The proportion of total folate, DFE, folic acid and food folate intake provided by different food categories to total intake is illustrated for males and females in Figures 4-5 and 4-6, respectively. Enriched grains (24.4%), vegetables (21.8%), and fortified RTE cereals and bars (12.5%) provided the majority of total folate for males, whereas vegetables (27.6%) provided the most total folate in females, followed by enriched grains (20.4%) and fortified RTE cereals and bars (13.3%). Enriched grains contributed the most to DFE intake for both males (26.2%) and females (22.0%), followed by vegetables (17.0%) and fortified RTE cereals and bars (16.4%) for males, and similarly, vegetables (21.6%) and fortified RTE cereals and bars (19.0%) for females. The food categories that supplied the most folic acid for males and females included enriched grains (41.1%; 41.9%, respectively), fortified RTE cereals and bars (29.3%; 36.0%, respectively), and combination foods (17.5%; 14.8%, respectively). The largest contributors of
food folate for males and females included vegetables (31.6%; 38.4%, respectively) and legumes and nuts (16.2%; 14.4%, respectively).

With the exception of vegetables, the greatest contributors of folic acid intake (i.e., enriched cereal-grain products, fortified cereals and bars, and combination foods that included “enriched” ingredients) were also the greatest contributors to DFE intake. Without folic acid fortification of these products, DFE intake would have been solely provided by food folate intake from dietary sources (e.g., vegetables, legumes and nuts, and fruit). Furthermore, DFE intake for males and females would have been 293 µg DFE/d and 249 µg DFE/d, respectively, which would have been insufficient to meet the RDA.

**Folate Intake from Fortified Ready-to-Eat Cereals**

Intake of DFE and folic acid consumed from fortified RTE cereals was compared to the total daily DFE and folic acid intake to determine what proportion of DFE and folic acid in the diet comes from fortified RTE cereals. The percentage of males and females who consumed fortified RTE cereals was 91.4% and 96.9%, respectively. Of these subjects, 50% and 58% of males and females, respectively, consumed highly fortified RTE cereals. For males, 41.6% of total DFE intake and 23.0% of total folic acid intake came from consumption of fortified RTE cereals (Figure 4-7); for females, 37.8% of total DFE intake and 20.9% of total folic acid intake came from consumption of fortified RTE cereals (Figure 4-8). The mean DFE intake from fortified RTE cereals alone for males and females was 103 µg DFE/d and 97 µg DFE/d, respectively. The mean folic acid intake from fortified RTE cereals for males and females was 59 µg/d and 56 µg/d, respectively.
Folate and Homocysteine Concentrations and Gender

Relative to objective 1, mean serum folate, RBC folate, and homocysteine concentrations were determined for males and females, adjusted for age, and are presented in Table 4-10. No significant differences in serum folate (P = 0.3) or RBC folate (P = 0.2) concentrations were detected between males and females. Mean serum folate and RBC folate concentrations for both genders were above the limits considered normal for the radiobinding assay (>13.6 nmol/L; >317 nmol/L, respectively) (Figures 4-9 and 4-10). Although males had a significantly higher mean homocysteine concentration compared to females (P <0.0001), mean homocysteine concentrations for males and females were in an acceptable range (<12.0 µmol/L) (Figure 4-11).

Each subject’s serum folate, RBC folate, and homocysteine concentrations were compared against the limits considered normal for each metabolite. Only one male subject fell below the recommended limit for serum folate (>13.6 nmol/L) with a concentration of 11.7 nmol/L. The participants whose values were below the minimum recommended concentration for RBC folate (>317 nmol/L) included 1 male subject (289 nmol/L) and 3 female subjects (179, 259, and 278 nmol/L). Nine male subjects had elevated (>12.0 µmol/L) homocysteine concentrations (12.4 to 45.7 µmol/L). Of these males, 4 subjects had homocysteine concentrations >14 µmol/L (21.6, 27.0, 29.6, 45.7 µmol/L). Three female subjects had high homocysteine concentrations (12.4, 12.9, 13.0 µmol/L). The one male subject with a low serum folate concentration (11.7 nmol/L) had a high homocysteine concentration (29.6 µmol/L) and normal RBC folate concentration (460 nmol/L). The male subject with the highest homocysteine concentration (45.7 µmol/L) had a borderline low serum folate concentration (14.9 nmol/L) and normal RBC folate concentration (589 nmol/L).
Folate and Homocysteine Concentrations and Genotype

Relative to objective 4, the relationship between MTHFR 677 C→T genotype and serum folate concentration and MTHFR 677 C→T genotype and RBC folate concentration also were determined. Mean serum and RBC folate concentrations by genotype for all subjects and for males and females are presented in Tables 4-11 and 4-12, respectively, and illustrated in Figures 4-12 and 4-13. Significant differences were not detected in serum folate concentrations among the genotype groups for all subjects or for males or females. However, for all participants, individuals with the CT or TT genotypes had significantly lower mean RBC folate concentrations (P <0.01) relative to the mean RBC folate of individuals with the CC genotype (Table 4-12). No significant difference was detected for mean RBC folate concentration between individuals with the CT and TT genotypes (P = 0.5). When mean RBC folate concentrations among genotype groups were compared within each gender, the trend for RBC folate concentrations by genotype for males was similar to the trend for all subjects. However, the same genotype effect on RBC folate was not detected for female subjects.

When mean homocysteine concentrations were compared among the MTHFR 677 C→T genotype groups for all subjects, significant differences (P <0.05) were detected between the CC versus CT genotypes and the CC versus TT genotypes. No significant differences were detected between individuals with the CT and TT genotypes (P = 0.4) (Table 4-13). As presented in Table 4-13, the genotype effect on homocysteine concentrations observed for all subjects was different when data were separated by gender. Significant differences in mean homocysteine concentrations were detected among genotype groups for males, but not for females. Male subjects with the TT or CT genotypes had the highest mean homocysteine concentrations (9.2
µmol/L; 8.5 µmol/L, respectively), whereas, male subjects with the CC genotype had the lowest mean homocysteine concentration (7.5 µmol/L). These data are also illustrated in Figure 4-14.

**Serum Folate and Homocysteine**

When the association between serum folate and homocysteine concentrations was evaluated, a significant inverse association was found for all participants (P <0.001), as well as within gender groups (i.e., males, P = 0.02; females, P = 0.02). These associations indicate that as the concentration of serum folate increased, homocysteine concentration decreased.

This association was further tested within each gender and by genotype group. For males, a significant association between serum folate and homocysteine concentration was not detected for either the CC or TT genotype groups (P = 0.5; P = 0.3, respectively). There was a trend toward a significant association for CT genotype group (P = 0.06). A significant association also was not detected for females within the CC genotype group (P = 0.4) or the TT genotype group (P = 0.1). However, a significant inverse association between serum folate and homocysteine concentration was detected for females within the CT genotype group (P = 0.03).

**Relationship Between Folate Intake and Status**

Relative to objective 4, the association between serum or RBC folate concentrations and folate/folic acid intake (µg DFE/d) was assessed using linear regression analysis to determine whether the level of DFE intake impacted serum or RBC folate concentrations. As hypothesized, a significant positive association between serum folate concentration and DFE intake for all subjects was detected (P = 0.03). When this relationship was analyzed by gender, there was insufficient power to detect whether significant differences existed for males or females (P = 0.2; P = 0.4, respectively). No relationship was detected between RBC folate and DFE intake for all subjects (P = 0.5) or by gender (P = 0.07).
The association between homocysteine concentration and folate/folic acid intake (µg DFE/d) also was examined to determine if the level of folate/folic acid intake impacted homocysteine concentration. A significant inverse association between homocysteine concentration and DFE intake was found for all participants (P < 0.0001), as well as for males (P = 0.02). The behavior of this relationship was not constant between genders as an association was not detected in females.

Folate Status and Fortified Ready-to-Eat Cereal Consumption

Although no significant difference was detected, serum folate concentration tended to be different (P = 0.08) between consumers and non-consumers of fortified RTE cereals for all subjects. Gender differences in serum folate and RBC folate concentrations between consumers and non-consumers of fortified RTE cereals were not detected. Comparison of homocysteine concentrations of consumers and non-consumers of fortified RTE cereals identified a significant difference in mean homocysteine concentration between female consumers (6.5 µmol/L) and non-consumers (8.4 µmol/L; P < 0.05). A significant difference in mean homocysteine concentration between male consumers (8.0 µmol/L) and non-consumers (8.4 µmol/L) was not detected (P > 0.5).
Table 4-1. Demographic characteristics of males and females.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Demographic variable</th>
<th>Males (n = 140)</th>
<th>Females (n = 162)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)\textsuperscript{b,c}</td>
<td>26.0 ± 8.2</td>
<td>25.3 ± 7.1</td>
<td>0.6</td>
</tr>
<tr>
<td>BMI (kg/m\textsuperscript{2})\textsuperscript{b,c}</td>
<td>24.5 ± 4.3</td>
<td>22.6 ± 3.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Education level (years)\textsuperscript{b,c}</td>
<td>16.0 ± 0.2</td>
<td>15.2 ± 2.5</td>
<td>0.4</td>
</tr>
<tr>
<td>Ethnicity (%)\textsuperscript{d}</td>
<td></td>
<td></td>
<td>0.4</td>
</tr>
<tr>
<td>White</td>
<td>58</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>Asian</td>
<td>17</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>African American</td>
<td>7</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Indian</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>11</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Marital Status (%)\textsuperscript{d}</td>
<td></td>
<td></td>
<td>0.7</td>
</tr>
<tr>
<td>Single</td>
<td>79</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>Married</td>
<td>15</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Divorced</td>
<td>4</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Separated</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Refused</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Student Status (%)\textsuperscript{d}</td>
<td></td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>Full-Time</td>
<td>67</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>Part-Time</td>
<td>4</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Not a student</td>
<td>9</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>19</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Employed (%)\textsuperscript{d}</td>
<td></td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>Yes</td>
<td>55</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>34</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>11</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}Due to rounding, percentages may not always sum to 100. \textsuperscript{b}Mean ± standard deviation (SD). \textsuperscript{c}One-way ANOVA was used for statistical comparisons between genders. \textsuperscript{d}Chi-square test was used for statistical comparisons between genders and demographic variables.
Table 4-2. Mean intake (µg/d) by folate category and gender.\textsuperscript{a,b,c}

<table>
<thead>
<tr>
<th>Folate group</th>
<th>Males (n = 140)</th>
<th>Females (n = 162)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total folate</td>
<td>462 (426, 501)</td>
<td>371 (344, 400)</td>
<td>0.0001</td>
</tr>
<tr>
<td>DFE</td>
<td>652 (603, 705)</td>
<td>512 (476, 551)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Folic acid</td>
<td>175 (156, 195)</td>
<td>128 (115, 142)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Food folate</td>
<td>293 (268, 321)</td>
<td>249 (229, 270)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Mean (5%, 95% CI).  \textsuperscript{b}One-way ANOVA was used for statistical comparisons between genders after adjusting for age.  \textsuperscript{c}P-values were based on normalized (log transformed) data. The results have been back-transformed to original scale.

Figure 4-1. Mean intake (µg/d) by folate category and gender. TF = total folate; DFE = dietary folate equivalents; FA = folic acid; FF = food folate. *Significant difference (P <0.01) in mean intake (µg/d) by folate category between males and females after adjusting for age.
Table 4-3. Mean intake (µg/1,000 kcals/d) by folate category and gender.\textsuperscript{a,b,c}

<table>
<thead>
<tr>
<th>Folate group</th>
<th>Males (n = 140)</th>
<th>Females (n = 162)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total folate</td>
<td>210 (197, 224)</td>
<td>233 (219, 247)</td>
<td>0.02</td>
</tr>
<tr>
<td>DFE</td>
<td>296 (279, 314)</td>
<td>321 (304, 340)</td>
<td>0.05</td>
</tr>
<tr>
<td>Folic acid</td>
<td>79 (72, 87)</td>
<td>80 (73, 88)</td>
<td>0.9</td>
</tr>
<tr>
<td>Food folate</td>
<td>133 (124, 143)</td>
<td>156 (146, 167)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Mean (5%, 95% CI).  \textsuperscript{b}One-way ANOVA was used for statistical comparisons between genders after adjusting for age.  \textsuperscript{c}P-values were based on normalized (log transformed) data. The results have been back-transformed to original scale.

![Graph](image)

Figure 4-2. Mean intake (µg/1,000 kcals/d) by folate category and gender. TF = total folate; DFE = dietary folate equivalents; FA = folic acid; FF = food folate. *Significant difference (P \leq 0.05) in mean intake (µg/1,000 kcals/d) by folate category between males and females after adjusting for age.
Table 4-4. Mean intake (µg/d) by folate category and genotype.\textsuperscript{a,b,c}

<table>
<thead>
<tr>
<th>Folate group</th>
<th>CC genotype (n = 151)</th>
<th>CT genotype (n = 118)</th>
<th>TT genotype (n = 31)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total folate</td>
<td>423 (391, 456)</td>
<td>395 (361, 429)</td>
<td>410 (344, 477)</td>
<td>0.7</td>
</tr>
<tr>
<td>DFE</td>
<td>581 (538, 625)</td>
<td>559 (512, 606)</td>
<td>583 (490, 675)</td>
<td>0.9</td>
</tr>
<tr>
<td>Folic acid</td>
<td>151 (135, 167)</td>
<td>141 (125, 158)</td>
<td>161 (126, 196)</td>
<td>0.9</td>
</tr>
<tr>
<td>Food folate</td>
<td>273 (250, 296)</td>
<td>262 (238, 287)</td>
<td>265 (218, 311)</td>
<td>0.8</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Mean (5%, 95% CI). \textsuperscript{b}One-way ANOVA was used for statistical comparisons between genders after adjusting for age. \textsuperscript{c}P-values were based on normalized (log transformed) data. The results have been back-transformed to original scale.

Table 4-5. Mean intake (µg/1,000 kcals/d) by folate category and genotype.\textsuperscript{a,b,c}

<table>
<thead>
<tr>
<th>Folate group</th>
<th>CC genotype (n = 151)</th>
<th>CT genotype (n = 118)</th>
<th>TT genotype (n = 31)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total folate</td>
<td>227 (213, 241)</td>
<td>215 (200, 229)</td>
<td>224 (195, 252)</td>
<td>0.6</td>
</tr>
<tr>
<td>DFE</td>
<td>312 (294, 330)</td>
<td>304 (285, 323)</td>
<td>317 (279, 356)</td>
<td>0.9</td>
</tr>
<tr>
<td>Folic acid</td>
<td>81 (74, 88)</td>
<td>77 (69, 84)</td>
<td>88 (71, 104)</td>
<td>0.9</td>
</tr>
<tr>
<td>Food folate</td>
<td>146 (136, 157)</td>
<td>143 (132, 154)</td>
<td>144 (123, 165)</td>
<td>0.9</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Mean (5%, 95% CI). \textsuperscript{b}One-way ANOVA was used for statistical comparisons between genders after adjusting for age. \textsuperscript{c}P-values were based on normalized (log transformed) data. The results have been back-transformed to original scale.
Figure 4-3. Percentage of subjects who consumed specific ranges of folate expressed as μg DFE/d.

Figure 4-4. Percentage of subjects who consumed specific ranges of folic acid (µg/d).
Table 4-6. Mean total folate contribution (µg/d) by dietary source and gender.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Dietary source</th>
<th>Males (n = 140)</th>
<th>Females (n = 162)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enriched cereal-grains</td>
<td>104 ± 6</td>
<td>94 ± 5</td>
</tr>
<tr>
<td>Fortified cereals and bars</td>
<td>66 ± 9</td>
<td>63 ± 8</td>
</tr>
<tr>
<td>Vegetable</td>
<td>117 ± 9</td>
<td>127 ± 9</td>
</tr>
<tr>
<td>Fruit</td>
<td>25 ± 2</td>
<td>27 ± 2</td>
</tr>
<tr>
<td>Juice (orange and grapefruit)</td>
<td>16 ± 2</td>
<td>14 ± 2</td>
</tr>
<tr>
<td>Legumes and nuts</td>
<td>58 ± 6</td>
<td>51 ± 5</td>
</tr>
<tr>
<td>Meat</td>
<td>4 ± 0</td>
<td>3 ± 0</td>
</tr>
<tr>
<td>Combination</td>
<td>18 ± 2</td>
<td>19 ± 2</td>
</tr>
<tr>
<td>Snacks</td>
<td>7 ± 2\textsuperscript{b}</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>Dairy</td>
<td>14 ± 1</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>Eggs</td>
<td>5 ± 1</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>24 ± 4</td>
<td>25 ± 4</td>
</tr>
<tr>
<td>Other</td>
<td>9 ± 1</td>
<td>9 ± 1</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Mean ± SE. One-way ANOVA was used for statistical comparisons between genders after adjusting for age and calories. \textsuperscript{b}Significantly lower than females (P = 0.01).

Table 4-7. Mean DFE contribution (µg DFE/d) by dietary source and gender.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Dietary source</th>
<th>Males (n = 140)</th>
<th>Females (n = 162)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enriched cereal-grains</td>
<td>158 ± 9</td>
<td>143 ± 9</td>
</tr>
<tr>
<td>Fortified cereals and bars</td>
<td>128 ± 17</td>
<td>129 ± 15</td>
</tr>
<tr>
<td>Vegetable</td>
<td>119 ± 10</td>
<td>130 ± 9</td>
</tr>
<tr>
<td>Fruit</td>
<td>25 ± 2</td>
<td>27 ± 2</td>
</tr>
<tr>
<td>Juice (orange and grapefruit)</td>
<td>16 ± 2</td>
<td>14 ± 2</td>
</tr>
<tr>
<td>Legumes and nuts</td>
<td>59 ± 6</td>
<td>52 ± 5</td>
</tr>
<tr>
<td>Meat</td>
<td>5 ± 1</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>Combination</td>
<td>66 ± 4</td>
<td>59 ± 4</td>
</tr>
<tr>
<td>Snacks</td>
<td>10 ± 2\textsuperscript{b}</td>
<td>18 ± 2</td>
</tr>
<tr>
<td>Dairy</td>
<td>14 ± 1</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>Eggs</td>
<td>5 ± 1</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>35 ± 5</td>
<td>36 ± 5</td>
</tr>
<tr>
<td>Other</td>
<td>9 ± 1</td>
<td>10 ± 1</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Mean ± SE. One-way ANOVA was used for statistical comparisons between genders after adjusting for age and calories. \textsuperscript{b}Significantly lower than females (P = 0.01).
Table 4-8. Mean folic acid contribution (µg/d) by dietary source and gender.

<table>
<thead>
<tr>
<th>Dietary source</th>
<th>Males (n = 140)</th>
<th>Females (n = 162)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enriched cereal-grains</td>
<td>78 ± 5</td>
<td>71 ± 5</td>
</tr>
<tr>
<td>Fortified cereals and bars</td>
<td>73 ± 10</td>
<td>73 ± 9</td>
</tr>
<tr>
<td>Vegetable</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Fruit</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Juice (orange and grapefruit)</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Legumes and nuts</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Meat</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Combination</td>
<td>26 ± 2</td>
<td>24 ± 2</td>
</tr>
<tr>
<td>Snacks</td>
<td>4 ± 1</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>Dairy</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Eggs</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>7 ± 2</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>Other</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

aMean ± SE. One-way ANOVA was used for statistical comparisons between genders after adjusting for age and calories.  bSignificantly lower than females (P = 0.02).

Table 4-9. Mean food folate contribution (µg/d) by dietary source and gender.

<table>
<thead>
<tr>
<th>Dietary source</th>
<th>Males (n = 140)</th>
<th>Females (n = 162)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enriched cereal-grains</td>
<td>25 ± 1</td>
<td>23 ± 1</td>
</tr>
<tr>
<td>Fortified cereals and bars</td>
<td>4 ± 1</td>
<td>4 ± 0</td>
</tr>
<tr>
<td>Vegetable</td>
<td>117 ± 9</td>
<td>127 ± 9</td>
</tr>
<tr>
<td>Fruit</td>
<td>25 ± 2</td>
<td>27 ± 2</td>
</tr>
<tr>
<td>Juice (orange and grapefruit)</td>
<td>16 ± 2</td>
<td>14 ± 2</td>
</tr>
<tr>
<td>Legumes and nuts</td>
<td>58 ± 6</td>
<td>51 ± 5</td>
</tr>
<tr>
<td>Meat</td>
<td>4 ± 0</td>
<td>3 ± 0</td>
</tr>
<tr>
<td>Combination</td>
<td>8 ± 1</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>Snacks</td>
<td>3 ± 1b</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>Dairy</td>
<td>14 ± 1</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>Eggs</td>
<td>5 ± 1</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>23 ± 3</td>
<td>27 ± 3</td>
</tr>
<tr>
<td>Other</td>
<td>9 ± 1</td>
<td>9 ± 1</td>
</tr>
</tbody>
</table>

aMean ± SE. One-way ANOVA was used for statistical comparisons between genders after adjusting for age and calories.  bSignificantly lower than females (P = 0.02).
Figure 4-5. Percentage of total intake derived from each food group by folate category for males. TF = total folate, DFE = dietary folate equivalents, FA = folic acid, FF = food folate. The relative contribution of each food group to TF, DFE, FA and FF intake was calculated by dividing the mean intake of each food group by total intake for each folate category (e.g., mean DFE intake from enriched cereal grains divided by total mean DFE intake).

Figure 4-6. Percentage of total intake derived from each food group by folate category for females. TF = total folate, DFE = dietary folate equivalents, FA = folic acid, FF = food folate. The relative contribution of each food group to TF, DFE, FA and FF intake was calculated by dividing the mean intake of each food group by total intake for each folate category (e.g., mean DFE intake from enriched cereal grains divided by total mean DFE intake).
Figure 4-7. Mean percent contribution of fortified RTE cereal to the total mean intake for each folate category for males. TF = total folate, DFE = dietary folate equivalents, FA = folic acid, FF = food folate.

Figure 4-8. Mean percent contribution of fortified RTE cereal to the total mean intake for each folate category for females. TF = total folate, DFE = dietary folate equivalents, FA = folic acid, FF = food folate.
Table 4-10. Comparison of serum and RBC folate, and homocysteine concentrations by gender\(^{a,b,c}\)

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum folate (nmol/L)</td>
<td>39.9 (37.8, 42.0)</td>
<td>41.7 (39.6, 43.8)</td>
<td>0.3</td>
</tr>
<tr>
<td>RBC folate (nmol/L)</td>
<td>810 (766, 857)</td>
<td>767 (727, 809)</td>
<td>0.2</td>
</tr>
<tr>
<td>Homocysteine (µmol/L)</td>
<td>8.0 (7.7, 8.4)</td>
<td>6.6 (6.3, 6.9)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

\(^{a}\)Concentrations expressed as mean (5%, 95% CI). \(^{b}\)One-way ANOVA was used for statistical comparisons between genders after adjusting for age. \(^{c}\)P-values were based on normalized (log transformed) data. The results have been back-transformed to original scale.
Figure 4-9. Mean serum folate concentration (nmol/L) by gender after adjusting for age. Normal serum folate status was defined as a serum folate concentration >13.6 nmol/L.

Figure 4-10. Mean RBC folate concentration (nmol/L) by gender after adjusting for age. Normal RBC folate status was defined as a RBC folate concentration >317 nmol/L.

Figure 4-11. Mean homocysteine concentration (µmol/L) by gender after adjusting for age. Normal homocysteine concentration defined as <12 µmol/L. *Significant difference (P <0.0001) in mean homocysteine concentration (µmol/L) between males and females.
Table 4-11. Comparison of mean serum folate concentrations (nmol/L) by genotype.\textsuperscript{a,b}

<table>
<thead>
<tr>
<th>Gender</th>
<th>CC genotype</th>
<th>CT genotype</th>
<th>TT genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>All subjects\textsuperscript{c}</td>
<td>42.5 (40.3, 44.7)</td>
<td>39.3 (37.1, 41.6)</td>
<td>38.5 (34.3, 42.6)</td>
</tr>
<tr>
<td>Males\textsuperscript{d}</td>
<td>41.7 (38.6, 44.9)</td>
<td>38.6 (35.4, 41.8)</td>
<td>36.9 (31.3, 42.4)</td>
</tr>
<tr>
<td>Females\textsuperscript{d}</td>
<td>43.3 (40.2, 46.3)</td>
<td>40.0 (36.8, 43.2)</td>
<td>40.2 (34.1, 46.3)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Mean (5\%, 95\% CI). \textsuperscript{b}GLM procedure was used for statistical comparisons of mean serum folate concentrations between genotypes for each gender after adjusting for age. P-values were based on normalized (log transformed) data. The results have been back-transformed to original scale. \textsuperscript{c}Unable to detect significant differences in serum folate concentrations among genotype groups (P >0.05). \textsuperscript{d}No significant differences detected in serum folate among genotype groups for males or females (P >0.1).

Table 4-12. Comparison of mean RBC folate concentrations (nmol/L) by genotype.\textsuperscript{a,b}

<table>
<thead>
<tr>
<th>Gender</th>
<th>CC genotype</th>
<th>CT genotype</th>
<th>TT genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>All subjects\textsuperscript{c,d}</td>
<td>848 (803, 895)</td>
<td>744 (700, 790)</td>
<td>713 (634, 801)</td>
</tr>
<tr>
<td>Males\textsuperscript{e,f}</td>
<td>878 (811, 944)</td>
<td>756 (693, 819)</td>
<td>733 (622, 845)</td>
</tr>
<tr>
<td>Females\textsuperscript{g,h}</td>
<td>821 (762, 880)</td>
<td>732 (673, 790)</td>
<td>691 (584, 798)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Mean (5\%, 95\% CI). \textsuperscript{b}GLM procedure was used for statistical comparisons of mean RBC folate concentrations between genotype groups after adjusting for age. P-values were based on normalized (log transformed) data. The results have been back-transformed to original scale. \textsuperscript{c}CC genotype significantly higher than CT genotype (P = 0.002). \textsuperscript{d}CC genotype significantly higher than TT genotype (P <0.01). \textsuperscript{e}CC genotype significantly higher than CT genotype (P = 0.01). \textsuperscript{f}CC genotype significantly higher than TT genotype (P = 0.05). \textsuperscript{g}CC genotype significantly higher than CT genotype (P = 0.04). \textsuperscript{h}Unable to detect a significant difference for CC versus TT genotype (P = 0.07).

Table 4-13. Comparison of mean homocysteine concentrations (µmol/L) by genotype.\textsuperscript{a,b}

<table>
<thead>
<tr>
<th>Gender</th>
<th>CC genotype</th>
<th>CT genotype</th>
<th>TT genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>All subjects\textsuperscript{c}</td>
<td>6.9 (6.6, 7.2)</td>
<td>7.5 (7.1, 7.8)</td>
<td>7.8 (7.1, 8.6)</td>
</tr>
<tr>
<td>Males\textsuperscript{d,e}</td>
<td>7.5 (7.0, 7.9)</td>
<td>8.5 (7.9, 9.0)</td>
<td>9.2 (8.0, 10.3)</td>
</tr>
<tr>
<td>Females\textsuperscript{g}</td>
<td>6.5 (6.1, 6.8)</td>
<td>6.6 (6.2, 7.1)</td>
<td>6.6 (5.8, 7.5)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Mean (5\%, 95\% CI). \textsuperscript{b}GLM procedure was used for statistical comparisons of mean homocysteine concentrations between genotypes for each gender after adjusting for age. P-values were based on normalized (log transformed) data. The results have been back-transformed to original scale. \textsuperscript{c}CT and TT genotypes significantly higher than CC genotype (P = 0.03). \textsuperscript{d}CT genotype significantly higher than CC genotype (P = 0.01). \textsuperscript{e}TT genotype significantly higher than CC genotype (P <0.01). \textsuperscript{f}No significant differences detected in homocysteine concentrations among genotype groups for females (P >0.05).
Figure 4-12. Mean serum folate concentration (nmol/L) by genotype after adjusting for age.

Figure 4-13. Mean RBC folate concentration (nmol/L) by genotype after adjusting for age.
*Significant difference (P < 0.01) in mean RBC folate concentration compared to all subjects with the CC genotype. **Significant difference (P ≤ 0.05) in mean RBC folate concentration compared to males with the CC genotype.
***Significant difference (P = 0.04) in mean RBC folate concentration compared to females with the CC genotype.
Figure 4-14. Mean homocysteine concentration (µmol/L) by genotype after adjusting for age. *Significant difference (P = 0.03) in mean homocysteine concentration compared to all subjects with the CC genotype. **Significant difference (P ≤ 0.01) in mean homocysteine concentration compared to males with the CC genotype.
CHAPTER 5
DISCUSSION AND CONCLUSIONS

The focus of this study was to assess folate intake and status of non-supplement consuming healthy young men and women and to estimate the relative contribution of folate/folic acid from different food categories to overall intake. Previous research studies have addressed the impact of folic acid enrichment of cereal-grain products and folic acid fortification of breakfast cereals on folate status of men and women (3-6, 13, 125, 150), but the present study is the first to include an estimate of the relative contribution of folate/folic acid from different food categories to overall intake. Information related to the contribution of different food categories to folate/folic acid intake can be used to develop targeted intervention strategies directed at improving folate/folic acid intake.

Recent updates of the USDA National Nutrient Database for Standard Reference (Release 17), which reflects folic acid fortification of foods, separates dietary folate intake into four categories: food folate, folic acid, total folate, and DFEs (8). Unlike most previous investigations that only reported total folate intake (i.e., µg of food folate plus µg of folic acid without adjustment for differences in bioavailability), our study provides separate data on folic acid and food folate intake enabling a comparison with the recommended level of folic acid for women of reproductive potential, as well as intake expressed as DFE, a calculated value essential for comparison to the RDA for males and females.

The majority of reports in the literature have used data from NHANES to evaluate the adequacy of folate intake in the US post-fortification (4, 13, 151). One of the limitations of that database is that nutrient intake data are estimated for each individual from a single 24-hour dietary recall. In the present study, the previously validated DHQ was used to assess dietary intake based on recall of food frequency over a 12 month period (144). Another limitation
associated with using the NHANES database is the fact that intake analysis data are reported as total folate intake (µg/d) and do not provide folate intake expressed as DFEs; consequently, the researchers using that database were unable to compare intake relative to the RDA.

A study by Dietrich et al. (13), in which total folate intake (µg/d) of males and females was compared before and after fortification, reported that the absolute increase in total folate (µg/d) were larger among men than women. These investigators attributed this to the fact that men, in general, have higher dietary intakes of folate than women. Although our analysis also indicated that males consumed more folate per day than females, the average total folate intake (µg/1,000 kcals/d) was significantly higher for females. Adjusting for calories allows for more equal comparison between genders as the DHQ revealed that males consume more daily calories, and for this reason, males are more likely to consume more folate/folic acid than females on any given day.

Based on data from NHANES 1999-2000, Briefel et al. (152) reported that among women of childbearing age (20 to 39 years), mean total folate intake was 327 µg/d. Dietrich et al. (13) also evaluated folate intake of NHANES 1999-2000 participants and found that the mean total folate intake of males and females combined (aged ≥20 years) was 351 µg/d. In comparison, our data revealed a slightly higher average total folate intake of 371 µg/d for women. The average total folate intake for men was 462 µg/d. However, since these data do not account for the form of the vitamin consumed (i.e., food folate versus folic acid from enriched and fortified products), it is not possible to compare these intake levels fairly, nor is there a way to determine if they would meet the current RDA for folate. Our data, however, revealed that the mean total intake expressed as DFEs for males and females (652 µg DFE/d; 512 µg DFE/d, respectively) exceeded the RDA of 400 µg DFE/d, with the majority of males (81%) and females
(68%) meeting the RDA. Interestingly, after adjusting for caloric intake, females in our study had a significantly higher average intake of food folate compared to males, but no difference was detected for folic acid intake between genders.

The present study estimated the actual and relative contribution of different food categories to overall folate/folic acid intake. This unique aspect of the present study identified the major contributors of daily folate intake (i.e., µg DFE/d) to be enriched cereal-grains products, fortified cereals and bars, and combination foods that include “enriched” ingredients for both males and females. When comparing the actual contribution of food categories to total folate intake, vegetables contributed the highest amount of total folate, followed by enriched cereal-grains products for men and women alike. The differences noted in average intake between total folate and DFE are due to the way DFEs are calculated, where more weight is given to folic acid than endogenous food folate (µg DFE/d = 1.7 x µg/d folic acid + µg/d food folate).

The IOM recommends that all women of reproductive potential consume 400 µg of folic acid daily from vitamin supplements and/or fortified foods in addition to consuming food folate from a varied diet to reduce the risk of having an NTD-affected pregnancy (7). Yang et al. (6) examined folic acid intake in women of childbearing age in the US using NHANES 2001-2002 data and nutrient intakes estimated from the USDA National Nutrient Database Standard Reference, Release 16. Mean consumption of folic acid from fortified foods was 128 µg/d in non-supplement consuming, non-pregnant women, and only 8% of the women reported consuming ≥400 µg/d of folic acid from fortified foods. Similar to the findings by Yang et al., the average intake of folic acid from fortified foods for women in the present study who were non-supplement users was 128 µg/d or approximately one-third of the amount recommended for
NTD risk reduction. Furthermore, only 3% of female subjects consumed ≥400 µg/d of folic acid from enriched and fortified foods. This falls well short of the Healthy People 2010 goal of 80% of women of reproductive potential achieving a daily folic acid intake of 400 µg. However, even though folic acid intake was inadequate relative to the amount recommended for NTD risk reduction, and only two-thirds of the women in our study exceeded the folate RDA, the estimated levels of folate/folic acid intake reported in our study were associated with normal blood folate status. Whether or not the level of intake that was associated with normal blood folate status in our study is adequate to achieve the level of NTD risk reduction associated with 400 µg of folic acid is not known.

Since folic acid enrichment of cereal-grain products was designed to augment folic acid intake from supplements, it is understandable that the majority of women would not be consuming the recommended 400 µg/d of folic acid from dietary sources alone. What the data from this investigation illustrate so clearly is that when women do not take supplements, which is the case for the majority of women, dependence on dietary sources of folic acid to meet the recommended intake is totally inadequate. The CDC, the March of Dimes, and the National Council on Folic Acid have organized the National Folic Acid Campaign for the prevention of NTDs, with the goal of teaching all women about the importance of folic acid and ensuring women consume 400 µg of folic acid daily (17). The campaign targets all women of reproductive age, the health care professionals who serve these women, and community advocacy groups (153). However, despite all of these major folic acid awareness campaigns, these data illustrate that more attention needs to be focused on increasing folic acid intake from dietary sources for women of childbearing age who do not take supplements.
In 2005, the CDC and the March of Dimes launched the Preconception Health and Health Care Initiative, the aims of which are to improve the health of mothers before pregnancy and maternal and infant health outcomes (130). As a result of this summit and initiative, strategies for improving preconception health incorporating folic acid awareness have been incorporated into numerous programs throughout the US, including areas in clinical practice, consumer roles, and public health (130, 154). However, many of these campaigns have proven to be ineffective as recent data from the 2005 March of Dimes Gallup Survey reported by the CDC suggested only 33% of the general population of women of reproductive age take a folic acid supplement daily (17). Therefore, the sole source of folic acid in the diets of the majority of this target group is enriched and fortified foods. Because only 3% of women in our study consumed 400 µg/d of folic acid from fortified foods alone, it can be concluded that non-supplement users are at risk for not meeting the recommended level of folic acid intake shown to result in NTD risk reduction. Women of reproductive potential who do not take supplements should be encouraged to consume adequate amounts of fortified or enriched cereal grain products daily to achieve the recommended level of folic acid intake.

The present study also evaluated the impact of folate intake on folate status of male and female non-supplement users. A significant positive association was detected between intake of DFE and serum folate concentrations for all subjects, and a significant inverse association was found between intake of DFE and homocysteine concentrations for all subjects and for male subjects only. No relationship was detected between intake of DFE and RBC folate concentrations for all subjects or by gender. The mean folate/folic acid intake observed in this study was associated with adequate mean serum folate concentrations for both males (39.9 nmol/L) and females (41.7 nmol/L). These concentrations are higher than reported by Jacques et
al. (4), who reported that the mean plasma folate concentration for both males and females who were non-supplement users in the Framingham Offspring Study (aged 32 to 80 years) was 23 nmol/L. Lawrence et al. (5) reported a median serum folate concentration of 42.4 nmol/L post-fortification; however, no information was given on subjects’ folic acid supplement use. Caudill et al. (155) investigated 135 women (aged 18 to 45 years) who were non-supplement users post-implementation of folic acid fortification. These women were reported to have a mean serum folate concentration of 50 nmol/L. Possible explanations for differences in blood folate concentrations among studies could be due to various ages of study participants, differences in analytical methodology for measuring blood folate, and the fact that folic acid fortification had been in effect longer when Caudill et al. completed their study and when blood samples used for folate analysis were collected in the current study.

Based on data from the NHANES, Pfeiffer et al. (156) reported that the prevalence of low serum folate concentrations (<7 nmol/L) for women of childbearing age (15 to 45 years) was <1%. Serum folate samples collected as part of the NHANES study were analyzed using the Bio-Rad QuantaPhase II radiobinding assay. This method yields lower serum folate values than the microbiological method, the latter of which uses a cutoff value of 13.6 nmol/L (61).

Although the cutoff value for serum folate concentration of 13.6 nmol/L was used in the present study, none of the women in this investigation fell below this level. If the Bio-Rad norm for serum folate that was established with the radiobinding assay had been used in this study, then none of the male or female subjects would have had a low serum folate concentration. Taken together, the data examining folate status provide convincing evidence to indicate that a significant proportion of the population, particularly women of reproductive age, have more than adequate folate status.
With respect to RBC folate, mean RBC folate concentrations in the present study for males (810 nmol/L) and females (767 nmol/L) were well above the lower limit of normal (i.e., 317 nmol/L) based on the value established for use with the radiobinding assay. RBC folate concentration is a better indicator of long-term folate status than serum folate concentration. Only 2% of females and <1% of males had RBC folate concentrations <317 nmol/L. Our data are similar to those reported by Choumenkovitch et al. (3) indicating that 96.1% of women >45 years who were non-supplement users had adequate RBC folate concentrations (≥362.6 nmol/L). Despite the fact that the majority of our female subjects did not meet the recommended level of intake for NTD risk reduction, 98% of them had adequate RBC folate concentrations. However, whether or not their status is sufficient to reduce NTD risk is unknown. Research from an Irish population indicated that RBC folate concentrations ≥908 nmol/L were associated with an eight-fold difference in NTD incidence compared to concentrations <341 nmol/L (85). However, these results may not be applicable to a US population so further research is warranted.

The benefits of adequate blood folate status may confer benefits related to chronic disease risk reduction. Mild folate deficiency has been associated with an increased plasma homocysteine concentration, an independent risk factor for vascular disease (100, 157, 158). Homocysteine is also recognized to be a “functional indicator of folate status” (63). The data from our study confirm that serum folate concentration is inversely related to plasma homocysteine concentrations. Although no significant differences in mean serum or RBC folate concentrations were detected between males and females, males had a significantly higher mean homocysteine concentration (8.0 µmol/L) compared to females (6.6 µmol/L). These data agree with findings reported by Ganji et al. (66) that plasma homocysteine concentrations are often higher in males than females. Of the 6.5% of males (n = 9) with elevated homocysteine (≥12
102 µmol/L), five subjects fell below the mean serum folate concentration for males, six were below the mean RBC folate concentration for males, and four subjects consumed <400 µg DFE/d. Although not evaluated as part of this study, a possible explanation for the elevated homocysteine concentrations of these subjects could be related to vitamin B12 deficiency (159, 160). Because elevated plasma homocysteine concentrations are considered a risk factor for cardiovascular disease, the association between homocysteine and folate broadens the health-related consequences of low folate status and has implications for both men and women. Therefore, marketing folic acid consumption should not be limited to prevention of NTDs as folate status plays a role in various health-related problems.

Our study also investigated the relationship between folate status, homocysteine and the MTHFR 677C→T polymorphism. Comparable to the findings of Brattstrom et al. (106), Kauwell et al. (59), and Shelnutt et al. (21), subjects with the TT genotype had higher homocysteine concentrations and lower RBC folate concentrations than individuals with the CC genotype. However, in contrast to these reports, serum folate concentrations in the present study population did not differ among genotype groups. One explanation for this could be due to the fact that the majority of our population consumed sufficient dietary folate to maintain normal serum folate concentrations. Previous research by Shelnutt et al. (21) determined that a predisposition for reduced blood folate and elevated plasma homocysteine concentration could be significantly improved in response to improved folate status. Other research has also confirmed the ability of increased food folate and folic acid intake to positively modify the negative effects of the MTHFR polymorphism on folate status (161, 162). Ashfield-Watt and colleagues (161), however, determined that individuals with the TT genotype require higher
intakes of folic acid from fortified foods or supplements than individuals with the CC or CT genotype to achieve a similar lowering effect on homocysteine concentrations.

Another aim of our study was to characterize the relative contribution of specific food categories to food folate/folic acid intake. The findings from this investigation indicate that the intake of folic acid from fortified RTE cereals alone represented 20% of the total folic acid intake for women of reproductive age who consumed fortified cereals. Seventy-eight percent of total folic acid in their diets was provided by a combination of enriched cereal-grain products and fortified cereals and bars, including RTE cereals. For females, RTE cereal consumption also accounted for 13.3% of total folate intake and 19.0% of DFE intake. Our findings are similar to those of Dietrich et al. (13) who reported that fortified RTE breakfast cereals provided 12.1% of total folate intake. However, estimates of folic acid intake from our study population may not be representative of women of reproductive potential because we used the data collected for a study designed to assess vitamin B12 intake from dietary sources. The protocol for that study excluded women who consumed highly fortified RTE cereals on a daily basis, but not those who consumed these products less frequently. These results may not be observed in other parts of the world where fortified RTE cereals are not a prevalent component of the diet.

Although actual intake of fortified cereals might be greater than reported in the present study, our study identified fortified RTE cereal as an important source of folic acid in the diet. Most RTE cereals contain 100 µg of folic acid per serving, and some highly fortified cereals contain as much as 400 µg per serving. Examples of specific brands of highly fortified RTE cereal include All-Bran®, Multigrain Cheerios®, Complete®, KASHI Heart-to-Heart®, Mueslix®, Product 19®, Smart Start®, Special K®, and Total® (all varieties). In this study, 58% of women who consumed fortified cereals reported consuming highly fortified RTE cereals. A note of
caution is that cereals are often consumed in amounts twice the labeled serving size (163); therefore, individuals who consume highly fortified breakfast cereals in large quantities have the potential to exceed the UL (1,000 µg/d of folic acid). Nevertheless, no subjects in this study were found to consume the UL for folic acid.

Since the majority of women of childbearing age are not taking folic acid supplements, promoting folic acid consumption from fortified RTE cereals and enriched cereal-grain products represents an important approach to the delivery of folic acid in the diet. Ensuring women consume the recommended quantities of folic acid daily is fundamental as more than half of all pregnancies are unplanned and because the development of NTDs occurs during the first 28 days of pregnancy, often before many women realize they are pregnant.

**Strengths and Limitations**

Weaknesses of the current investigation include limitations associated with the use of a FFQ to assess dietary folate intake, including the fact that there is no option for individuals to add personal responses related to food intake on the questionnaire. More accurate approaches to assess dietary intake include weighed food records or multiple-day food records. However, these methods are time consuming and tedious and are generally discouraged for larger studies. The DHQ used in this study has been validated as an appropriate method of assessing usual dietary intake (144, 164). Nevertheless, this subgroup may not be representative of men and women in the US population in contrast to NHANES, a population based study designed to be representative of the US population. Furthermore, the criteria used for the study from which our data were obtained attempted to exclude women who consumed highly fortified RTE cereals on a daily basis. This may have resulted in an underestimation of folic acid intake from fortified RTE cereals.
Another limitation of this study was the lack of statistical power due to variability and limited observations when comparing folate status of subjects by MTHFR 677C→T genotype within the same gender group. Another limitation of this study is that differences in measured blood folate concentrations have been noted between the radiobinding assay, the method used in this study, and the microbiological assay method used by many other investigators. Fazili et al. (61) reported that the vitamin B12/folate radioassay kit may underestimate serum folate concentrations compared to the LC-MS/MS and microbiological assay. An advantage of using the radioassay in this study is the fact that this was the method used for analysis of blood folate concentrations in the most recent NHANES, making comparisons between the data in this study and data reported in the NHANES study possible.

Strengths of the present study included the ability to estimate DFE intake, thus enabling us to compare intake to the RDA, and the ability to compare the relative contribution of specific food categories to total folate/folic acid intake in non-supplement users. Moreover, the fact that supplement users were excluded from the study made it possible to assess the impact of dietary folate/folic acid intake on folate status. It would have been ideal to compare folate status between consumers and non-consumers of RTE cereals; however, since 90% of our subjects consumed these products, it was not feasible to make this comparison.

Summary

This study supports previous research that has shown a positive relationship between folate/folic acid intake and folate status. The estimated average dietary folate/folic acid intake of our study population exceeded the current RDA, and average blood folate concentrations were well within the acceptable range. Although male subjects had a significantly higher mean homocysteine concentration compared to females, the majority of all subjects had plasma
homocysteine concentrations within the acceptable limit. In relation to the MTHFR 677 C→T polymorphism, the data from all subjects are consistent with previous studies in which the TT genotype was associated with significantly lower RBC folate and higher homocysteine concentrations compared to the CC genotype.

Based on the findings from this study, it appears that folic acid fortified foods are important contributors to the diet in terms of meeting the folate RDA of non-supplement users. The major food categories that contributed toward meeting the RDA for males and females were enriched cereal-grain products, fortified cereals and bars and combination foods that include “enriched” ingredients. This highlights the integral role of folic acid fortification in achieving an adequate intake (based on the RDA) and status of this vitamin in non-supplement users; however, despite the relative contribution of folic acid fortified foods toward meeting the RDA, intake of these foods was not sufficient among our study population to meet the IOM recommendation (400 µg/d of folic acid) for women of reproductive potential. The extremely low proportion of females (3%) who met this recommendation is disappointing considering the extensive efforts to promote awareness and behavior change among women of reproductive potential.

**Practical Application**

Since public health educational programs and strategies designed to market daily folic acid supplementation to women of reproductive age have not been effective, women might be more responsive to food advertisements, including promotion of fortified cereals and snack bars as good to excellent dietary sources of folic acid. Rather than solely promoting folic acid supplementation, researchers, dietitians, health educators and food industry manufacturers should forge efforts to create and market educational outreach programs promoting intake of fortified
RTE cereals, breakfast and snack bars, and enriched cereal-grain products as easy alternatives to help women consume 100% of the RDA, meet the IOM recommendation for folic acid and maintain adequate folate status. However, folic acid fortification will not ensure adequate folic acid intake in target women unless women are educated to change the way they eat. Health professionals and health educators could promote eating one serving of cereal that has been fortified with 100% of the daily value of folic acid as a means of consuming the recommended amount of folic acid daily.

Health professionals and researchers should make an effort to explore the reasons why women of reproductive age are not consuming sufficient levels of folic acid to prevent NTDs. These future findings may allow researchers to better understand barriers to folic acid consumption in this population. Previous surveys have shown that the biggest barrier to overcome with women is the lack of knowledge about folic acid usage. Many women who have heard of the vitamin do not know that they need to take it before pregnancy to reduce NTD risk. Therefore, educational awareness about the importance of consuming supplemental folic acid and/or folic acid-fortified foods every day is another objective that should be taken into consideration. Targeted messages aimed at younger women, regardless of pregnancy plans, should include campaigns focused on promoting consumption of fortified RTE cereals to help increase the number of women of childbearing age who consume adequate folic acid daily.
APPENDIX A
SUBJECT DATA COLLECTION FORM

Introduction

I am calling in regard to your interest in our nutrition study; do you have a few minutes right now?

This is a UF Nutrition department study and involves coming in one morning for about 1 hour for a fasting blood sample, we take about 1 ½ ounces of blood, and you only need to fast 8 hours. We will give you a breakfast snack right afterward, and then give a brief explanation of a food frequency questionnaire you will be taking home. You will be asked to mail it back in the provided envelope, and once we receive the questionnaire you would get paid the $50. I just have to ask you some questions to see if you are eligible for our study and to get background information, OK?

How old are you? 18-49
Do you smoke? no
Are you pregnant or breastfeeding? no
Do you take any prescription medications other than oral contraceptives? no

If not within the age range or if they answer yes to any question:
I am very sorry, but you do not meet our exclusion criteria, but thank you for your interest.

Now I just have a few questions about your diet to see what specific category of our study you would fit in to. Please answer as best you can, estimates are ok and consider all instances of when you might eat the items I will ask about, even if only occasionally.

Do you take a multi-vitamin, complex, red star nutritional yeast, or any other supplement or additive ever?

If they take a multivitamin, B complex, red star nutritional yeast, complete the session through all diet info but do not record. Conclude by confirming their name and saying “This has been a preliminary screening call, your information will be reviewed by the principal investigator based on need, and our selection criteria at this time. If you are chosen you will be called again to schedule an appointment over the next two weeks. Thank you very much for your interest and your time.

Do you eat breakfast cereals? (If so) What Kind do you eat mostly?

If they eat a 100% fortified cereal or eats a 50% cereal daily complete the through all diet info but do not record. Conclude by confirming their name and saying “This has been a preliminary screening call, your information will be reviewed by the principal investigator based on need, and our selection criteria at this time. If you are chosen you will be called again to schedule an appointment over the next two weeks. Thank you very much for your interest and your time.
If the interviewee fulfills all selection criteria continue with the questionnaire, record info on moderate/non-fortified cereal consumption below.

**Do you eat breakfast cereals?**
- Yes
- No

<table>
<thead>
<tr>
<th>Name/Brand</th>
<th>Quantity</th>
<th>Frequency</th>
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**Are you a vegan, vegetarian or meat eater?**
- Vegan – this means you eat NO animal derived foods intentionally (if they eat small amount like in cake then OK)
- Vegetarian – this means you eat NO beef, chicken, turkey, pork, or fish

**How often do you eat ...**

<table>
<thead>
<tr>
<th></th>
<th>Never (&lt;1 x/mo)</th>
<th>Rarely (1-4 x/mo)</th>
<th>Occasionally (2-4 x/wk)</th>
<th>Frequently (5-7 x/wk)</th>
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<tbody>
<tr>
<td>Beef</td>
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<td>Chicken</td>
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<td>Turkey</td>
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<td>Pork</td>
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<td>Fish</td>
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<td>Eggs</td>
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<td>Cheese</td>
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<td>Cow’s Milk</td>
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<td>Yogurt</td>
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<tr>
<td>Other Dairy</td>
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**Do you follow a restricted diet such as:**
- No red meat
- Lactose-free
- Kosher
- Weight loss
- Weight gain
- Low salt
- Low fat
- Low cholesterol
- Low carbohydrate
- Hypoallergenic

(If so) How long have you consumed this type of diet?

________________________________________________________________________

**Have you made any major dietary changes within the last 3 years?**
- No
- Yes; How long ago did you make changes and what changes did you make?

________________________________________________________________________

**Do you consume alcoholic beverages?**
- NO
- YES
  - How often/quantity
Health Information

I am going to ask you a few questions about your health to determine if you are eligible for our study. I will be recording this information, but it will be kept confidential and is this ok with you? _______

<table>
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<tr>
<th>Height:</th>
<th>Weight:</th>
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</table>

Have you do you currently have any of the following?  

<table>
<thead>
<tr>
<th>Condition</th>
<th>NO</th>
<th>YES</th>
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<tbody>
<tr>
<td>Alcoholism</td>
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<tr>
<td>Anemia</td>
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<td>Blood clots</td>
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<td>Bronchitis</td>
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<td>Cystic Fibrosis</td>
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<td>Dermatitis</td>
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<td>Diabetes</td>
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<td>Eating disorders/Chronic nausea or vomiting</td>
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<td>Food allergy</td>
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<td>Gall bladder disease</td>
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<td>GI problems/ Lactose intolerance</td>
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<td>Gout</td>
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<td>Migraines</td>
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<td>Hemorrhoids</td>
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<td>Hepatitis/Liver disease</td>
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<td>Heart disease/High cholesterol/High blood pressure</td>
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<td>HIV</td>
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<td>Kidney disease</td>
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<td>Neurological disorder</td>
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<td>Obesity</td>
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<td>Seizures/Stroke</td>
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<td>Thyroid problem</td>
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<td>Tumors/Cancer</td>
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<td>Ulcers</td>
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<td>Other</td>
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Have you been hospitalized within the last 5 years?  

<table>
<thead>
<tr>
<th>Cause</th>
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Do you have a history of more than 1 miscarriage?  

- Yes
- No

If you are selected to participate in this study are you willing to sign an informed consent understanding we have access to medical information on you?  

- Yes
- No
Demographic Information

What is your birth date? _______ / _______ / _______
Month   Day   Year

How would you describe your race or ethnic background?
- White
- Black or African American
- American Indian or Alaska Native
- Hispanic or Latino
- Asian
- Native Hawaiian or Other Pacific Islander
- Other __________________________________________

What is the highest level of school or training that you have completed? [Circle only one response]

- Grade school  01  02  03  04  05  06  07  08
- High school   09  10  11  12
- Technical school or college  13  14  15  16
- Graduate or professional  17  18  19  20+
- Don’t know     X

Marital status?
- Single/never married
- Married
- Separated
- Divorced
- Widowed

Are you a full-time or part-time student? Are you employed?
- Full time
- Part time
- Not a student
- Yes
- No
- Student employee
Contact Information

<table>
<thead>
<tr>
<th>Name</th>
<th>M / F</th>
<th>Last</th>
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<th>Middle</th>
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<td>City</td>
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Name of person and phone number to call in case of an emergency if you are invited to participate in this study:

                                     

If we need to contact you, and can not reach you where/with who can a message be left?

                                     

How did you hear about our study?

                                     
APPENDIX B
DIETARY HISTORY QUESTIONNAIRE

NATIONAL INSTITUTES OF HEALTH

Diet History Questionnaire

Today's date:

<table>
<thead>
<tr>
<th>MONTH</th>
<th>DAY</th>
<th>YEAR</th>
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In what month were you born?

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<th>MONTH</th>
<th>DAY</th>
<th>YEAR</th>
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In what year were you born?

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<th>YEAR</th>
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<td>2006</td>
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Are you male or female?

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<th></th>
<th>Male</th>
<th>Female</th>
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<tbody>
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<td>Male</td>
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<tr>
<td>Female</td>
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BEFORE TURNING THE PAGE, PLEASE COMPLETE THE FOLLOWING QUESTIONS.

BAR CODE LABEL OR SUBJECT ID HERE
1. Over the past 12 months, how often did you drink tomato juice or vegetable juice?

- NEVER (GO TO QUESTION 2)
- 1 time per month or less
- 2–3 times per month
- 1–2 times per week
- 3–4 times per week
- 5–6 times per week

1a. Each time you drank tomato juice or vegetable juice, how much did you usually drink?

- Less than ¾ cup (6 ounces)
- ¾ to 1¼ cups (6 to 10 ounces)
- More than 1¼ cups (10 ounces)

2. Over the past 12 months, how often did you drink orange juice?

- NEVER (GO TO QUESTION 3)
- 1 time per month or less
- 2–3 times per month
- 1–2 times per week
- 3–4 times per week
- 5–6 times per week

2a. Each time you drank orange juice, how much did you usually drink?

- Less than ¾ cup (6 ounces)
- ¾ to 1¼ cups (6 to 10 ounces)
- More than 1¼ cups (10 ounces)

3. Over the past 12 months, how often did you drink other 100% fruit juice or 100% fruit juice mixtures (such as apple, grape, pineapple, grapefruit or others)?

- NEVER (GO TO QUESTION 4)
- 1 time per month or less
- 2–3 times per month
- 1–2 times per week
- 3–4 times per week
- 5–6 times per week

3a. Each time you drank other fruit juice or fruit juice mixtures, how much did you usually drink?

- Less than ¾ cup (6 ounces)
- ¾ to 1¼ cups (6 to 12 ounces)
- More than 1¼ cups (12 ounces)

Over the past 12 months...

4. How often did you drink other fruit drinks (such as cranberry cocktail, Hi-C, lemonade, or Kool-Aid, diet or regular)?

- NEVER (GO TO QUESTION 5)
- 1 time per month or less
- 2–3 times per month
- 1–2 times per week
- 3–4 times per week
- 5–6 times per week

4a. Each time you drank fruit drinks, how much did you usually drink?

- Less than 1 cup (8 ounces)
- 1 to 2 cups (8 to 16 ounces)
- More than 2 cups (16 ounces)

4b. How often were your fruit drinks diet or sugar-free drinks?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

5. How often did you drink milk, including lactose-free milk (but NOT milk substitutes) as a beverage (NOT in coffee, NOT in cereal)? (Please include chocolate milk, flavored milk like Ovaltine or Quick, and hot chocolate.)

- NEVER (GO TO QUESTION 6)
- 1 time per month or less
- 2–3 times per month
- 1–2 times per week
- 3–4 times per week
- 5–6 times per week

5a. Each time you drank milk as a beverage, how much did you usually drink?

- Less than 1 cup (8 ounces)
- 1 to 1½ cups (8 to 12 ounces)
- More than 1½ cups (12 ounces)

Please continue on next page.

5b. What kind of milk did you usually drink?
6. How often did you drink a milk substitute such as soy or rice milk as a beverage (NOT in coffee, NOT in cereal)? (Please include milk substitute used to make other beverages such as chocolate “milk”, Ovaltine, Quick and hot chocolate.)

☐ NEVER (GO TO QUESTION 7)

☐ 1 time per month or less ☐ 1 time per day
☐ 2–3 times per month ☐ 2–3 times per day
☐ 1–2 times per week ☐ 4–5 times per day
☐ 3–4 times per week ☐ 6 or more times per day
☐ 5–6 times per week

6a. Each time you drank a milk substitute as a beverage, how much did you usually drink?

☐ Less than 1 cup (8 ounces)
☐ 1 to 1½ cups (8 to 12 ounces)
☐ More than 1½ cups (12 ounces)

6b. What kind of milk substitute did you usually drink?

☐ Soy milk (8th Continent)
☐ Soy milk (Whitewave - Silk)
☐ Soy milk (VitaSoy)
☐ Soy milk (other brand)
☐ Rice milk

7. How often did you drink meal replacement, energy, or high-protein beverages such as Instant Breakfast, Ensure, Slimfast, AdvantEdge, Boost or others?

☐ NEVER (GO TO QUESTION 8)

☐ 1 time per month or less ☐ 1 time per day
☐ 2–3 times per month ☐ 2–3 times per day
☐ 1–2 times per week ☐ 4–5 times per day
☐ 3–4 times per week ☐ 6 or more times per day
☐ 5–6 times per week

7a. Each time you drank meal replacement, energy, or high protein beverages, how much did you usually drink?

☐ Less than 1 cup (8 ounces)
☐ 1 to 1½ cups (8 to 12 ounces)
☐ More than 1½ cups (12 ounces)

8. Over the past 12 months, did you drink soft drinks, soda, or pop?

☐ NO (GO TO QUESTION 9)

☐ YES

8a. How often did you drink soft drinks, soda, or pop IN THE SUMMER?

☐ NEVER

☐ 1 time per month or less ☐ 1 time per day
☐ 2–3 times per month ☐ 2–3 times per day
☐ 1–2 times per week ☐ 4–5 times per day
☐ 3–4 times per week ☐ 6 or more times per day
☐ 5–6 times per week

8b. How often did you drink soft drinks, soda, or pop DURING THE REST OF THE YEAR?

☐ NEVER

☐ 1 time per month or less ☐ 1 time per day
☐ 2–3 times per month ☐ 2–3 times per day
☐ 1–2 times per week ☐ 4–5 times per day
☐ 3–4 times per week ☐ 6 or more times per day
☐ 5–6 times per week

8c. Each time you drank soft drinks, soda, or pop, how often were these soft drinks, soda, or pop diet or sugar-free?

☐ Almost never or never
☐ About ¼ of the time
☐ About ½ of the time
☐ About ¾ of the time
☐ Almost always or always
8e. How often were these soft drinks, soda, or pop caffeine-free? (See list of caffeine and caffeine-free sodas provided by the researcher.)

☐ Almost never or never
☐ About ¼ of the time
☐ About ½ of the time
☐ About ¾ of the time
☐ Almost always or always

9. Over the past 12 months, did you drink beer?

☐ NO (GO TO QUESTION 10)

☐ YES

9a. How often did you drink beer IN THE SUMMER?

☐ NEVER
☐ 1 time per month or less
☐ 2–3 times per month
☐ 1–2 times per week
☐ 3–4 times per week
☐ 5–6 times per week

9b. How often did you drink beer DURING THE REST OF THE YEAR?

☐ NEVER
☐ 1 time per month or less
☐ 2–3 times per month
☐ 1–2 times per week
☐ 3–4 times per week
☐ 5–6 times per week

9c. Each time you drank beer, how much did you usually drink?

☐ Less than a 12-ounce can or bottle
☐ 1 to 3 12-ounce cans or bottles
☐ More than 3 12-ounce cans or bottles

Over the past 12 months...

10. How often did you drink wine or wine coolers?

☐ NEVER (GO TO QUESTION 11)

☐ 1 time per month or less
☐ 2–3 times per month
☐ 1–2 times per week
☐ 3–4 times per week
☐ 5–6 times per week

10a. Each time you drank wine or wine coolers, how much did you usually drink?

☐ Less than 5 ounces or less than 1 glass
☐ 5 to 12 ounces or 1 to 2 glasses
☐ More than 12 ounces or more than 2 glasses

11. How often did you drink liquor or mixed drinks?

☐ NEVER (GO TO QUESTION 12)

☐ 1 time per month or less
☐ 2–3 times per month
☐ 1–2 times per week
☐ 3–4 times per week
☐ 5–6 times per week

11a. Each time you drank liquor or mixed drinks, how much did you usually drink?

☐ Less than 1 shot of liquor
☐ 1 to 3 shots of liquor
☐ More than 3 shots of liquor

12. Over the past 12 months, did you eat oatmeal, grits, or other cooked cereal?

☐ NO (GO TO QUESTION 13)

☐ YES

12a. How often did you eat oatmeal, grits, or other cooked cereal IN THE WINTER?

☐ NEVER

☐ 1–6 times per winter
☐ 7–11 times per winter
☐ 1 time per month
☐ 2–3 times per month
☐ 1 time per week

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12b. How often did you eat oatmeal, grits, or other cooked cereal DURING THE REST OF THE YEAR?

- NEVER
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

12c. Each time you ate oatmeal, grits, or other cooked cereal, how much did you usually eat?

- Less than ¾ cup
- ¾ to 1¼ cups
- More than 1¼ cups

13. How often did you eat cold cereal?

- NEVER (GO TO QUESTION 14)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

13a. Each time you ate cold cereal, how much did you usually eat?

- Less than 1 cup
- 1 to 2½ cups
- More than 2½ cups

13b. How often was the cold cereal you ate a fortified cereal? (See list of fortified cereals provided by the researcher.)

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

13c. How often was the cold cereal you ate any other type of cold cereal (such as Corn Flakes, Rice Krispies, Frosted Flakes, Fruit Loops, Cap'n Crunch, or others)?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

13d. Was milk or a milk substitute added to your cold cereal?

- NO (GO TO QUESTION 14)
- YES

13e. What kind of milk or milk substitute was usually added?

- Whole milk (including lactose-free variety)
- 2% fat milk (including lactose-free variety)
- 1% fat milk (including lactose-free variety)
- Skim, nonfat, or ½% fat milk (including lactose-free variety)
- Soy milk (8th Continent)
- Soy milk (Whitewave - Silk)
- Soy milk (VitaSoy)
- Soy milk (other brand)
- Rice milk
- Other

13f. Each time milk or a milk substitute was added to your cold cereal, how much was usually added?

- Less than ½ cup
- ½ to 1 cup
- More than 1 cup

14. How often did you eat applesauce?

- NEVER (GO TO QUESTION 15)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

Please continue on next page.
19c. Each time you ate peaches, nectarines, or plums, how much did you usually eat?

☐ Less than 1 fruit or less than ½ cup
☐ 1 to 2 fruits or ½ to ¾ cup
☐ More than 2 fruits or more than ¾ cup

20. How often did you eat grapes?

☐ NEVER (GO TO QUESTION 21)
☐ 1–6 times per year ☐ 2 times per week
☐ 7–11 times per year ☐ 3–4 times per week
☐ 1 time per month ☐ 5–6 times per week
☐ 2–3 times per month ☐ 1 time per day
☐ 1 time per week ☐ 2 or more times per day

20a. Each time you ate grapes, how much did you usually eat?

☐ Less than ½ cup or less than 10 grapes
☐ ½ to 1 cup or 10 to 30 grapes
☐ More than 1 cup or more than 30 grapes

21. Over the past 12 months, did you eat cantaloupe?

☐ NO (GO TO QUESTION 22)
☐ YES

21a. How often did you eat fresh cantaloupe WHEN IN SEASON? (See list for description of "in season").

☐ NEVER
☐ 1–6 times per season ☐ 2 times per week
☐ 7–11 times per season ☐ 3–4 times per week
☐ 1 time per month ☐ 5–6 times per week
☐ 2–3 times per month ☐ 1 time per day
☐ 1 time per week ☐ 2 or more times per day

21b. How often did you eat fresh or frozen cantaloupe DURING THE REST OF THE YEAR?

☐ NEVER
☐ 1–6 times per year ☐ 2 times per week
☐ 7–11 times per year ☐ 3–4 times per week
☐ 1 time per month ☐ 5–6 times per week
☐ 2–3 times per month ☐ 1 time per day
☐ 1 time per week ☐ 2 or more times per day

22. Over the past 12 months, did you eat melon, other than cantaloupe (such as watermelon or honeydew)?

☐ NO (GO TO QUESTION 23)
☐ YES

22a. How often did you eat fresh melon, other than cantaloupe (such as watermelon or honeydew) WHEN IN SEASON? (See list for description of “in season”.)

☐ NEVER
☐ 1–6 times per season ☐ 2 times per week
☐ 7–11 times per season ☐ 3–4 times per week
☐ 1 time per month ☐ 5–6 times per week
☐ 2–3 times per month ☐ 1 time per day
☐ 1 time per week ☐ 2 or more times per day

22b. How often did you eat fresh or frozen melon, other than cantaloupe (such as watermelon or honeydew) DURING THE REST OF THE YEAR?

☐ NEVER
☐ 1–6 times per year ☐ 2 times per week
☐ 7–11 times per year ☐ 3–4 times per week
☐ 1 time per month ☐ 5–6 times per week
☐ 2–3 times per month ☐ 1 time per day
☐ 1 time per week ☐ 2 or more times per day

22c. Each time you ate melon other than cantaloupe, how much did you usually eat?

☐ Less than ½ cup or 1 small wedge
☐ ½ to 2 cups or 1 medium wedge
☐ More than 2 cups or 1 large wedge
23. Over the past 12 months, did you eat strawberries?

☐ NO (GO TO QUESTION 24)

☐ YES

23a. How often did you eat fresh strawberries WHEN IN SEASON? (See list for description of “in season”.)

☐ NEVER

☐ 1–6 times per season

☐ 7–11 times per season

☐ 1 time per month

☐ 2–3 times per month

☐ 1 time per week

23b. How often did you eat fresh or frozen strawberries DURING THE REST OF THE YEAR?

☐ NEVER

☐ 1–6 times per year

☐ 7–11 times per year

☐ 1 time per month

☐ 2–3 times per month

☐ 1 time per week

23c. Each time you ate strawberries, how much did you usually eat?

☐ Less than ¼ cup or less than 3 berries

☐ ¼ to ½ cup or 3 to 8 berries

☐ More than ½ cup or more than 8 berries

24. Over the past 12 months, did you eat oranges, tangerines, or tangelos?

☐ NO (GO TO QUESTION 25)

☐ YES

24a. How often did you eat fresh oranges, tangerines, or tangelos WHEN IN SEASON? (See list for description of “in season”.)

☐ NEVER

☐ 1–6 times per season

☐ 7–11 times per season

☐ 1 time per month

☐ 2–3 times per month

☐ 1 time per week

24b. How often did you eat oranges, tangerines, or tangelos (fresh or canned) DURING THE REST OF THE YEAR?

☐ NEVER

☐ 1–6 times per year

☐ 7–11 times per year

☐ 1 time per month

☐ 2–3 times per month

☐ 1 time per week

24c. Each time you ate oranges, tangerines, or tangelos, how many did you usually eat?

☐ Less than 1 fruit

☐ 1 fruit

☐ More than 1 fruit

25. Over the past 12 months, did you eat grapefruit?

☐ NO (GO TO QUESTION 26)

☐ YES

25a. How often did you eat fresh grapefruit WHEN IN SEASON? (See list for description of “in season”.)

☐ NEVER

☐ 1–6 times per season

☐ 7–11 times per season

☐ 1 time per month

☐ 2–3 times per month

☐ 1 time per week

25b. How often did you eat grapefruit (fresh or canned) DURING THE REST OF THE YEAR?

☐ NEVER

☐ 1–6 times per year

☐ 7–11 times per year

☐ 1 time per month

☐ 2–3 times per month

☐ 1 time per week

25c. Each time you ate grapefruit, how much did you usually eat?

☐ Less than ½ grapefruit

☐ ½ grapefruit

☐ More than ½ grapefruit
26. How often did you eat other kinds of fruit such as pineapple, mangoes, blueberries, or others?

- NEVER (GO TO QUESTION 27)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

26a. Each time you ate other kinds of fruit, how much did you usually eat?

- Less than ¼ cup
- ¼ to ½ cup
- More than ¼ cup

27. How often did you eat COOKED greens (such as spinach, turnip, collard, mustard, chard, or kale)?

- NEVER (GO TO QUESTION 28)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

27a. Each time you ate COOKED greens, how much did you usually eat?

- Less than ½ cup
- ½ to 1 cup
- More than ½ cup

28. How often did you eat RAW greens (such as spinach, turnip, collard, mustard, chard, or kale)? (We will ask about lettuce later.)

- NEVER (GO TO QUESTION 29)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

28a. Each time you ate RAW greens, how much did you usually eat?

- Less than ¼ cup or less than 2 baby carrots
- ¼ to ½ cup or 2 to 5 baby carrots
- More than ½ cup or more than 5 baby carrots

29. How often did you eat coleslaw?

- NEVER (GO TO QUESTION 30)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

29a. Each time you ate coleslaw, how much did you usually eat?

- Less than ¼ cup
- ¼ to ½ cup
- More than ¼ cup

30. How often did you eat sauerkraut or cabbage (other than coleslaw)?

- NEVER (GO TO QUESTION 31)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

30a. Each time you ate sauerkraut or cabbage, how much did you usually eat?

- Less than ¼ cup
- ¼ to 1 cup
- More than 1 cup

31. How often did you eat carrots (fresh, canned, or frozen)?

- NEVER (GO TO QUESTION 32)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

31a. Each time you ate carrots, how much did you usually eat?

- Less than ¼ cup or less than 2 baby carrots
- ¼ to ½ cup or 2 to 5 baby carrots
- More than ½ cup or more than 5 baby carrots
32. How often did you eat **string beans** or **green beans** (fresh, canned, or frozen)?

- NEVER (GO TO QUESTION 33)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

32a. Each time you ate **string beans** or **green beans**, how much did you usually eat?

- Less than ½ cup
- ½ to 1 cup
- More than 1 cup

33. How often did you eat **peas** (fresh, canned, or frozen)?

- NEVER (GO TO QUESTION 34)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

33a. Each time you ate **peas**, how much did you usually eat?

- Less than ¼ cup
- ¼ to ½ cup
- More than ½ cup

34. Over the past 12 months, did you eat **corn**?

- NO (GO TO QUESTION 35)
- YES

34a. How often did you eat **fresh corn WHEN IN SEASON**? (See list for description of “in season”).

- NEVER
- 1–6 times per season
- 7–11 times per season
- 1 time per month
- 2–3 times per month
- 1 time per week

34b. How often did you eat **corn** (fresh, canned, or frozen) DURING THE REST OF THE YEAR?

- NEVER
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

34c. Each time you ate **corn**, how much did you usually eat?

- Less than 1 ear or less than ½ cup
- 1 ear or ¼ to 1 cup
- More than 1 ear or more than 1 cup

35. Over the past 12 months, how often did you eat **broccoli** (fresh or frozen)?

- NEVER (GO TO QUESTION 36)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

35a. Each time you ate **broccoli**, how much did you usually eat?

- Less than ¼ cup
- ¼ to 1 cup
- More than 1 cup

36. How often did you eat **cauliflower** or **Brussels sprouts** (fresh or frozen)?

- NEVER (GO TO QUESTION 37)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

36a. Each time you ate **cauliflower** or **Brussels sprouts**, how much did you usually eat?

- Less than ¼ cup
- ½ to ½ cup
- More than ½ cup
37. How often did you eat mixed vegetables?

☐ NEVER (GO TO QUESTION 38)

☐ 1–6 times per year
☐ 7–11 times per year
☐ 1 time per month
☐ 2–3 times per month
☐ 1 time per week

☐ 2 times per week
☐ 3–4 times per week
☐ 5–6 times per week
☐ 1 time per day
☐ 2 or more times per day

37a. Each time you ate mixed vegetables, how much did you usually eat?

☐ Less than ½ cup
☐ ½ to 1 cup
☐ More than 1 cup

38. How often did you eat onions?

☐ NEVER (GO TO QUESTION 39)

☐ 1–6 times per year
☐ 7–11 times per year
☐ 1 time per month
☐ 2–3 times per month
☐ 1 time per week

☐ 2 times per week
☐ 3–4 times per week
☐ 5–6 times per week
☐ 1 time per day
☐ 2 or more times per day

38a. Each time you ate onions, how much did you usually eat?

☐ Less than 1 slice or less than 1 tablespoon
☐ 1 slice or 1 to 4 tablespoons
☐ More than 1 slice or more than 4 tablespoons

39. Now think about all the cooked vegetables you ate in the past 12 months and how they were prepared. How often were your vegetables cooked with some sort of fat, including oil spray? (Please do not include potatoes.)

☐ NEVER (GO TO QUESTION 40)

☐ 1–6 times per year
☐ 7–11 times per year
☐ 1 time per month
☐ 2–3 times per month
☐ 1 time per week

☐ 2 times per week
☐ 3–4 times per week
☐ 5–6 times per week
☐ 1 time per day
☐ 2 or more times per day

Over the past 12 months...

39a. Which fats were usually added to your vegetables during cooking? (Please do not include potatoes. Mark all that apply.)

☐ Margarine (including low-fat)
☐ Butter (including low-fat)
☐ Lard, fatback, or bacon fat
☐ Olive oil
☐ Canola or rapeseed oil
☐ Oil spray, such as Pam or others
☐ Other kinds of oils
☐ Corn oil
☐ None of the above

40. Now, thinking again about all the cooked vegetables you ate in the past 12 months, how often was some sort of fat, sauce, or dressing added after cooking or at the table? (Please do not include potatoes.)

☐ NEVER (GO TO QUESTION 41)

☐ 1–6 times per year
☐ 7–11 times per year
☐ 1 time per month
☐ 2–3 times per month
☐ 1–2 times per week

☐ 2 times per week
☐ 3–4 times per week
☐ 5–6 times per week
☐ 1 time per day
☐ 2 or more times per day

40a. Which fats, sauces, or dressings were usually added after cooking or at the table? (Please do not include potatoes. Mark all that apply.)

☐ Margarine including low-fat
☐ Cheese sauce
☐ Butter including low-fat
☐ White sauce
☐ Lard, fatback, or bacon fat
☐ Other
☐ Salad dressing

40b. If margarine, butter, lard, fatback, or bacon fat was added to your cooked vegetables after cooking or at the table, how much did you usually add?

☐ Did not usually add these
☐ Less than 1 teaspoon
☐ 1 to 3 teaspoons
☐ More than 3 teaspoons

40c. If salad dressing, cheese sauce, or white sauce was added to your cooked vegetables after cooking or at the table, how much did you usually add?

☐ Did not usually add these
☐ Less than 1 tablespoon
☐ 1 to 3 tablespoons
☐ More than 3 tablespoons
41. Over the past 12 months, how often did you eat **sweet peppers** (green, red, or yellow)?

- [ ] NEVER (GO TO QUESTION 42)
  - [ ] 1–6 times per year
  - [ ] 7–11 times per year
  - [ ] 1 time per month
  - [ ] 2–3 times per month
  - [ ] 1 time per week
  - [ ] 2 or more times per day

41a. Each time you ate **sweet peppers**, how much did you usually eat?

- [ ] Less than 1/8 pepper
- [ ] 1/8 to ¼ pepper
- [ ] More than ¼ pepper

42. Over the past 12 months, did you eat **fresh tomatoes** (including those in salads)?

- [ ] NO (GO TO QUESTION 43)
- [ ] YES

42a. How often did you eat **fresh tomatoes** (including those in salads) **WHEN IN SEASON**? (See description of “in season”.)

- [ ] NEVER
  - [ ] 1–6 times per season
  - [ ] 7–11 times per season
  - [ ] 1 time per month
  - [ ] 2–3 times per month
  - [ ] 1 time per week
  - [ ] 2 or more times per week

42b. How often did you eat **fresh tomatoes** (including those in salads) **DURING THE REST OF THE YEAR**?

- [ ] NEVER
  - [ ] 1–6 times per year
  - [ ] 7–11 times per year
  - [ ] 1 time per month
  - [ ] 2–3 times per month
  - [ ] 1 time per week
  - [ ] 2 or more times per week

42c. Each time you ate **fresh tomatoes**, how much did you usually eat?

- [ ] Less than ¼ tomato
- [ ] ¼ to ½ tomato
- [ ] More than ½ tomato

43. How often did you eat **lettuce salads** (with or without other vegetables)?

- [ ] NEVER (GO TO QUESTION 44)
  - [ ] 1–6 times per year
  - [ ] 7–11 times per year
  - [ ] 1 time per month
  - [ ] 2–3 times per month
  - [ ] 1 time per week
  - [ ] 2 or more times per day

43a. Each time you ate **lettuce salads**, how much did you usually eat?

- [ ] Less than ¼ cup
- [ ] ¼ to 1¼ cups
- [ ] More than 1¼ cups

44. How often did you eat **salad dressing** (including low-fat) on salads?

- [ ] NEVER (GO TO QUESTION 45)
  - [ ] 1–6 times per year
  - [ ] 7–11 times per year
  - [ ] 1 time per month
  - [ ] 2–3 times per month
  - [ ] 1 time per week
  - [ ] 2 or more times per day

44a. Each time you ate **salad dressing** on salads, how much did you usually eat?

- [ ] Less than 2 tablespoons
- [ ] 2 to 4 tablespoons
- [ ] More than 4 tablespoons

45. How often did you eat **sweet potatoes** or **yams**?

- [ ] NEVER (GO TO QUESTION 46)
  - [ ] 1–6 times per year
  - [ ] 7–11 times per year
  - [ ] 1 time per month
  - [ ] 2–3 times per month
  - [ ] 1 time per week
  - [ ] 2 or more times per day

45a. Each time you ate **sweet potatoes** or **yams**, how much did you usually eat?

- [ ] 1 small potato or less than ¼ cup
- [ ] 1 medium potato or ¼ to ¾ cup
- [ ] 1 large potato or more than ¾ cup
46. How often did you eat French fries, home fries, hash browned potatoes, or tater tots?

- NEVER (GO TO QUESTION 47)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

46a. Each time you ate French fries, home fries, hash browned potatoes, or tater tots how much did you usually eat?

- Less than 10 fries or less than 1/2 cup
- 10 to 25 fries or 1/2 to 1 cup
- More than 25 fries or more than 1 cup

47. How often did you eat potato salad?

- NEVER (GO TO QUESTION 48)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

47a. Each time you ate potato salad, how much did you usually eat?

- Less than 1/4 cup
- 1/2 to 1 cup
- More than 1 cup

48. How often did you eat baked, boiled, or mashed potatoes?

- NEVER (GO TO QUESTION 49)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

48a. Each time you ate baked, boiled, or mashed potatoes, how much did you usually eat?

- 1 small potato or less than 1/2 cup
- 1 medium potato or 1/2 to 1 cup
- 1 large potato or more than 1 cup

Over the past 12 months...

48b. How often was sour cream (including low-fat) added to your potatoes, EITHER IN COOKING OR AT THE TABLE?

- Almost never or never (GO TO QUESTION 48d)
- About 1/4 of the time
- About 1/2 of the time
- About 3/4 of the time
- Almost always or always

48c. Each time sour cream was added to your potatoes, how much was usually added?

- Less than 1 tablespoon
- 1 to 3 tablespoons
- More than 3 tablespoons

48d. How often was margarine (including low-fat) added to your potatoes, EITHER IN COOKING OR AT THE TABLE?

- Almost never or never
- About 1/4 of the time
- About 1/2 of the time
- About 3/4 of the time
- Almost always or always

48e. How often was butter (including low-fat) added to your potatoes, EITHER IN COOKING OR AT THE TABLE?

- Almost never or never
- About 1/4 of the time
- About 1/2 of the time
- About 3/4 of the time
- Almost always or always

48f. Each time margarine or butter was added to your potatoes, how much was usually added?

- Never added
- Less than 1 teaspoon
- 1 to 3 teaspoons
- More than 3 teaspoons

48g. How often was cheese or cheese sauce added to your potatoes, EITHER IN COOKING OR AT THE TABLE?

- Almost never or never (GO TO QUESTION 49)
- About 1/4 of the time
- About 1/2 of the time
- About 3/4 of the time
- Almost always or always
48h. Each time cheese or cheese sauce was added to your potatoes, how much was usually added?

☐ Less than 1 tablespoon  ☐ 1 to 3 tablespoons  ☐ More than 3 tablespoons

49. How often did you eat salsa?

☐ NEVER (GO TO QUESTION 50)
☐ 1–6 times per year  ☐ 2 times per week
☐ 7–11 times per year  ☐ 3–4 times per week
☐ 1 time per month  ☐ 5–6 times per week
☐ 2–3 times per month  ☐ 1 time per day
☐ 1 time per week  ☐ 2 or more times per day

49a. Each time you ate salsa, how much did you usually eat?

☐ Less than 1 tablespoon  ☐ 1 to 5 tablespoons  ☐ More than 5 tablespoons

50. How often did you eat catsup?

☐ NEVER (GO TO QUESTION 51)
☐ 1–6 times per year  ☐ 2 times per week
☐ 7–11 times per year  ☐ 3–4 times per week
☐ 1 time per month  ☐ 5–6 times per week
☐ 2–3 times per month  ☐ 1 time per day
☐ 1 time per week  ☐ 2 or more times per day

50a. Each time you ate catsup, how much did you usually eat?

☐ Less than 1 teaspoon  ☐ 1 to 6 teaspoons  ☐ More than 6 teaspoons

51. How often did you eat stuffing, dressing, or dumplings?

☐ NEVER (GO TO QUESTION 52)
☐ 1–6 times per year  ☐ 2 times per week
☐ 7–11 times per year  ☐ 3–4 times per week
☐ 1 time per month  ☐ 5–6 times per week
☐ 2–3 times per month  ☐ 1 time per day
☐ 1 time per week  ☐ 2 or more times per day

51a. Each time you ate stuffing, dressing, or dumplings, how much did you usually eat?

☐ Less than ½ cup  ☐ ½ to 1 ⅔ cups  ☐ More than 1 ⅔ cups

52. How often did you eat chili made with beef? (DO NOT include chili made with soy or vegetable protein substitute. We will ask about this later.)

☐ NEVER (GO TO QUESTION 52b)
☐ 1–6 times per year  ☐ 2 times per week
☐ 7–11 times per year  ☐ 3–4 times per week
☐ 1 time per month  ☐ 5–6 times per week
☐ 2–3 times per month  ☐ 1 time per day
☐ 1 time per week  ☐ 2 or more times per day

52a. Each time you ate chili made with beef, how much did you usually eat?

☐ Less than ½ cup  ☐ ½ to 1 ⅔ cups  ☐ More than 1 ⅔ cups

52b. How often did you eat chili made with meat other than beef? (DO NOT include chili made with soy or vegetable protein substitutes. We will ask about these later.)

☐ NEVER (GO TO QUESTION 52d)
☐ 1–6 times per year  ☐ 2 times per week
☐ 7–11 times per year  ☐ 3–4 times per week
☐ 1 time per month  ☐ 5–6 times per week
☐ 2–3 times per month  ☐ 1 time per day
☐ 1 time per week  ☐ 2 or more times per day

52c. Each time you ate chili made with meat other than beef, how much did you usually eat?

☐ Less than ½ cup  ☐ ½ to 1 ⅔ cups  ☐ More than 1 ⅔ cups

52d. How often did you eat chili without meat? (DO NOT include chili made with a soy or vegetable protein meat substitute. We will ask about meat substitutes later.)

☐ NEVER (GO TO QUESTION 53)
☐ 1–6 times per year  ☐ 2 times per week
☐ 7–11 times per year  ☐ 3–4 times per week
☐ 1 time per month  ☐ 5–6 times per week
☐ 2–3 times per month  ☐ 1 time per day
☐ 1 time per week  ☐ 2 or more times per day
52e. Each time you ate chili made without meat, how much did you usually eat?
- Less than ¼ cup
- ⅜ to 1⅜ cups
- More than 1⅜ cups

53. How often did you eat Mexican foods (such as tacos, tostadas, burritos, tamales, fajitas, enchiladas, quesadillas, and chimichangas) made with beef? (DO NOT include Mexican foods made with soy or vegetable protein substitutes. We will ask about these later.)
- NEVER (GO TO QUESTION 53b)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week
- 2 or more times per day

53a. Each time you ate Mexican foods made with beef, how much did you usually eat?
- Less than 1 taco, burrito, etc.
- 1 to 2 tacos, burritos, etc.
- More than 2 tacos, burritos, etc.

53b. How often did you eat Mexican foods (such as tacos, tostadas, burritos, tamales, fajitas, enchiladas, quesadillas, and chimichangas) made with meat other than beef? (DO NOT include Mexican foods made with soy or vegetable protein substitutes. We will ask about these later.)
- NEVER (GO TO QUESTION 53d)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week
- 2 or more times per day

53c. Each time you ate Mexican foods made with meat other than beef how much did you usually eat?
- Less than ¼ cup
- ⅜ to 1⅜ cups
- More than 1⅜ cups

53d. How often did you eat Mexican foods (such as tacos, tostadas, burritos, tamales, fajitas, enchiladas, quesadillas, and chimichangas) made without meat? (DO NOT include Mexican foods made with a soy or vegetable protein meat substitute.)
- NEVER (GO TO QUESTION 54)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week
- 2 or more times per day

53e. Each time you ate Mexican foods made without meat, how much did you usually eat?
- Less than ½ cup
- ½ to 1⅗ cups
- More than 1⅗ cups

54. How often did you eat cooked dried beans (such as baked beans, pinto beans, kidney beans, black-eyed peas, lima beans, lentils, soybeans, refried beans, or chick peas/garbanzo beans, including hummus)? (Please don’t include bean soups or chili.)
- NEVER (GO TO QUESTION 55)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week
- 2 or more times per day

54a. Each time you ate beans, how much did you usually eat?
- Less than ½ cup
- ½ to 1 cup
- More than 1 cup

54b. How often were the beans you ate refried beans, beans prepared with any type of fat, or with meat added?
- Almost never or never
- About ⅓ of the time
- About ⅔ of the time
- Almost always or always
55. How often did you eat other kinds of vegetables such as asparagus, mushrooms, zucchini or others?

- NEVER (GO TO QUESTION 56)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

55a. Each time you ate other kinds of vegetables, how much did you usually eat?

- Less than ¼ cup
- ¼ to ½ cup
- More than ½ cup

56. How often did you eat rice or other cooked grains (such as bulgur, cracked wheat, or millet)?

- NEVER (GO TO QUESTION 57)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

56a. Each time you ate rice or other cooked grains, how much did you usually eat?

- Less than ¼ cup
- ¼ to 1½ cups
- More than 1½ cups

56b. How often was butter, margarine, or oil added to your rice IN COOKING OR AT THE TABLE?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

57. How often did you eat pancakes, waffles, or French toast?

- NEVER (GO TO QUESTION 58)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

57a. Each time you ate pancakes, waffles, or French toast, how much did you usually eat?

- Less than 1 medium piece
- 1 to 3 medium pieces
- More than 3 medium pieces

57b. How often was margarine (including low-fat) added to your pancakes, waffles, or French toast AFTER COOKING OR AT THE TABLE?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

57c. How often was butter (including low-fat) added to your pancakes, waffles, or French toast AFTER COOKING OR AT THE TABLE?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

57d. Each time margarine or butter was added to your pancakes, waffles, or French toast, how much was usually added?

- Never added
- Less than 1 teaspoon
- 1 to 3 teaspoons
- More than 3 teaspoons

57e. How often was syrup added to your pancakes, waffles, or French toast?

- Almost never or never (GO TO QUESTION 58)
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

57f. Each time syrup was added to your pancakes, waffles, or French toast, how much was usually added?

- Less than 1 tablespoon
- 1 to 4 tablespoons
- More than 4 tablespoons
58. How often did you eat **lasagna, stuffed shells, stuffed manicotti, ravioli, or tortellini made with beef**? *(DO NOT include spaghetti or other pasta or products made with soy or vegetable protein substitutes. We will ask about these later.)*

- NEVER (GO TO QUESTION 58b)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

58a. Each time you ate **lasagna, stuffed shells, stuffed manicotti, ravioli, or tortellini made with beef**, how much did you usually eat?

- Less than 1 cup
- 1 to 2 cups
- More than 2 cups

58b. How often did you eat **lasagna, stuffed shells, stuffed manicotti, ravioli, or tortellini made with meat other than beef**? *(DO NOT include spaghetti or other pasta or products made with soy or vegetable protein substitutes. We will ask about these later.)*

- NEVER (GO TO QUESTION 58d)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

58c. Each time you ate **lasagna, stuffed shells, stuffed manicotti, ravioli, or tortellini made with meat other than beef**, how much did you usually eat?

- Less than 1 cup
- 1 to 2 cups
- More than 2 cups

58d. How often did you eat **lasagna, stuffed shells, stuffed manicotti, ravioli, or tortellini made without meat**? *(DO NOT include spaghetti or other pasta or products made with a soy or vegetable protein substitutes. We will ask about these later.)*

- NEVER (GO TO QUESTION 59)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

58e. Each time you ate **lasagna, stuffed shells, stuffed manicotti, ravioli, or tortellini made without meat**, how much did you usually eat?

- Less than 1 cup
- 1 to 2 cups
- More than 2 cups

59. How often did you eat **macaroni and cheese**?

- NEVER (GO TO QUESTION 60)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

59a. Each time you ate **macaroni and cheese**, how much did you usually eat?

- Less than 1 cup
- 1 to 1½ cups
- More than 1½ cups

60. How often did you eat **pasta salad or macaroni salad**?

- NEVER (GO TO QUESTION 61)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

60a. Each time you ate **pasta salad or macaroni salad**, how much did you usually eat?

- Less than ¼ cup
- ¼ to 1 cup
- More than 1 cup

Over the past 12 months...

60b. Each time you ate **pasta salad or macaroni salad**, how much did you usually eat?

- Less than ¼ cup
- ¼ to 1 cup
- More than 1 cup
61. Other than the pastas listed in Questions 58, 59, and 60, how often did you eat pasta, spaghetti, or other noodles?

- NEVER (GO TO QUESTION 62)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week
- 2 or more times per day

61a. Each time you ate pasta, spaghetti, or other noodles, how much did you usually eat?
- Less than 1 cup
- 1 to 3 cups
- More than 3 cups

61b. How often did you eat your pasta, spaghetti, or other noodles with tomato sauce or spaghetti sauce made with beef? (DO NOT include tomato or spaghetti sauce made with soy or vegetable protein substitutes. We will ask about these later.)
- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

61c. How often did you eat your pasta, spaghetti, or other noodles with tomato sauce or spaghetti sauce made with meat other than beef? (DO NOT include tomato or spaghetti sauce made with soy or vegetable protein substitutes. We will ask about these later.)
- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

61d. How often did you eat your pasta, spaghetti, or other noodles with tomato sauce or spaghetti sauce made WITHOUT meat? (DO NOT include tomato sauce or spaghetti sauce made with a soy or vegetable protein meat substitute. We will ask about these later.)
- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

61e. How often did you eat your pasta, spaghetti, or other noodles with margarine, butter, oil, or cream sauce?
- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

62. How often did you eat bagels or English muffins?

- NEVER (GO TO INTRODUCTION TO QUESTION 63)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week
- 2 or more times per day

62a. Each time you ate bagels or English muffins, how many did you usually eat?
- Less than 1 bagel or English muffin
- 1 bagel or English muffin
- More than 1 bagel or English muffin

62b. How often was margarine (including low-fat) added to your bagels or English muffins?
- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

62c. How often was butter (including low-fat) added to your bagels or English muffins?
- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

62d. Each time margarine or butter was added to your bagels or English muffins, how much was usually added?
- Never added
- Less than 1 teaspoon
- 1 to 2 teaspoons
- More than 2 teaspoons
62e. How often was cream cheese (including low-fat) spread on your bagels or English muffins?

- Almost never or never (GO TO INTRODUCTION TO QUESTION 63)
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

Over the past 12 months...

62f. Each time cream cheese was added to your bagels or English muffins, how much was usually added?

- Less than 1 tablespoon
- 1 to 2 tablespoons
- More than 2 tablespoons

The next questions ask about your intake of breads other than bagels or English muffins. First, we will ask about bread you ate as part of sandwiches only. Then we will ask about all other bread you ate.

63. How often did you eat breads or rolls AS PART OF SANDWICHES (including burger and hot dog rolls)?

- NEVER (GO TO QUESTION 64)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

63a. Each time you ate breads or rolls AS PART OF SANDWICHES, how many did you usually eat?

- 1 slice or ½ roll
- 2 slices or 1 roll
- More than 2 slices or more than 1 roll

63b. How often were the breads or rolls that you used for your sandwiches white bread (including burger and hot dog rolls)?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

63c. How often was mayonnaise or mayonnaise-type dressing (including low-fat) added to your sandwich bread or rolls?

- Almost never or never (GO TO QUESTION 63e)
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

63d. Each time mayonnaise or mayonnaise-type dressing was added to your sandwich breads or rolls, how much was usually added?

- Less than 1 teaspoon
- 1 to 3 teaspoons
- More than 3 teaspoons

63e. How often was margarine (including low-fat) added to your sandwich bread or rolls?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

63f. How often was butter (including low-fat) added to your sandwich bread or rolls?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

63g. Each time margarine or butter was added to your sandwich breads or rolls, how much was usually added?

- Never added
- Less than 1 teaspoon
- 1 to 2 teaspoons
- More than 2 teaspoons

64. How often did you eat breads or dinner rolls, NOT AS PART OF SANDWICHES?

- NEVER (GO TO QUESTION 65)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

65. How often did you eat breads or dinner rolls, NOT AS PART OF SANDWICHES?

- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week
- 2 or more times per day
64a. Each time you ate **breads** or **dinner rolls**, **NOT AS PART OF SANDWICHES**, how much did you usually eat?
- [ ] 1 slice or 1 dinner roll
- [ ] 2 slices or 2 dinner rolls
- [ ] More than 2 slices or 2 dinner rolls

*Over the past 12 months...*

64b. How often were the breads or rolls you ate **white bread**?
- [ ] Almost never or never
- [ ] About ¼ of the time
- [ ] About ½ of the time
- [ ] About ¾ of the time
- [ ] Almost always or always

64c. How often was **margarine** (including low-fat) added to your breads or rolls?
- [ ] Almost never or never
- [ ] About ¼ of the time
- [ ] About ½ of the time
- [ ] About ¾ of the time
- [ ] Almost always or always

64d. How often was **butter** (including low-fat) added to your breads or rolls?
- [ ] Almost never or never
- [ ] About ¼ of the time
- [ ] About ½ of the time
- [ ] About ¾ of the time
- [ ] Almost always or always

64e. Each time **margarine** or **butter** was added to your breads or rolls, how much was usually added?
- [ ] Never added
- [ ] Less than 1 teaspoon
- [ ] 1 to 2 teaspoons
- [ ] More than 2 teaspoons

64g. Each time **cream cheese** was added to your breads or rolls, how much was usually added?
- [ ] Less than 1 tablespoon
- [ ] 1 to 2 tablespoons
- [ ] More than 2 tablespoons

65. How often did you eat **jam**, **jelly**, or **honey** on bagels, muffins, bread, rolls, or crackers?
- [ ] NEVER (GO TO QUESTION 66)
- [ ] 1–6 times per year
- [ ] 7–11 times per year
- [ ] 1 time per month
- [ ] 2–3 times per month
- [ ] 1 time per week
- [ ] 2 or more times per week

65a. Each time you ate **jam**, **jelly**, or **honey**, how much did you usually eat?
- [ ] Less than 1 teaspoon
- [ ] 1 to 3 teaspoons
- [ ] More than 3 teaspoons

66. How often did you eat **peanut butter** or **other nut butter**?
- [ ] NEVER (GO TO QUESTION 67)
- [ ] 1–6 times per year
- [ ] 7–11 times per year
- [ ] 1 time per month
- [ ] 2–3 times per month
- [ ] 1 time per day
- [ ] 1 time per week
- [ ] 2 or more times per day

66a. Each time you ate **peanut butter** or **other nut butter**, how much did you usually eat?
- [ ] Less than 1 tablespoon
- [ ] 1 to 2 tablespoons
- [ ] More than 2 tablespoons

67. How often did you eat **roast beef** or **steak IN SANDWICHES, SUBS or WRAPS**?
- [ ] NEVER (GO TO QUESTION 68)
- [ ] 1–6 times per year
- [ ] 7–11 times per year
- [ ] 1 time per month
- [ ] 2–3 times per month
- [ ] 1 time per week
- [ ] 2 or more times per day
67a. Each time you ate roast beef or steak in sandwiches, subs or wraps, how much did you usually eat?

☐ Less than 1 slice or less than 2 ounces
☐ 1 to 2 slices or 2 to 4 ounces
☐ More than 2 slices or more than 4 ounces

Over the past 12 months...

68. How often did you eat turkey or chicken cold cuts (such as loaf, luncheon meat, turkey ham, turkey salami, or turkey pastrami), including those used in sandwiches, subs or wraps? (DO NOT include turkey or chicken cold cuts made with soy or vegetable protein substitutes. We will ask about these later.)

☐ NEVER (GO TO QUESTION 69)
☐ 1–6 times per year  ☐ 2 times per week
☐ 7–11 times per year  ☐ 3–4 times per week
☐ 1 time per month  ☐ 5–6 times per week
☐ 2–3 times per month  ☐ 1 time per day
☐ 1 time per week  ☐ 2 or more times per day

68a. Each time you ate turkey or chicken cold cuts, how much did you usually eat?

☐ Less than 1 slice
☐ 1 to 3 slices
☐ More than 3 slices

69. How often did you eat luncheon or deli-style ham including luncheon or deli-style ham used in sandwiches, subs or wraps? (DO NOT include luncheon or deli-style ham made with soy or vegetable protein substitutes. We will ask about these later.)

☐ NEVER (GO TO QUESTION 70)
☐ 1–6 times per year  ☐ 2 times per week
☐ 7–11 times per year  ☐ 3–4 times per week
☐ 1 time per month  ☐ 5–6 times per week
☐ 2–3 times per month  ☐ 1 time per day
☐ 1 time per week  ☐ 2 or more times per day

69a. Each time you ate luncheon or deli-style ham, how much did you usually eat?

☐ Less than 1 slice
☐ 1 to 3 slices
☐ More than 3 slices

69b. How often was the luncheon or deli-style ham you ate light, low-fat, or fat-free?

☐ Almost never or never
☐ About ¼ of the time
☐ About ½ of the time
☐ About ¾ of the time
☐ Almost always or always

70. How often did you eat other cold cuts or luncheon meats (such as beef bologna, corned beef, pastrami, or others, including low-fat)? Include other cold cuts and luncheon meats used in sandwiches, subs or wraps. (DO NOT include ham, turkey, salami, chicken cold cuts or cold cuts made with soy or vegetable protein.)

☐ NEVER (GO TO QUESTION 71)
☐ 1–6 times per year  ☐ 2 times per week
☐ 7–11 times per year  ☐ 3–4 times per week
☐ 1 time per month  ☐ 5–6 times per week
☐ 2–3 times per month  ☐ 1 time per day
☐ 1 time per week  ☐ 2 or more times per day

70a. Each time you ate other cold cuts or luncheon meats, how much did you usually eat?

☐ Less than 1 slice
☐ 1 to 3 slices
☐ More than 3 slices

70b. How often were the other cold cuts or luncheon meats you ate light, low-fat, or fat-free cold cuts or luncheon meats? (Please do not include ham, turkey, or chicken cold cuts.)

☐ Almost never or never
☐ About ¼ of the time
☐ About ½ of the time
☐ About ¾ of the time
☐ Almost always or always

Please continue on next page.
71. How often did you eat canned tuna (including in salads, sandwiches, or casseroles)?

- NEVER (GO TO QUESTION 72)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week
- 2 or more times per day

71a. Each time you ate canned tuna, how much did you usually eat?

- Less than ¼ cup or less than 2 ounces
- ¼ to ½ cup or 2 to 3 ounces
- More than ½ cup or more than 3 ounces

71b. How often was the canned tuna you ate water-packed tuna?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

72. How often did you eat GROUND chicken or turkey? (DO NOT include soy or vegetable protein substitutes. We will ask about these later.)

- NEVER (GO TO QUESTION 73)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week
- 2 or more times per day

72a. Each time you ate GROUND chicken or turkey, how much did you usually eat?

- Less than 2 ounces or less than ½ cup
- 2 to 4 ounces or ½ to 1 cup
- More than 4 ounces or more than 1 cup

73. How often did you eat beef hamburgers or cheeseburgers? (DO NOT include soy or vegetable protein substitutes. We will ask about these later.)

- NEVER (GO TO QUESTION 74)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week
- 2 or more times per day

73a. Each time you ate beef hamburgers or cheeseburgers, how much did you usually eat?

- Less than 1 patty or less than 2 ounces
- 1 patty or 2 to 4 ounces
- More than 1 patty or more than 4 ounces

73b. How often were the beef hamburgers or cheeseburgers you ate made with lean ground beef?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

74. How often did you eat ground beef in mixtures (such as meatballs, casseroles, or meatloaf)? (DO NOT include soy or vegetable protein substitutes. We will ask about these later.)

- NEVER (GO TO QUESTION 75)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week
- 2 or more times per day

74a. Each time you ate ground beef in mixtures, how much did you usually eat?

- Less than 3 ounces or less than ½ cup
- 3 to 8 ounces or ½ to 1 cup
- More than 8 ounces or more than 1 cup
75. How often did you eat **hot dogs** or **frankfurters**?  
(Do NOT include sausages or vegetarian hot dogs.)

- [ ] NEVER (GO TO QUESTION 76)
- [ ] 1–6 times per year
- [ ] 7–11 times per year
- [ ] 1 time per month
- [ ] 2–3 times per month
- [ ] 1 time per week
- [ ] 2 or more times per day

75a. Each time you ate **hot dogs** or **frankfurters**, how many did you usually eat?  
- [ ] Less than 1 hot dog
- [ ] 1 to 2 hot dogs
- [ ] More than 2 hot dogs

75b. How often were the hot dogs or frankfurters you ate **light** or **low-fat hot dogs**?  
- [ ] Almost never or never
- [ ] About ¼ of the time
- [ ] About ½ of the time
- [ ] About ¾ of the time
- [ ] Almost always or always

77. How often did you eat **roast beef** or **pot roast**?  
(Do NOT include roast beef or pot roast in sandwiches, subs or wraps. Do NOT include soy or vegetable protein substitutes. We will ask about these later.)

- [ ] NEVER (GO TO QUESTION 78)
- [ ] 1–6 times per year
- [ ] 7–11 times per year
- [ ] 1 time per month
- [ ] 2–3 times per month
- [ ] 1 time per week
- [ ] 2 or more times per day

77a. Each time you ate **roast beef** or **pot roast** (including in mixtures), how much did you usually eat?  
- [ ] Less than 2 ounces
- [ ] 2 to 5 ounces
- [ ] More than 5 ounces

78. How often did you eat **steak** (beef)?  
(Do NOT include steak in sandwiches, subs or wraps.)

- [ ] NEVER (GO TO QUESTION 79)
- [ ] 1–6 times per year
- [ ] 7–11 times per year
- [ ] 1 time per month
- [ ] 2–3 times per month
- [ ] 1 time per week
- [ ] 2 or more times per day

78a. Each time you ate **steak** (beef), how much did you usually eat?  
- [ ] Less than 3 ounces
- [ ] 3 to 7 ounces
- [ ] More than 7 ounces

78b. How often was the steak you ate **lean steak**?  
- [ ] Almost never or never
- [ ] About ¼ of the time
- [ ] About ½ of the time
- [ ] About ¾ of the time
- [ ] Almost always or always

79. How often did you eat **pork** or **beef spareribs**?  
(Do NOT include roast beef or pot roast in sandwiches, subs or wraps. Do NOT include soy or vegetable protein substitutes. We will ask about these later.)

- [ ] NEVER (GO TO QUESTION 80)
- [ ] 1–6 times per year
- [ ] 7–11 times per year
- [ ] 1 time per month
- [ ] 2–3 times per month
- [ ] 1 time per week
- [ ] 2 or more times per day
79a. Each time you ate pork or beef spareribs, how much did you usually eat?

☐ Less than 4 ribs  ☐ 4 to 12 ribs  ☐ More than 12 ribs

80. How often did you eat roast turkey, turkey cutlets, or turkey nuggets (including in sandwiches)? (DO NOT include soy or vegetable protein substitutes. We will ask about these later.)

☐ NEVER (GO TO QUESTION 81)

☐ 1–6 times per year  ☐ 2 times per week
☐ 7–11 times per year  ☐ 3–4 times per week
☐ 1 time per month  ☐ 5–6 times per week
☐ 2–3 times per month  ☐ 1 time per day
☐ 1 time per week  ☐ 2 or more times per day

80a. Each time you ate roast turkey, turkey cutlets, or turkey nuggets, how much did you usually eat? (Please note: 4 to 8 turkey nuggets = 3 ounces.)

☐ Less than 2 ounces  ☐ 2 to 4 ounces  ☐ More than 4 ounces

81. How often did you eat chicken as part of salads, sandwiches, casseroles, stews, or other mixtures? (DO NOT include soy or vegetable protein substitutes. We will ask about these later.)

☐ NEVER (GO TO QUESTION 82)

☐ 1–6 times per year  ☐ 2 times per week
☐ 7–11 times per year  ☐ 3–4 times per week
☐ 1 time per month  ☐ 5–6 times per week
☐ 2–3 times per month  ☐ 1 time per day
☐ 1 time per week  ☐ 2 or more times per day

Over the past 12 months...

81a. Each time you ate chicken as part of salads, sandwiches, casseroles, stews, or other mixtures, how much did you usually eat?

☐ Less than ½ cup  ☐ ½ to 1½ cups  ☐ More than 1½ cups

82. How often did you eat baked, broiled, roasted, stewed, or fried chicken (including nuggets)? (DO NOT include chicken in mixtures or products made with soy or vegetable protein substitutes. We will ask about these later.)

☐ NEVER (GO TO QUESTION 83)

☐ 1–6 times per year  ☐ 2 times per week
☐ 7–11 times per year  ☐ 3–4 times per week
☐ 1 time per month  ☐ 5–6 times per week
☐ 2–3 times per month  ☐ 1 time per day
☐ 1 time per week  ☐ 2 or more times per day

82a. Each time you ate baked, broiled, roasted, stewed, or fried chicken (including nuggets), how much did you usually eat?

☐ Less than 2 drumsticks or wings, less than 1 breast or thigh, or less than 4 nuggets
☐ 2 drumsticks or wings, 1 breast or thigh, or 4 to 8 nuggets
☐ More than 2 drumsticks or wings, more than 1 breast or thigh, or more than 8 nuggets

82b. How often was the chicken you ate fried chicken (including deep fried) or chicken nuggets?

☐ Almost never or never  ☐ About ¼ of the time  ☐ About ½ of the time  ☐ About ¾ of the time  ☐ Almost always or always

82c. How often was the chicken you ate WHITE meat?

☐ Almost never or never  ☐ About ¼ of the time  ☐ About ½ of the time  ☐ About ¾ of the time  ☐ Almost always or always

82d. How often did you eat chicken WITH skin?

☐ Almost never or never  ☐ About ¼ of the time  ☐ About ½ of the time  ☐ About ¾ of the time  ☐ Almost always or always
83. How often did you eat **baked ham** or **ham steak**?

- **NEVER (GO TO QUESTION 84)**
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

83a. Each time you ate **baked ham** or **ham steak**, how much did you usually eat?

- Less than 1 ounce
- 1 to 3 ounces
- More than 3 ounces

84. How often did you eat **pork** (including chops, roasts, sausage and in mixed dishes)? *(DO NOT include ham, or ham steak.)*

- **NEVER (GO TO QUESTION 85)**
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

84a. Each time you ate **pork**, how much did you usually eat?

- Less than 2 ounces or less than 1 chop
- 2 to 5 ounces or 1 chop
- More than 5 ounces or more than 1 chop

85. How often did you eat **gravy** on meat, chicken, potatoes, rice, etc.?

- **NEVER (GO TO QUESTION 86)**
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

85a. Each time you ate **gravy** on meat, chicken, potatoes, rice, etc., how much did you usually eat?

- Less than 1/8 cup
- 1/8 to 1/2 cup
- More than 1/2 cup

86. How often did you eat **liver** (all kinds) or **liverwurst**?

- **NEVER (GO TO QUESTION 87)**
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

86a. Each time you ate **liver** or **liverwurst**, how much did you usually eat?

- Less than 1 ounce
- 1 to 4 ounces
- More than 4 ounces

87. How often did you eat **bacon** (including low-fat but **not** imitation)? *(DO NOT include soy or vegetable protein substitutes. We will ask about these later.)*

- **NEVER (GO TO QUESTION 88)**
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

87a. Each time you ate **bacon**, how much did you usually eat?

- Fewer than 2 slices
- 2 to 3 slices
- More than 3 slices

87b. How often was the bacon you ate **light, low-fat, or lean bacon**?

- Almost never or never
- About 1/2 of the time
- About 1/3 of the time
- About 1/3 of the time
- Almost always or always

**Please continue on next page.**
88. How often did you eat beef sausage (including low-fat)? (DO NOT include soy or vegetable protein substitutes. We will ask about these later.)

- [ ] NEVER (GO TO QUESTION 89)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

88a. Each time you ate beef sausage, how much did you usually eat?

- [ ] Less than 1 patty or 2 links
- [ ] 1 to 3 patties or 2 to 5 links
- [ ] More than 3 patties or 5 links

88b. How often was the beef sausage you ate light, low-fat, or lean beef sausage?

- [ ] Almost never or never
- [ ] About ¼ of the time
- [ ] About ½ of the time
- [ ] Almost always or always

88c. Each time you ate beef sausage, how much did you usually eat?

- [ ] Less than 1 patty or 2 links
- [ ] 1 to 3 patties or 2 to 5 links
- [ ] More than 3 patties or 5 links

89. How often did you eat fish sticks, fried fish, or fried seafood (not including clams, shrimp, or other shellfish or soy or vegetable protein fish substitutes)?

- [ ] NEVER (GO TO QUESTION 89b)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

89a. Each time you ate fish sticks, fried fish, or fried seafood how much did you usually eat?

- [ ] Less than 2 ounces or less than 1 fillet
- [ ] 2 to 7 ounces or 1 fillet
- [ ] More than 7 ounces or more than 1 fillet

89b. How often did you eat clams, mussels, oysters, or scallops?

- [ ] NEVER (GO TO QUESTION 89d)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

89c. Each time you ate clams, mussels, oysters, or scallops how much did you usually eat?

- [ ] Less than 2 ounces
- [ ] 2 to 7 ounces
- [ ] More than 7 ounces

89d. How often did you eat shrimp, crab, or lobster? (DO NOT include imitation products or products made with soy or vegetable protein substitutes.)

- [ ] NEVER (GO TO QUESTION 90)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

89e. Each time you ate shrimp, crab, or lobster how much did you usually eat?

- [ ] Less than 2 ounces or less than 1 fillet
- [ ] 2 to 7 ounces or 1 fillet
- [ ] More than 7 ounces or more than 1 fillet

90. How often did you eat fish or seafood (not including clams, shrimp, or other shellfish or soy or vegetable protein fish substitutes) that was NOT FRIED? (Include intake from fish sushi.)

- [ ] NEVER (GO TO QUESTION 90b)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week

90a. Each time you ate fish or seafood that was NOT FRIED, how much did you usually eat?

- [ ] Less than 2 ounces or less than 1 fillet
- [ ] 2 to 5 ounces or 1 fillet
- [ ] More than 5 ounces or more than 1 fillet

90b. How often did you eat shellfish (all kinds) that was NOT FRIED?

- [ ] NEVER (GO TO QUESTION 91)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week
90c. Each time you ate shellfish (all kinds) that was NOT FRIED how much did you usually eat?

☐ Less than 2 ounces
☐ 2 to 7 ounces
☐ More than 7 ounces

Over the past 12 months...

Now think about all the meat, poultry, and fish you ate in the past 12 months and how they were prepared.

91. How often was oil, butter, margarine, or other fat used to FRY, SAUTE, BASTE, OR MARINATE any meat, poultry, or fish you ate? (Please do not include deep frying.)

☐ NEVER (GO TO QUESTION 92)

☐ 1–6 times per year ☐ 2 times per week
☐ 7–11 times per year ☐ 3–4 times per week
☐ 1 time per month ☐ 5–6 times per week
☐ 2–3 times per month ☐ 1 time per day
☐ 1 time per week ☐ 2 or more times per day

91a. Which of the following fats were regularly used to prepare your meat, poultry, or fish? (Mark all that apply.)

☐ Margarine (including low-fat)
☐ Corn oil
☐ Butter (including low-fat)
☐ Canola or rapeseed oil
☐ Lard, fatback, or bacon fat
☐ Oil spray, such as Pam or others
☐ Olive oil
☐ Other kinds of oils
☐ None of the above

92. How often did you eat tofu?

☐ NEVER (GO TO QUESTION 93)

☐ 1–6 times per year ☐ 2 times per week
☐ 7–11 times per year ☐ 3–4 times per week
☐ 1 time per month ☐ 5–6 times per week
☐ 2–3 times per month ☐ 1 time per day
☐ 1 time per week ☐ 2 or more times per day

92a. Each time you ate tofu, how much did you usually eat?

☐ Less than ¼ cup or less than 2 ounces
☐ ¼ to ½ cup or 2 to 4 ounces
☐ More than ½ cup or more than 4 ounces

93. Over the past 12 months, did you eat soups?

☐ NO (GO TO QUESTION 94)

☐ YES

93a. How often did you eat soup DURING THE WINTER?

☐ NEVER

☐ 1–6 times per winter ☐ 2 times per week
☐ 7–11 times per winter ☐ 3–4 times per week
☐ 1 time per month ☐ 5–6 times per week
☐ 2–3 times per month ☐ 1 time per day
☐ 1 time per week ☐ 2 or more times per day

93b. How often did you eat soup DURING THE REST OF THE YEAR?

☐ NEVER

☐ 1–6 times per year ☐ 2 times per week
☐ 7–11 times per year ☐ 3–4 times per week
☐ 1 time per month ☐ 5–6 times per week
☐ 2–3 times per month ☐ 1 time per day
☐ 1 time per week ☐ 2 or more times per day

93c. Each time you ate soup, how much did you usually eat?

☐ Less than 1 cup
☐ 1 to 2 cups
☐ More than 2 cups

93d. How often were the soups you ate bean soups?

☐ Almost never or never
☐ About ¼ of the time
☐ About ½ of the time
☐ About ¾ of the time
☐ Almost always or always

93e. How often were the soups you ate cream soups (including chowders)?

☐ Almost never or never
☐ About ¼ of the time
☐ About ½ of the time
☐ About ¾ of the time
☐ Almost always or always
Over the past 12 months...

93f. How often were the soups you ate tomato or vegetable soups?

☐ Almost never or never
☐ About ¼ of the time
☐ About ½ of the time
☐ About ¾ of the time
☐ Almost always or always

93g. How often were the soups you ate broth soups (including chicken) with or without noodles or rice?

☐ Almost never or never
☐ About ¼ of the time
☐ About ½ of the time
☐ About ¾ of the time
☐ Almost always or always

94. How often did you eat pizza?

☐ NEVER (GO TO QUESTION 95)

☐ 1–6 times per year
☐ 7–11 times per year
☐ 1 time per month
☐ 2–3 times per month
☐ 1 time per week
☐ 2 or more times per day

94a. Each time you ate pizza, how much did you usually eat?

☐ Less than 1 slice or less than 1 mini pizza
☐ 1 to 3 slices or 1 mini pizza
☐ More than 3 slices or more than 1 mini pizza

94b. How often did you eat pizza with pepperoni, sausage, or meat other than beef? (DO NOT include pizza made with soy or vegetable protein substitutes. We will ask about these later.)

☐ Almost never or never
☐ About ¼ of the time
☐ About ½ of the time
☐ About ¾ of the time
☐ Almost always or always

94c. How often did you eat pizza with ground beef or beef meatballs? (DO NOT include pizza made with soy or vegetable protein substitutes. We will ask about these later.)

☐ Almost never or never
☐ About ¼ of the time
☐ About ½ of the time
☐ About ¾ of the time
☐ Almost always or always

95. How often did you eat crackers?

☐ NEVER (GO TO QUESTION 96)

☐ 1–6 times per year
☐ 7–11 times per year
☐ 1 time per month
☐ 2–3 times per month
☐ 1 time per week
☐ 2 or more times per day

Please continue on next page.
95a. Each time you ate crackers, how many did you usually eat?

☐ Fewer than 4 crackers
☐ 4 to 10 crackers
☐ More than 10 crackers

96. How often did you eat corn bread or corn muffins?

☐ NEVER (GO TO QUESTION 97)
☐ 1–6 times per year
☐ 7–11 times per year
☐ 1 time per month
☐ 2–3 times per month
☐ 1 time per week

96a. Each time you ate corn bread or corn muffins, how much did you usually eat?

☐ Less than 1 piece or muffin
☐ 1 to 2 pieces or muffins
☐ More than 2 pieces or muffins

97. How often did you eat biscuits?

☐ NEVER (GO TO QUESTION 98)
☐ 1–6 times per year
☐ 7–11 times per year
☐ 1 time per month
☐ 2–3 times per month
☐ 1 time per week

97a. Each time you ate biscuits, how many did you usually eat?

☐ Fewer than 1 biscuit
☐ 1 to 2 biscuits
☐ More than 2 biscuits

98. How often did you eat potato chips, tortilla chips, or corn chips (including low-fat, fat-free, or low-salt)?

☐ NEVER (GO TO QUESTION 99)
☐ 1–6 times per year
☐ 7–11 times per year
☐ 1 time per month
☐ 2–3 times per month
☐ 1 time per week

98a. Each time you ate potato chips, tortilla chips, or corn chips, how much did you usually eat?

☐ Fewer than 10 chips or less than 1 cup
☐ 10 to 25 chips or 1 to 2 cups
☐ More than 25 chips or more than 2 cups

98b. How often were the chips you ate Wow chips or other chips made with fat substitute (Olean or Olestra)?

☐ Almost never or never
☐ About ¼ of the time
☐ About ½ of the time
☐ About ¾ of the time
☐ Almost always or always

98c. How often were the chips you ate other low-fat or fat-free chips?

☐ Almost never or never
☐ About ¼ of the time
☐ About ½ of the time
☐ About ¾ of the time
☐ Almost always or always

99. How often did you eat popcorn (including low-fat)?

☐ NEVER (GO TO QUESTION 100)
☐ 1–6 times per year
☐ 7–11 times per year
☐ 1 time per month
☐ 2–3 times per month
☐ 1 time per week

99a. Each time you ate popcorn, how much did you usually eat?

☐ Less than 2 cups, popped
☐ 2 to 5 cups, popped
☐ More than 5 cups, popped

100. How often did you eat pretzels?

☐ NEVER (GO TO QUESTION 101)
☐ 1–6 times per year
☐ 7–11 times per year
☐ 1 time per month
☐ 2–3 times per month
☐ 1 time per week
100a. Each time you ate pretzels, how many did you usually eat?

☐ Fewer than 5 average twists
☐ 5 to 20 average twists
☐ More than 20 average twists

100b. How often did you eat pretzels?

☐ NEVER (GO TO QUESTION 102)
☐ 1–6 times per year
☐ 7–11 times per year
☐ 1 time per month
☐ 2–3 times per month
☐ 1 time per week
☐ 2 or more times per day

101. How often did you eat peanuts, walnuts, seeds, or other nuts?

☐ NEVER (GO TO QUESTION 102)
☐ 1–6 times per year
☐ 7–11 times per year
☐ 1 time per month
☐ 2–3 times per month
☐ 1 time per week
☐ 2 or more times per day

101a. Each time you ate peanuts, walnuts, seeds, or other nuts, how much did you usually eat?

☐ Less than ¼ cup
☐ ¼ to ½ cup
☐ More than ½ cup

102. How often did you eat an energy, high-protein, or breakfast bar?

☐ NEVER (GO TO QUESTION 103)
☐ 1–6 times per year
☐ 7–11 times per year
☐ 1 time per month
☐ 2–3 times per month
☐ 1 time per week
☐ 2 or more times per day

102a. Which type of energy, high-protein or breakfast bar did you usually eat? (Mark as many that apply.)

☐ Nutri-Grain Bar/Kellogg’s Granola Bar
☐ Power Bar
☐ Power Bar Performance/Luna Bar
☐ Power Bar Protein Plus
☐ Power Bar Harvest
☐ Power Bar Pria
☐ Balance Bar
☐ Slimfast Bar
☐ Zone Bar
☐ Other

102b. Each time you ate an energy, high-protein, or breakfast bar, how much did you usually eat?

☐ Less than 1 bar
☐ 1 bar
☐ More than 1 bar

103. How often did you eat yogurt (NOT including frozen yogurt)?

☐ NEVER (GO TO QUESTION 104)
☐ 1–6 times per year
☐ 7–11 times per year
☐ 1 time per month
☐ 2–3 times per month
☐ 1 time per week
☐ 2 or more times per day

103a. Each time you ate yogurt, how much did you usually eat?

☐ Less than ½ cup or less than 1 container
☐ ½ to 1 cup or 1 container
☐ More than 1 cup or more than 1 container

104. How often did you eat cottage cheese (including low-fat)?

☐ NEVER (GO TO QUESTION 105)
☐ 1–6 times per year
☐ 7–11 times per year
☐ 1 time per month
☐ 2–3 times per month
☐ 1 time per week
☐ 2 or more times per day

104a. Each time you ate cottage cheese, how much did you usually eat?

☐ Less than ¼ cup
☐ ¼ to 1 cup
☐ More than 1 cup

105. How often did you eat cheese (including low-fat; including on cheeseburgers or in sandwiches, subs or wraps)? (DO NOT include cheese made from soy or vegetable protein.)

☐ NEVER (GO TO QUESTION 106)
☐ 1–6 times per year
☐ 7–11 times per year
☐ 1 time per month
☐ 2–3 times per month
☐ 1 time per week
☐ 2 or more times per day

105a. Each time you ate cheese, how much did you usually eat?

☐ Less than ½ ounce or less than 1 slice
☐ ½ to 1½ ounces or 1 slice
☐ More than 1½ ounces or more than 1 slice
105b. How often was the cheese you ate light or low-fat cheese?

☐ Almost never or never
☐ About ¼ of the time
☐ About ½ of the time
☐ About ¾ of the time
☐ Almost always or always

105c. How often was the cheese you ate fat-free cheese?

☐ Almost never or never
☐ About ¼ of the time
☐ About ½ of the time
☐ About ¾ of the time
☐ Almost always or always

106. How often did you eat frozen yogurt, or sherbet (including low-fat or fat-free)? Please do not include Tofutti.

☐ NEVER (GO TO QUESTION 107)

☐ 1–6 times per year
☐ 7–11 times per year
☐ 1 time per month
☐ 2–3 times per month
☐ 1 time per week

☐ 2 times per week
☐ 3–4 times per week
☐ 5–6 times per week
☐ 1 time per day
☐ 2 or more times per day

106a. Each time you ate frozen yogurt, or sherbet, how much did you usually eat?

☐ Less than ½ cup or less than 1 scoop
☐ ½ to 1 cup or 1 to 2 scoops
☐ More than 1 cup or more than 2 scoops

107. How often did you eat ice cream or ice cream bars, (including low-fat or fat-free)?

☐ NEVER (GO TO QUESTION 108)

☐ 1–6 times per year
☐ 7–11 times per year
☐ 1 time per month
☐ 2–3 times per month
☐ 1 time per week

☐ 2 times per week
☐ 3–4 times per week
☐ 5–6 times per week
☐ 1 time per day
☐ 2 or more times per day

107a. Each time you ate ice cream or ice cream bars, how much did you usually eat?

☐ Less than ½ cup or less than 1 scoop
☐ ½ to 1½ cups or 1 to 2 scoops
☐ More than 1½ cups or more than 2 scoops

107b. How often was the ice cream you ate light, low-fat, or fat-free ice cream?

☐ Almost never or never
☐ About ¼ of the time
☐ About ½ of the time
☐ About ¾ of the time
☐ Almost always or always

Over the past 12 months...

108. How often did you eat cake (including low-fat or fat-free)?

☐ NEVER (GO TO QUESTION 109)

☐ 1–6 times per year
☐ 7–11 times per year
☐ 1 time per month
☐ 2–3 times per month
☐ 1 time per week

☐ 2 times per week
☐ 3–4 times per week
☐ 5–6 times per week
☐ 1 time per day
☐ 2 or more times per day

108a. Each time you ate cake, how much did you usually eat?

☐ Less than 1 medium piece
☐ 1 medium piece
☐ More than 1 medium piece

108b. How often was the cake you ate light, low-fat, or fat-free cake?

☐ Almost never or never
☐ About ¼ of the time
☐ About ½ of the time
☐ About ¾ of the time
☐ Almost always or always

109. How often did you eat cookies or brownies (including low-fat or fat-free)?

☐ NEVER (GO TO QUESTION 110)

☐ 1–6 times per year
☐ 7–11 times per year
☐ 1 time per month
☐ 2–3 times per month
☐ 1 time per week

☐ 2 times per week
☐ 3–4 times per week
☐ 5–6 times per week
☐ 1 time per day
☐ 2 or more times per day

109a. Each time you ate cookies or brownies, how much did you usually eat?

☐ Less than 2 cookies or 1 small brownie
☐ 2 to 4 cookies or 1 medium brownie
☐ More than 4 cookies or 1 large brownie
109b. How often were the cookies or brownies you ate light, low-fat, or fat-free cookies or brownies?

- [ ] Almost never or never
- [ ] About ¼ of the time
- [ ] About ½ of the time
- [ ] About ¾ of the time
- [ ] Almost always or always

110. How often did you eat doughnuts, sweet rolls, Danish, or pop-tarts?

- [ ] NEVER (GO TO QUESTION 111)
- [ ] 1–6 times per year
- [ ] 7–11 times per year
- [ ] 1 time per month
- [ ] 2–3 times per month
- [ ] 1 time per week
- [ ] 2 or more times per day

110a. Each time you ate doughnuts, sweet rolls, Danish, or pop-tarts, how much did you usually eat?

- [ ] Less than 1 piece
- [ ] 1 to 2 pieces
- [ ] More than 2 pieces

111. How often did you eat sweet muffins or dessert breads (including low-fat or fat-free)?

- [ ] NEVER (GO TO QUESTION 112)
- [ ] 1–6 times per year
- [ ] 7–11 times per year
- [ ] 1 time per month
- [ ] 2–3 times per month
- [ ] 1 time per week
- [ ] 2 or more times per day

111a. Each time you ate sweet muffins or dessert breads, how much did you usually eat?

- [ ] Less than 1 medium piece
- [ ] 1 medium piece
- [ ] More than 1 medium piece

111b. How often were the sweet muffins or dessert breads you ate light, low-fat, or fat-free sweet muffins or dessert breads?

- [ ] Almost never or never
- [ ] About ¼ of the time
- [ ] About ½ of the time
- [ ] About ¾ of the time
- [ ] Almost always or always

112. How often did you eat fruit crisp, cobbler, or strudel?

- [ ] NEVER (GO TO QUESTION 113)
- [ ] 1–6 times per year
- [ ] 7–11 times per year
- [ ] 1 time per month
- [ ] 2–3 times per month
- [ ] 1 time per week
- [ ] 2 or more times per day

Over the past 12 months...

112a. Each time you ate fruit crisp, cobbler, or strudel, how much did you usually eat?

- [ ] Less than ½ cup
- [ ] ½ to 1 cup
- [ ] More than 1 cup

113. How often did you eat pie?

- [ ] NEVER (GO TO QUESTION 114)
- [ ] 1–6 times per year
- [ ] 7–11 times per year
- [ ] 1 time per month
- [ ] 2–3 times per month
- [ ] 1 time per week
- [ ] 2 or more times per day

113a. Each time you ate pie, how much did you usually eat?

- [ ] Less than ¼ of a pie
- [ ] About ¼ of a pie
- [ ] More than ¼ of a pie

The next four questions ask about the kinds of pie you ate. Please read all four questions before answering.

113b. How often were the pies you ate fruit pie (such as apple, blueberry, others)?

- [ ] Almost never or never
- [ ] About ¼ of the time
- [ ] About ½ of the time
- [ ] About ¾ of the time
- [ ] Almost always or always

113c. How often were the pies you ate cream, pudding, custard, key lime or meringue pie?

- [ ] Almost never or never
- [ ] About ¼ of the time
- [ ] About ½ of the time
- [ ] About ¾ of the time
- [ ] Almost always or always
113d. How often were the pies you ate pumpkin or sweet potato pie?
- □ Almost never or never
- □ About ¼ of the time
- □ About ½ of the time
- □ About ¾ of the time
- □ Almost always or always

113e. How often were the pies you ate pecan pie?
- □ Almost never or never
- □ About ¼ of the time
- □ About ½ of the time
- □ About ¾ of the time
- □ Almost always or always

114. How often did you eat chocolate candy?
- □ NEVER (GO TO QUESTION 115)
- □ 1–6 times per year
- □ 7–11 times per year
- □ 1 time per month
- □ 2–3 times per month
- □ 1 time per week
- □ 2 or more times per day

114a. Each time you ate chocolate candy, how much did you usually eat?
- □ Less than 1 average bar or less than 1 ounce
- □ 1 average bar or 1 to 2 ounces
- □ More than 1 average bar or more than 2 ounces

115. How often did you eat other candy?
- □ NEVER (GO TO QUESTION 116)
- □ 1–6 times per year
- □ 7–11 times per year
- □ 1 time per month
- □ 2–3 times per month
- □ 1 time per week
- □ 2 or more times per day

115a. Each time you ate other candy, how much did you usually eat?
- □ Fewer than 2 pieces
- □ 2 to 9 pieces
- □ More than 9 pieces

116. How often did you eat eggs or egg whites, (NOT counting egg substitutes or eggs in baked goods and desserts)? (Please include eggs in salads, quiche, and soufflés.)
- □ NEVER (GO TO QUESTION 116b)
- □ 1–6 times per year
- □ 7–11 times per year
- □ 1 time per month
- □ 2–3 times per month
- □ 1 time per week
- □ 2 or more times per day

Over the past 12 months...

116a. Each time you ate eggs, how many did you usually eat?
- □ 1 egg
- □ 2 eggs
- □ 3 or more eggs

116b. Over the past 12 months, how often did you eat an egg substitute product (NOT counting egg substitute product used in baked goods and desserts)? (Please include egg substitute used in quiche, soufflés, and egg dishes.)
- □ NEVER (GO TO QUESTION 116e)
- □ 1–6 times per year
- □ 7–11 times per year
- □ 1 time per month
- □ 2–3 times per month
- □ 1 time per week
- □ 2 or more times per day

116c. Which of the following brands do you eat most often (mark as many that apply)?
- Morningstar Farms Scrambles
- Morningstar Farms Better n’ Eggs
- Eggbeaters
- Second Nature
- Other

116d. Each time you ate egg substitute product, how much did you usually eat?
- □ Less than ½ cup
- □ ½ to 1 cup
- □ More than 1 cup
116e. How often were the eggs or egg substitute product you ate cooked in oil, butter, or margarine?

☐ Almost never or never
☐ About ¼ of the time
☐ About ½ of the time
☐ About ¾ of the time
☐ Almost always or always

116f. How often were the eggs you ate part of egg salad?

☐ Almost never or never
☐ About ¼ of the time
☐ About ½ of the time
☐ About ¾ of the time
☐ Almost always or always

117. How many cups of coffee, caffeinated or decaffeinated, did you drink?

☐ NEVER (GO TO QUESTION 118)

☐ Less than 1 cup per month
☐ 1–3 cups per month
☐ 1 cup per week
☐ 2–4 cups per week
☐ 5–6 cups per week
☐ 1 cup per day
☐ 2–3 cups per day
☐ 4–5 cups per day
☐ 6 or more cups per day

117a. How often was the coffee you drank decaffeinated?

☐ Almost never or never
☐ About ¼ of the time
☐ About ½ of the time
☐ About ¾ of the time
☐ Almost always or always

118. How many glasses of ICED tea, caffeinated or decaffeinated, did you drink?

☐ NEVER (GO TO QUESTION 119)

☐ Less than 1 cup per month
☐ 1–3 cups per month
☐ 1 cup per week
☐ 2–4 cups per week
☐ 5–6 cups per week
☐ 1 cup per day
☐ 2–3 cups per day
☐ 4–5 cups per day
☐ 6 or more cups per day

119a. How often was the iced tea you drank decaffeinated or herbal tea?

☐ Almost never or never
☐ About ¼ of the time
☐ About ½ of the time
☐ About ¾ of the time
☐ Almost always or always

119. How many cups of HOT tea, caffeinated or decaffeinated, did you drink?

☐ NEVER (GO TO QUESTION 120)

☐ Less than 1 cup per month
☐ 1–3 cups per month
☐ 1 cup per week
☐ 2–4 cups per week
☐ 5–6 cups per week
☐ 1 cup per day
☐ 2–3 cups per day
☐ 4–5 cups per day
☐ 6 or more cups per day

119a. How often was the hot tea you drank decaffeinated or herbal tea?

☐ Almost never or never
☐ About ¼ of the time
☐ About ½ of the time
☐ About ¾ of the time
☐ Almost always or always

Over the past 12 months...

120. How often did you add sugar or honey to your coffee or tea?

☐ NEVER (GO TO QUESTION 121)

☐ Less than 1 time per month
☐ 1–3 times per month
☐ 1 cup per week
☐ 2–4 times per week
☐ 5–6 times per week
☐ 1 time per day
☐ 2–3 times per day
☐ 4–5 times per day
☐ 6 or more times per day

120a. Each time sugar or honey was added to your coffee or tea, how much was usually added?

☐ Less than 1 teaspoon
☐ 1 to 3 teaspoons
☐ More than 3 teaspoons

121. How often did you add artificial sweetener to your coffee or tea?

☐ NEVER (GO TO QUESTION 122)

☐ Less than 1 time per month
☐ 1–3 times per month
☐ 1 time per week
☐ 2–4 times per week
☐ 5–6 times per week
☐ 1 time per day
☐ 2–3 times per day
☐ 4–5 times per day
☐ 6 or more times per day
121a. What kind of **artificial sweetener** did you usually use?

- [ ] Equal or aspartame
- [ ] Sweet N Low or saccharin

122. How often was **non-dairy creamer** added to your coffee or tea?

- [ ] NEVER (GO TO QUESTION 123)
- [ ] Less than 1 time per month
- [ ] 1–3 times per month
- [ ] 1 time per week
- [ ] 2–4 times per week

122a. Each time **non-dairy creamer** was added to your coffee or tea, how much was usually used?

- [ ] Less than 1 teaspoon
- [ ] 1 to 3 teaspoons
- [ ] More than 3 teaspoons

122b. What kind of **non-dairy creamer** did you usually use?

- [ ] Regular powdered
- [ ] Low-fat or fat-free powdered
- [ ] Regular liquid
- [ ] Low-fat or fat-free liquid

123. How often was **cream** or **half and half** added to your coffee or tea?

- [ ] NEVER (GO TO QUESTION 124)
- [ ] Less than 1 time per month
- [ ] 1–3 times per month
- [ ] 1 time per week
- [ ] 2–4 times per week

123a. Each time **cream** or **half and half** was added to your coffee or tea, how much was usually added?

- [ ] Less than 1 tablespoon
- [ ] 1 to 2 tablespoons
- [ ] More than 2 tablespoons

124. How often was **milk** added to your coffee or tea?

- [ ] NEVER (GO TO QUESTION 125)
- [ ] Less than 1 time per month
- [ ] 1–3 times per month
- [ ] 1 time per week
- [ ] 2–4 times per week
- [ ] 6 or more times per day

124a. Each time **milk** was added to your coffee or tea, how much was usually added?

- [ ] Less than 1 tablespoon
- [ ] 1 to 3 tablespoons
- [ ] More than 3 tablespoons

124b. What kind of **milk** was usually added to your coffee or tea?

- [ ] Whole milk (including lactose-free variety)
- [ ] 2% milk (including lactose-free variety)
- [ ] 1% milk (including lactose-free variety)
- [ ] Skim, nonfat, or ½% milk (including lactose-free variety)
- [ ] Evaporated or condensed (canned) milk
- [ ] Soy milk
- [ ] Rice milk
- [ ] Other

**Over the past 12 months…**

125. How often was **sugar** or **honey** added to foods you ate? (Please do not include sugar in coffee, tea, other beverages, or baked goods.)

- [ ] NEVER (GO TO INTRODUCTION TO QUESTION 126)
- [ ] 1–6 times per year
- [ ] 7–11 times per year
- [ ] 1 time per month
- [ ] 2–3 times per month
- [ ] 1 time per week
- [ ] 2 or more times per day

125a. Each time **sugar** or **honey** was added to foods you ate, how much was usually added?

- [ ] Less than 1 teaspoon
- [ ] 1 to 3 teaspoons
- [ ] More than 3 teaspoons
The following questions are about the kinds of margarine, mayonnaise, sour cream, cream cheese, and salad dressing that you eat. If possible, please check the labels of these foods to help you answer.

126. Over the past 12 months, did you eat margarine?

☐ NO (GO TO QUESTION 127)
☐ YES

126a. How often was the margarine you ate regular-fat margarine (stick or tub)?

☐ Almost never or never
☐ About ¼ of the time
☐ About ½ of the time
☐ About ¾ of the time
☐ Almost always or always

126b. How often was the margarine you ate light or low-fat margarine (stick or tub)?

☐ Almost never or never
☐ About ¼ of the time
☐ About ½ of the time
☐ About ¾ of the time
☐ Almost always or always

126c. How often was the margarine you ate fat-free margarine?

☐ Almost never or never
☐ About ¼ of the time
☐ About ½ of the time
☐ About ¾ of the time
☐ Almost always or always

127. Over the past 12 months, did you eat butter?

☐ NO (GO TO QUESTION 128)
☐ YES

127a. How often was the butter you ate light or low-fat butter?

☐ Almost never or never
☐ About ¼ of the time
☐ About ½ of the time
☐ About ¾ of the time
☐ Almost always or always

128. Over the past 12 months, did you eat mayonnaise or mayonnaise-type dressing?

☐ NO (GO TO QUESTION 129)
☐ YES

128a. How often was the mayonnaise you ate regular-fat mayonnaise?

☐ Almost never or never
☐ About ¼ of the time
☐ About ½ of the time
☐ About ¾ of the time
☐ Almost always or always

128b. How often was the mayonnaise you ate light or low-fat mayonnaise?

☐ Almost never or never
☐ About ¼ of the time
☐ About ½ of the time
☐ About ¾ of the time
☐ Almost always or always

128c. How often was the mayonnaise you ate fat-free mayonnaise?

☐ Almost never or never
☐ About ¼ of the time
☐ About ½ of the time
☐ About ¾ of the time
☐ Almost always or always

129. Over the past 12 months, did you eat sour cream?

☐ NO (GO TO QUESTION 130)
☐ YES

129a. How often was the sour cream you ate regular-fat sour cream?

☐ Almost never or never
☐ About ¼ of the time
☐ About ½ of the time
☐ About ¾ of the time
☐ Almost always or always

129b. How often was the sour cream you ate light, low-fat, or fat-free sour cream?

☐ Almost never or never
☐ About ¼ of the time
☐ About ½ of the time
☐ About ¾ of the time
☐ Almost always or always
130. Over the past 12 months, did you eat cream cheese?

☐ NO (GO TO QUESTION 131)

☐ YES

130a. How often was the cream cheese you ate regular-fat cream cheese?

☐ Almost never or never
☐ About ¼ of the time
☐ About ½ of the time
☐ About ¾ of the time
☐ Almost always or always

130b. How often was the cream cheese you ate light, low-fat, or fat-free cream cheese?

☐ Almost never or never
☐ About ¼ of the time
☐ About ½ of the time
☐ About ¾ of the time
☐ Almost always or always

131. Over the past 12 months, did you eat salad dressing?

☐ NO (GO TO INTRODUCTION TO QUESTION 132)

☐ YES

131a. How often was the salad dressing you ate regular-fat salad dressing (including oil and vinegar dressing)?

☐ Almost never or never
☐ About ¼ of the time
☐ About ½ of the time
☐ About ¾ of the time
☐ Almost always or always

131b. How often was the salad dressing you ate light or low-fat salad dressing?

☐ Almost never or never
☐ About ¼ of the time
☐ About ½ of the time
☐ About ¾ of the time
☐ Almost always or always

131c. How often was the salad dressing you ate fat-free salad dressing?

☐ Almost never or never
☐ About ¼ of the time
☐ About ½ of the time
☐ About ¾ of the time
☐ Almost always or always

The following two questions ask you to summarize your usual intake of vegetables and fruits. Please do not include salads, potatoes, or juices.

132. Over the past 12 months, how many servings of vegetables (not including salad or potatoes) did you eat per week or per day?

☐ Less than 1 per week  2 per day
☐ 1–2 per week  3 per day
☐ 3–4 per week  4 per day
☐ 5–6 per week  5 or more per day
☐ 1 per day

Over the past 12 months...

133. Over the past 12 months, how many servings of fruit (not including juices) did you eat per week or per day?

☐ Less than 1 per week  2 per day
☐ 1–2 per week  3 per day
☐ 3–4 per week  4 per day
☐ 5–6 per week  5 or more per day
☐ 1 per day

134. Over the past month, which of the following foods did you eat AT LEAST THREE TIMES? (Mark all that apply.)

☐ Avocado, guacamole
☐ Cheesecake
☐ Chocolate, fudge, or butterscotch fudge, or syrups
☐ Chow mein toppings
☐ Croissants
☐ Dried apricots
☐ Egg rolls
☐ Granola bars
☐ Hot peppers
☐ Jello, gelatin
☐ Milkshakes or ice-cream sodas
☐ Olives
☐ Oysters
☐ Pickles or pickled vegetables or fruit
☐ Plantains
☐ Pork neckbones, hock, head, feet
☐ Pudding or custard
☐ Veal, venison, lamb
☐ Whipped cream, regular
☐ Whipped cream, substitute
☐ NONE

Please continue on next page.
135. For **ALL** of the past 12 months, have you followed any type of **vegetarian diet**?

- [ ] NO (GO TO INTRODUCTION TO QUESTION 136)
- [ ] YES

135a. Which of the following foods did you **TOTALLY EXCLUDE** from your diet? *(Mark all that apply.)*

- [ ] Beef, veal
- [ ] Pork, lamb
- [ ] Poultry (chicken, turkey, duck)
- [ ] Fish and seafood
- [ ] Shellfish
- [ ] Eggs (please do not include egg substitutes)
- [ ] Dairy products (milk, cheese, etc.) (please do not include milk/dairy substitutes)

136. Over the past 12 months, did you eat any **meat substitute** products **made with soy or vegetable protein**?

- [ ] NO Thank you **very much** for completing this questionnaire! Because we want to be able to use all the information you have provided, we would greatly appreciate it if you would please take a moment to review each page making sure that you:
  - Did not skip any pages
  - Crosssed out the incorrect answer and circled the correct answer if you made any changes

- [ ] YES (GO TO QUESTION 137)

137. How often did you eat **breakfast patties or breakfast links** made with soy or vegetable protein?

- [ ] NEVER (GO TO QUESTION 138)

```
- [ ] 1–6 times per year
- [ ] 7–11 times per year
- [ ] 1 time per month
- [ ] 2–3 times per month
- [ ] 1 time per week
- [ ] 2 or more times per day
```

137a. Which of the following brands do you eat most often (mark as many that apply)?

- [ ] Morningstar Farms
- [ ] Worthington
- [ ] Loma Linda
- [ ] Other (such as Boca, Quorn, etc.)

137b. Each time you ate a **breakfast patty** or **breakfast link made with soy or vegetable protein**, how much did you usually eat?

```
- [ ] Less than 1 patty or 2 links
- [ ] 1 to 3 patties or 2 to 5 links
- [ ] More than 3 patties or 5 links
```

138. How often did you eat **breakfast strips** *(imitation bacon)* made with soy or vegetable protein?

- [ ] NEVER (GO TO QUESTION 139)

```
- [ ] 1–6 times per year
- [ ] 7–11 times per year
- [ ] 1 time per month
- [ ] 2–3 times per month
- [ ] 1 time per week
- [ ] 2 or more times per day
```

138a. Which of the following brands do you eat most often? (Mark as many that apply.)

- [ ] Morningstar Farms
- [ ] Worthington
- [ ] Loma Linda
- [ ] Other (such as Boca, Quorn, etc.)

138b. Each time you ate **breakfast strips** *(imitation bacon)* made with soy or vegetable protein, how much did you usually eat?

```
- [ ] Fewer than 2 slices
- [ ] 2 to 3 slices
- [ ] More than 3 slices
```

139. How often did you eat **burgers made with soy or vegetable protein**?

- [ ] NEVER (GO TO QUESTION 140)

```
- [ ] 1–6 times per year
- [ ] 7–11 times per year
- [ ] 1 time per month
- [ ] 2–3 times per month
- [ ] 1 time per week
- [ ] 2 or more times per day
```

138c. Each time you ate **breakfast strips** *(imitation bacon)* made with soy or vegetable protein, how much did you usually eat?

```
- [ ] Fewer than 2 slices
- [ ] 2 to 3 slices
- [ ] More than 3 slices
```

140. How often did you eat **burgers made with soy or vegetable protein**?
139a. Which of the following brands do you eat most often? (Mark as many that apply.)

☐ Morningstar Farms
☐ Worthington
☐ Loma Linda
☐ Other (such as Boca, Quorn, etc.)

139b. Each time you ate a burger made with soy or vegetable protein, how much did you usually eat?

☐ Less than 1 patty or less than 2 ounces
☐ 1 patty or 2 to 4 ounces
☐ More than 1 patty or more than 4 ounces

140. How often did you eat imitation meat dinner entrees (not including imitation chicken or fish) made with soy or vegetable protein?

☐ NEVER (GO TO QUESTION 141)

1–6 times per year
7–11 times per year
1 time per month
2–3 times per month
1 time per week
2 or more times per day

140a. Which of the following brands do you eat most often (mark as many that apply)?

☐ Morningstar Farms
☐ Worthington
☐ Loma Linda
☐ Other (such as Boca, Quorn, etc.)

140b. Each time you ate an imitation meat dinner entree (not including imitation chicken or fish) made with soy or vegetable protein, how much did you usually eat?

☐ Less than 1 portion or ½ cup
☐ One portion or 1 cup
☐ More than one portion or more than 1 cup

141. How often did you eat imitation chicken/turkey patties, nuggets, wings or other imitation chicken/turkey product made with soy or vegetable protein?

☐ NEVER (GO TO QUESTION 142)

1–6 times per year
7–11 times per year
1 time per month
2–3 times per month
1 time per week
2 or more times per day

141a. Which of the following brands do you eat most often? (Mark as many that apply.)

☐ Morningstar Farms
☐ Worthington
☐ Loma Linda
☐ Other (such as Boca, Quorn, etc.)

141b. Each time you ate an imitation chicken/turkey patties, nuggets, wings or other imitation chicken/turkey product made with soy or vegetable protein, how much did you usually eat?

☐ Less than 1 patty or less than 2 ounces
☐ 1 patty or 2 to 4 ounces
☐ More than 1 patty or more than 4 ounces

142. How often did you eat entrees like chili, Mexican foods (tacos, burritos, tostados, enchiladas, etc) lasagna, manicotti, ravioli, stuffed shells, tortellini spaghetti with meat sauce, meatballs or casserole made with soy or vegetable protein products like Burger Crumbles?

☐ NEVER (GO TO QUESTION 143)

1–6 times per year
7–11 times per year
1 time per month
2–3 times per month
1 time per week
2 or more times per day

142a. Which of the following brands do you eat most often? (Mark as many that apply.)

☐ Morningstar Farms
☐ Worthington
☐ Loma Linda
☐ Other (such as Boca, Quorn, etc.)

142b. Each time you ate an entreé like chili, Mexican foods (tacos, burritos, tostados, enchiladas, etc) lasagna, manicotti, ravioli, stuffed shells, tortellini spaghetti with meat sauce, meatballs or casserole made with soy or vegetable protein products like Burger Crumbles, how much did you usually eat?

☐ Less than ½ cup
☐ ½ to 1 cup
☐ More than 1 cup
143. How often did you eat **cold cut substitutes** (such as meatless salami, meatless bologna, meatless chicken roll, meatless smoked turkey, meatless corned beef) made with soy or vegetable protein?

☐ NEVER (GO TO END)

☐ 1–6 times per year  ☐ 2 times per week
☐ 7–11 times per year  ☐ 3–4 times per week
☐ 1 time per month  ☐ 5–6 times per week
☐ 2–3 times per month  ☐ 1 time per day
☐ 1 time per week  ☐ 2 or more times per day

143a. Which of the following brands do you eat most often? (Mark as many that apply.)

☐ Morningstar Farms
☐ Worthington
☐ Loma Linda
☐ Other (such as Boca, Quorn, etc.)

143b. Each time you ate **cold cut substitutes** (such as meatless salami, meatless bologna, meatless chicken roll, meatless smoked turkey, meatless corned beef) made with soy or vegetable protein, how much did you usually eat?

☐ Less than 3 slices or less than 2 ounces
☐ 3 to 6 slices or 2 to 4 ounces
☐ More than 6 slices or more than 4 ounces

Thank you very much for completing this questionnaire! Because we want to be able to use all the information you have provided, we would greatly appreciate it if you would please take a moment to review each page making sure that you:

- Did not skip any pages
- Crossed out the incorrect answer and circled the correct answer if you made any changes
APPENDIX C
DIETARY HISTORY QUESTIONNAIRE INSTRUCTIONS MATERIALS

Please read these instructions prior to beginning the Diet History Questionnaire. These additional instructions combined with the “General Instructions” (found on the first page of the Diet History Questionnaire) will guide you while completing the questionnaire.

ADDITIONAL INSTRUCTIONS

1. When answering each question, think about your diet over the past year and NOT the past few weeks.

2. Several questions refer you to additional handouts that have been provided in your packet. Please be sure to use these handouts when you get to these questions.

<table>
<thead>
<tr>
<th>Question Number</th>
<th>Handout</th>
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<tbody>
<tr>
<td>8e</td>
<td>Caffeinated versus Non-caffeinated Beverages</td>
</tr>
<tr>
<td>13b</td>
<td>Fortified Cereal</td>
</tr>
<tr>
<td>19a, 21a, 22a, 23a, 24a, 25a, 34a, 42a</td>
<td>Seasonal Fruits and Vegetables</td>
</tr>
</tbody>
</table>

If you have any questions while completing the questionnaire, please contact Amanda Brown at 352-392-1991 ext 246. Please leave a voice message with your name, contact number, and best time or way to reach you.
Remember when answering these questions, we want you to think about your diet over the past year and NOT the past few weeks. Here are a few examples, that you may find helpful when completing the questionnaire.

1. Please look at question #57 on page 16. I go to IHOP for breakfast on the first Sunday of every month of the year. I always order one medium size Belgian waffle. I never make waffles, pancakes, or French toast at home. I only eat waffles at IHOP. Since there are 12 months in a year, I eat 12 waffles a year or 1 waffle a month. If you look at question #57, I would mark an "X" for 1 time per month. For question #57a, I would answer 1 to 3 medium pieces.

2. Please look at question #107 on page 31. I only eat ice cream during the summer months of June, July, and August. During that time, I eat 1 cup of ice cream twice a week. That means I eat ice cream 8 times during each of those months. So that adds up to 24 times. Since I do not eat ice cream during the other months, I can say that I only have ice cream 24 times a year. If I remember that there are 12 months in a year that means, on average, I only have ice cream twice a month. If you look at question #107 on page 31, I would mark an “X” for 2-3 times a month. For question #107a, I would mark ½ to 1 ½ cups or 1 to 2 scoops.
**Directions on How to Change Your Answer Choice**

Over the past 12 months, how often did you drink *tomato juice* or *vegetable juice*?

- a. [X] 1 time per month or less
- b. [ ] 2-3 times per month
- c. [ ] 1-2 times per week
- d. [ ] 3-4 times per week
- e. [ ] 5-6 times per week

You select “a” as your first answer, but then you change your mind.

**Step #1**
Cross out answer “a”

**Step #2**
Mark an “X” in the box of your new choice and circle that answer too.
Please use this list for Question 8e

Caffeinated versus non-caffeinated beverages

**Caffeinated**
- A & W Creme Soda
- Barq’s Root Beer
- Canada Dry Cola
- Coffee
- Diet A & W Creme Soda
- Diet Coke with Lemon
- Diet Cherry Coke
- Cherry Coke
- Cherry Pepsi
- Coke
- Diet Coke
- Diet Barq’s Root Beer
- Diet Dr. Pepper
- Diet Mountain Dew
- Diet Pepsi
- Diet RC Cola
- Diet Shasta Cola
- Diet Snapple Flavored teas
- Diet Sunkist Orange
- Dr. Pepper
- Hot Chocolate Mix
- Jolt
- Lipton Brisk
- Mellow Yellow
- Mountain Dew
- Mountain Dew Code Red
- Nestea Sweetened & Unsweetened Iced Teas
- Pepsi
- Pepsi One
- Pepsi Twist
- RC Cola
- Shasta Cola
- Snapple Flavored teas
- Sunkist Orange
- Surge
- Tea: Iced, Brewed, Instant, Green

**Non-caffeinated**
- 7-Up
- A & W Root Beer
- Diet 7-Up
- Diet A & W Root Beer
- Diet Sprite
- Minute Maid Orange
- Sprite
Please use this list for Question 13b

Fortified Cereals

All-Bran – Buds
All-Bran – Extra Fiber
All-Bran – Original
Cheerios – Multigrain
Complete Oat Bran Flakes
Complete Wheat Bran Flakes
Just Right Fruit and Nut
KASHI Heart to Heart
Kellogg’s Low Fat Granola no raisins
Kellogg’s Low Fat Granola with raisins
Mueslix
Product 19
Smart Start
Smart Start Soy Protein
Special K
Total - Original
Total - Whole Grain
Total Brown Sugar and Oat
Total Corn Flakes
Total Raisin Bran
Please use this list for Questions
19a, 21a, 22a, 23a, 24a, 25a, 34a, 42a

# Seasonal Fruits and Vegetables

<table>
<thead>
<tr>
<th>Fruit or Vegetable</th>
<th>Months When In Season</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cantaloupe</td>
<td>March to July</td>
</tr>
<tr>
<td><strong>Corn</strong></td>
<td>August to June</td>
</tr>
<tr>
<td>Grapefruit</td>
<td>September to June</td>
</tr>
<tr>
<td>Honeydew Melon</td>
<td>June to October</td>
</tr>
<tr>
<td>Nectarines</td>
<td>July to September</td>
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<tr>
<td>Oranges</td>
<td>October to June</td>
</tr>
<tr>
<td>Peaches</td>
<td>June to September</td>
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<td>Plums</td>
<td>June to October</td>
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<td>Strawberries</td>
<td>October to June</td>
</tr>
<tr>
<td>Tangelos</td>
<td>November to February</td>
</tr>
<tr>
<td>Tomatoes</td>
<td>September to June</td>
</tr>
</tbody>
</table>
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Melanie was born in Escondido, California. She received her Bachelor of Science in nutrition applied sciences from the Pennsylvania State University in 2005. After graduation, Melanie was accepted into the University of Florida’s combined Master of Science – Dietetic Internship Program. During graduate school, she served as the Gainesville District Dietetic Association Newsletter Co-Editor for 1½ years, and as the team leader for a school-based nutrition intervention project that she and her classmates developed and implemented at Talbot Elementary School during the 2005 to 2006 school year. She and several classmates also had the opportunity to present their work as part of the 2006 Florida Dietetic Association Annual Symposium Poster Session. In recognition of her academic accomplishments, Melanie recently was awarded a scholarship from the Agricultural Women’s Club. Melanie plans to move to Philadelphia after she graduates from the University of Florida. Her immediate career goal is to pass the Registration Examination for Dietitians and work as a registered dietitian specializing in pediatrics.