

EFFECT OF FOLATE AND VITAMIN B12 STATUS AND RELATED GENETIC
POLYMORPHISMS ON CONGENITAL HEART DEFECT RISK: A PILOT STUDY

By

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To my wife and parents

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LIST OF ABBREVIATIONS

1-C	one-carbon
5, 10-MTHF	5, 10-methylenetetrahydrofolate
5-MTHF	5-methyltetrahydrofolate
10-CHO-THF	10-formyltetrahydrofolate
Adenosyl-Cbl	adenosylcobalamin
AHA	American Heart Association
AI	Adequate Intake
ANOVA	Analysis of variance
B6	pyridoxal phosphate
B12	vitamin B12
BMI	body mass index
BWIS	Baltimore Washington Infant Study
Cbl	cobalamin
CBS	cystathionine B-synthase
CH ₃ -Cbl	methylcobalamin
CH ₃ -THF	methyltetrahydrofolate
CHD	congenital heart defects
CLP	cleft palate
CI	confidence interval
CN-Cbl	cyanocobalamin
Cyanobacteria	blue-green algae
d	day
DHF	dihydrofolate
DFEs	Dietary Folate Equivalents

DNA	Deoxyribonucleic acid
DRIs	Dietary Reference Intakes
dTMP	thymidylate
dUMP	deoxyuridylate
EAR	Estimated Average Requirements
FDA	Food and Drug Administration
g	gram
HCL	hydrochloric acid
Hct	hematocrit
Hcy	homocysteine
Hgb	hemoglobin
IF	intrinsic factor
IM	intramuscular injection
IOM	Institute of Medicine
MCH	mean corpuscular hemoglobin
MCHC	mean cell hemoglobin concentration
MCV	mean corpuscular volume
min	minutes
μ M	micromole
MMA	methylmalonic acid
MTHFR	methyltetrahydrofolate reductase
MTR	methionine synthase
MTRR	methionine synthase reductase
NCCs	neural crest cells
nM	nanomole

nmol/L	nanomoles per liter
NO	nitric oxide
NTD	neural tube defect
Oh-Cbl	hydroxocobalamin
PCC	population-based case-control studies
pmol/L	pica mol per litter
RBC	red blood cell
RCT	randomized control intervention trial
RDA	Recommended Dietary Allowance
ROS	reactive oxygen species
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SHMT	serine hydroxymethyltransferase
TC	transcobalamin
TDT	transmission disequilibrium test
THF	tetrahydrofolate
UL	Tolerable upper level

Abstract of Thesis Presented to the Graduate School
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One-carbon (1-C) metabolism reactions require folate as the central substrate that provides essential one carbon moieties. One carbon groups provided by folate are vital for many reactions including those required for amino acid metabolism, purine and pyrimidine synthesis, DNA methylation, and the formation of S-adenosylmethionine (SAM). Low folate status can alter this central role in maintaining 1-C metabolism which can lead to birth defects. Birth defects are the leading cause of death in newborn infants, with congenital heart defects (CHDs) accounting for one death in every three defect-related infant deaths. Congenital heart defect risk may possibly be related to folate and vitamin B12 status and this risk may be exacerbated in the presence of polymorphisms (MTHFR 677 C→T and 1298 A→C, MTRR 66 A→G and TC 776 C→G) related to folate and vitamin B12 metabolism. To assess the roles of folate and vitamin B12 status and associated genetic polymorphisms on CHD risk, a case-control pilot study was conducted including 390 non-pregnant mothers. Blood samples were obtained from subjects and were analyzed to identify biochemical variables and genotypes. Serum folate and vitamin B12 concentrations were inversely associated ($p < 0.05$) with CHD risk in the case group relative to the control group in subjects who had a delivery within 3 years from the time the blood was drawn. The MTHFR 677 CT and TT genotype groups were combined and compared between

cases and controls resulting in a significant ($P= 0.0356$) increase in risk for CHD in the case group. Future studies need to be conducted to provide a careful assessment of status at the appropriate time during gestation or within a very short time following the index pregnancy to allow a more accurate measure of status indicators that are likely to interact with polymorphisms affecting folate and vitamin B12.

CHAPTER 1 INTRODUCTION

Folate and vitamin B12 play critical roles in one-carbon (1-C) metabolism including deoxyribonucleic acid (DNA) synthesis and methylation, and homocysteine (Hcy) remethylation. Folate is required for the formation of 5-methyltetrahydrofolate (5-MTHF), a substrate critical for donating methyl groups to Hcy in a co-dependent manner with vitamin B12 serving as a cofactor. Folate is the 1-C donor required for the formation of S-adenosylmethionine (SAM) from methionine. While folate is involved in the formation of the methyl donor substrate, vitamin B12 is required to facilitate the transfer of the methyl group from the 5-MTHF to Hcy.

Impaired folate or vitamin B12 status can lead to impaired generation of substrates needed for the de novo synthesis of DNA and cell division. It will also affect formation and maturation of red and white blood cells in the bone marrow. Abnormalities in 1-C metabolism may result in elevated concentrations of Hcy and reduced DNA methylation, both of which possibly can affect development of embryos. Both nutrients alone and together have been linked to increased Hcy concentration, which is a risk factor for pregnancy complications with evidence for an association with congenital malformations.

Folate and vitamin B12 metabolism can be adversely affected by the presence of genetic polymorphisms, which are common mutations that may affect the structure and function of folate/vitamin B12-dependent enzymes and transport proteins. Certain polymorphisms (677 C→T plus 1298 A→C) are responsible for the reduced activity of the methyltetrahydrofolate reductase (MTHFR) enzyme which may lead to impaired Hcy remethylation and DNA methylation. The MTHFR 677C→T polymorphism has been associated with an increased risk of neural tube defects (NTDs) especially when folate status is low.

Congenital heart defect (CHD) risk may possibly be related to folate and vitamin B12 status and this risk may be exacerbated in the presence of MTHFR polymorphisms. Methionine synthase reductase (MTRR) is an enzyme required for the activation of methionine synthase (MTR) for the remethylation of Hcy to methionine which may be negatively impacted by a genetic polymorphism (MTRR 66 A→G). Investigation of the association of the MTRR 66 A→G polymorphism with CHD risk is warranted since impaired activity of MTRR may negatively affect 1-C metabolism. The MTRR 66 A→G was previously found to be positively associated with NTDs when vitamin B12 status was low, which provides some basis for a possible link to CHD since the embryonic origins of NTDs and CHDs are similar.

Transcobalamin (TC) 776 C→G is a polymorphism affecting the protein responsible for the cellular uptake of vitamin B12. It is proposed that individuals with TC 776 GG genotype may have an impaired ability to transport vitamin B12, which may reduce cellular uptake and lead to abnormal function including embryonic development. The critical role of TC in vitamin B12 function in 1-C metabolism necessitates the assessment of its effect on risk of CHD, however, only a limited number of studies have investigated the role of TC 776 C→G and CHD risk and the conclusions from these studies are not definitive due to limitations of sample size and other factors. A comprehensive investigation of the association of folate and vitamin B12 status and related genetic polymorphisms with CHD risk has not been conducted and is the long term goal of future studies to follow this initial pilot study.

Hypotheses

- low folate and/or vitamin B12 status are associated with increased risk for CHD;
- genotype status for the MTHFR 677C → T and 1298 A → C polymorphisms and the MTRR gene 66 A → G and the TC 776 C → G polymorphisms will exacerbate the effect of low folate and/or vitamin B12 status on CHD risk;
- congenital heart defect risk associated with either folate and/or vitamin B12 related polymorphisms will be exacerbated by low folate and/or vitamin B12 status.

Overall Goal

The primary goal of this study was to assess the roles of folate and vitamin B12 status and associated genetic polymorphisms on CHD risk in women with affected pregnancies compared to women who delivered infants with no birth defects.

Specific Objectives

- assess the association between maternal folate and vitamin B12 status biomarkers (serum and red blood cell (RBC) folate, vitamin B12, methylmalonic acid (MMA) and Hcy) and infant CHD risk;
- assess the association between maternal MTHFR 677 C→T and 1298 A→C and MTRR 66 A→G and TC 776 C→G genotypes on infant CHD risk;
- determine the interaction between maternal folate and vitamin B12 related polymorphisms and folate and vitamin B12 status indicators on infant CHD risk.

CHAPTER 2 LITERATURE REVIEW

Folate and Vitamin B12

Folate

Chemistry

Folate is a water-soluble vitamin. The generic name for the vitamin is folate and it is related to a family of substances containing pteridine rings joined with p-aminobenzoic acid and one or more glutamic acid molecules (1) (Figure 2-1). The form of naturally occurring folate depends on the side chain composition in terms of the number of glutamic acid molecules as well as the specific one-carbon moiety attached to the vitamin. The folate molecule can vary in structure through reduction of the pteridine moiety to dihydrofolate (DHF) and tetrahydrofolate (THF), elongation of the glutamate chain, and substitution of the 1-C unit at the N-5, N-10, or both positions (2,3). Folic acid, which rarely occurs in nature, is the fully oxidized monoglutamate form of the vitamin folate, and it is used extensively in food fortification and supplementation due to its chemical stability (1).

Bioavailability

Bioavailability may be defined as the proportion of an ingested nutrient that is used and stored in the body. Bioavailability of food folate is considerably dependent on the individual's ability to digest, absorb and metabolize the vitamin. The bioavailability of folate depends on the food source and often it is incomplete (1). The bioavailability of food folate is estimated using folic acid as the standard since folic acid exhibits nearly complete absorption (4), and is considered to be 100% bioavailable. The bioavailability of folate from a mixed diet is approximately 50% or less of the bioavailability of folic acid (5). When folic acid is consumed

with food, the bioavailability is reduced by ~ 15% compared to when it is consumed alone as a supplement, therefore folic acid in fortified foods is estimated to be ~85% bioavailable (6).

Metabolism (Absorption, transport, storage and excretion)

Folic acid is a fully oxidized monoglutamate form of folate and can be absorbed readily at the brush border membrane of the jejunum. However, food folate needs to be converted into the monoglutamate form by the folylpoly- γ -glutamate carboxypeptidase enzyme also known as folate conjugase (7,8). The monoglutamate form of folate is transported into the enterocyte via a pH-dependent carrier-mediated mechanism (9). At high concentrations of folate ($>10 \mu\text{mol/L}$), ion-mediated transport becomes the means of transport into the cell (10). After the entry into the mucosal cell, the monoglutamate form of folate is reduced and a portion is converted to methyltetrahydrofolate (CH₃-THF) before it's released into circulation (11).

Most folate in the plasma is bound to albumin in the form of 5-CH₃-THF, and a smaller amount is bound to a high affinity folate-binding protein (2). Cellular uptake of folate by cells is mediated by membrane-associated folate-binding proteins (12). After its uptake, 5-CH₃-THF is demethylated by MTR and then is converted into a polyglutamyl form by folypolyglutamate synthase (13). The metabolism of folate is divided into pathways occurring in either the cytoplasm or mitochondria. Both pathways facilitate the regeneration of THF through the reduction of various folate coenzymes. This allows THF to accept a 1-C unit from the folate pool, which is donated by both the cytosolic and mitochondrial pathways, leading to purine synthesis, DNA methylation, DNA synthesis, and Hcy remethylation to methionine (Figure 2-2)

While folate in the monoglutamate form is subsequently taken up by the cells, it is the polyglutamate form that helps sequester folate inside the cell due to the polarity of the side chains (13). Tissues do not store large amounts of folate beyond their metabolic needs. The total body content of folate is estimated to be between 15 to 30 mg (14). A small amount of the

dietary folate is excreted in the urine (15). The small quantity of free folate in the plasma is filtered through the glomerulus but mostly reabsorbed in the proximal renal tubules (16). The amount of folate excreted in the urine is estimated to be similar to the amount excreted in the feces (1).

Vitamin B12

Chemistry

Vitamin B12, also known as cobalamin (Cbl), refers to a family of substances composed of a central cobalt atom surrounded by macrocyclic corrin tetrapyrrole which are four reduced pyrrole rings connected by nucleotide side chains attached to the central atom (Figure 2-3). There are various forms of the vitamin B12 molecule, including methylcobalamin (CH₃-Cbl), cyanocobalamin (CN-Cbl), adenosylcobalamin (adenosyl-Cbl) and hydroxocobalamin (OH-Cbl).

The commercially produced form of vitamin B12 is known as CN-Cbl, which is generally used in supplements. The major form of vitamin B12 in blood is CH₃-Cbl, with smaller amounts as adenosyl-Cbl and OH-Cbl (17). Vitamin B12 in the form of OH-Cbl is widely used as an intramuscular injection (IM). Hydroxocobalamin is retained longer in the body than CN-Cbl. In the human body and higher animals, vitamin B12 is utilized in two major metabolic processes where it serves as a coenzyme. The first vitamin B12 dependent reaction is the conversion of methylmalonyl-CoA to succinyl-CoA, which uses adenosyl-Cbl as a cofactor, and the second conversion is the remethylation of Hcy to methionine, which uses CH₃-Cbl as a cofactor (18). Cobalamin will be referred to subsequently as vitamin B12.

Bioavailability

Vitamin B12 it is a product of microbial synthesis in all higher animals, and the sole source of vitamin B12 is from animal-related sources. Although some edible algae and blue-green algae (cyanobacteria), contain large amounts of vitamin B12, vitamin B12 from these two

sources appears to be inactive in mammals (19). Bioactivity of each of the forms of vitamin B12 is different. The various forms of vitamin B12 will have different dissociation, absorption, and protein carrier binding qualities which result in various degrees of bioavailability. As the intrinsic factor (IF)-mediated intestinal absorption system is estimated to be saturated at about 1.5–2.0 µg per meal under normal physiologic conditions, vitamin B12 bioavailability significantly decreases with increasing intake of vitamin B12 per meal (20). Recently reported by Watanabe, the bioavailability of vitamin B12 in healthy humans from fish, meat, and chicken averaged 42%, 56%–89%, and 61%–66%, respectively, while vitamin B12 in eggs appeared to be poorly absorbed (<9%) relative to other animal food products (20).

Metabolism (Absorption, transport, storage and excretion)

Release of vitamin B12 from proteins must occur prior to absorption. Saliva contains a vitamin B12 binding protein referred to as an R protein (18). Saliva activity starts the dissociation of vitamin B12 from the protein to which it is bound. Further dissociation of vitamin B12 occurs in the stomach with the action of hydrochloric acid (HCL) and pepsin. The dissociated vitamin B12 enters the duodenum from the stomach, bound to the R-protein. A protein synthesized by the gastric parietal cells referred to as IF binds to vitamin B12 forming the vitamin B12 intrinsic factor complex (IF-Cbl). This happens after the stomach acid is neutralized and the R protein is removed by digestive enzymes (21).

In the ileum, a specific IF-Cbl receptor initiates the uptake of vitamin B12 complex into the enterocytes. During uptake, the IF-Cbl complex is internalized by receptor mediated endocytosis where vitamin B12 is then processed and subsequently bound to TC and released into the circulation as TC-Cbl (22). Transcobalamin accounts for approximately 20% of all vitamin B12 bound in circulation, while haptocorrin accounts for the remaining 80% (23). However, only TC-Cbl has receptors on the cells surface which makes it essential for cellular

uptake, therefore TC-Cbl is considered the bioavailable form of the vitamin. Once TC-Cbl is taken up by receptor-mediated endocytosis, the lysosomal activity releases vitamin B12 from the complex into the tissue.

Vitamin B12 can be stored in the body for long periods of time. The liver is the main storage tissue accounting for ~60% of total storage while muscle tissues account for ~30%. There are approximately 2-5 mg of vitamin B12 stored in the body predominately in the form of adenosyl-Cbl and CH₃-Cbl. As stated earlier the IF-mediated intestinal absorption system is estimated to be saturated at about 1.5–2.0 µg per meal under normal physiologic conditions, therefore an increase in dose will result in an increase in urinary loss. Other factors such as bioavailability and diet content can also affect excretion of the vitamin.

Biochemical Functions of Folate and Vitamin B12

One-carbon metabolism reactions require folate as the central substrate that provides essential one carbon moieties. The methyl group is vital for many reactions including amino acid metabolism, purine and pyrimidine synthesis, and the formation of S-adenosylmethionine (SAM) (1). The formation of SAM in the 1-C cycle is critical for a large number of methylation reactions. The production of creatine, phospholipids, and neurotransmitters from SAM is important for normal physiological function. More importantly, SAM is a methylating agent for more than 100 compounds including proteins, RNA and DNA (24).

Folate in the polyglutamate form of THF is converted to 5, 10-methylenetetrahydrofolate (5, 10-MTHF) by accepting a methyl group from the conversion of serine to glycine via serine hydroxymethyltransferase (SHMT) (Figure 2-4) reaction 3. This reaction is important because it provides the methyl group needed for the production of thymidylate (dTMP) from deoxyuridylate (dUMP) by thymidylate synthase (1) (Figure 2-4) reaction 1. Folate in the form of DHF is reduced back to THF by dihydrofolate reductase. Folate also is involved in de novo

synthesis of adenine and guanine from 10-formylTHF (10-CHO-THF) resulting in purine synthesis and regeneration of THF (Figure 2-4) reactions 4, 12 and 13. These reactions collectively underline the critical role of folate in pyrimidine and purine synthesis. Thus, impaired folate status can be detrimental to the body since DNA synthesis required for cellular growth may be suppressed.

Another critical role for folate in 1-C metabolism is DNA methylation. DNA methylation depends on the reduction of 5, 10-MTHF to 5-MTHF by MTHFR. Methylenetetrahydrofolate reductase is an important enzyme since its only function in the body is in the above step, which is critical for the remethylation of Hcy to form methionine. Methionine serves as the dominant precursor for SAM. The coenzyme 5-MTHF is the methyl donor, and vitamin B12 is the cofactor needed for transferring the methyl group from 5-MTHF to Hcy to form methionine (remethylation), a reaction that requires the MTR enzyme (Figure 2-4) reactions 5, 6 and 7. The MTR reaction requires vitamin B12 as a cofactor for the remethylation of Hcy to methionine. During the MTR reaction, transfer of the methyl group from methylcobalamin-III results in the formation of the highly reactive cobalamin-I, which may become oxidized to cobalamin-II, resulting in MTR inactivation (25). Methionine synthase reductase is required for the reductive methylation of cobalamin-II (with SAM providing the methyl group), which reactivates MTR (26).

If either folate or vitamin B12 are deficient, MTR cannot remethylate Hcy to form methionine which leads to megaloblastic changes in rapidly dividing cells, such as bone marrow in addition to elevation of Hcy and depletion of SAM (1). The initial methyl group that was accepted by folate is the same methyl group that is donated by SAM to over 100 transmethylation reactions (1). When SAM donates its methyl group, it is converted to S-

adenosylhomocysteine (SAH), which then is hydrolyzed back into Hcy and adenosine by SAH hydrolase (1)(Figure 2-4) reactions 9 and 10.

Dietary protein is another source of methionine; however, the tightly regulated remethylation of Hcy to methionine also contributes to the body's need for this essential amino acid. When methionine is converted back to Hcy via the hydrolysis of SAH an elevation of Hcy may occur if the MTR reaction is impaired. For this reason, Hcy conversion to methionine needs to be optimized to maintain a balance between Hcy and the methionine pool and to prevent hyperhomocysteinaemia. Homocysteine can also be catabolized into cystathionine through the condensation with cysteine by pyridoxal phosphate (vitamin B6) and cystathionine B-synthase (CBS) (18) (Figure 2-4) reaction 11. However, this transsulfuration step is dependent on SAM activation and a surplus of methionine (1). When vitamin B12 and folate are deficient, cystathionine levels are elevated (27).

Polymorphisms

Polymorphisms are mutations that are present in more than 1% of the population. The relationship among the four polymorphisms occurring in genes involved in 1-C metabolism and the risk for CHD will be discussed in a subsequent section. Methyltetrahydrofolate reductase 677 C→T is the most widely studied polymorphism in which a transition of cytosine to thymine occurs in the gene for MTHFR at base pair 677, resulting in an alanine to valine substitution in the enzyme (28,29). A second polymorphism of the MTHFR gene occurs at base pair 1298 due to the substitution of cytosine for adenine. This substitution results in a coding change which that alanine is substituted for glutamate in the protein product (30,31). Both mutations alter the activity of the MTHFR enzyme which leads to lower concentration of 5-MTHF and less substrate available for the remethylation of Hcy. As discussed previously, the initial step in the

remethylation pathway leading to DNA methylation starts early with the reduction of 5, 10-MTHF to 5-MTHF via the MTHFR enzyme (Figure 2-4) reaction 5.

While these two polymorphisms MTHFR 677 C→T and 1298 A→C affect the activity of MTHFR and folate metabolism, two other polymorphisms related to vitamin B12 have also been investigated for their possible role as risk factors for CHD. The first is MTRR 66 A→G in which methionine replaces isoleucine in the enzyme protein. This mutation affects the activity of the MTR enzyme and results in lower remethylation of Hcy into methionine (Figure 2-4) reaction 6. The MTRR 66 A→G polymorphism was associated with an increase in plasma Hcy, with the GG genotype having a greater effect than the AG genotype (32). Also, it was found that MTRR 66 GG genotype was associated with an increased risk for having an NTD-affected pregnancy when maternal vitamin B12 concentrations were low (26).

The second polymorphism is the TC 776 C→G, which can affect the total amount of vitamin B12 carried in the blood and the amount that the cell takes up. As mentioned earlier, cells only have receptors for vitamin B12 bound to TC, and therefore TC-Cbl serves as the bioavailable form of the vitamin. Vitamin B12 bound to haptocorrin is not bioavailable because cells lack receptors for this carrier protein.

Several base pair substitutions that may occur in the gene that encodes the TC protein lead to decreased uptake of vitamin B12 (33). The most common polymorphism in the TC gene is a cytosine-to-guanine transition at base pair 776 (TC 776 C→G) that results in replacement of proline with arginine (34). This TC 776 C→G polymorphism negatively affects the serum holo-TC concentration and studies suggest that the TC 776 C→G polymorphism may affect TC binding affinity for vitamin B12 and the ability to transport vitamin B12 into tissues (35-38). Miler et al. observed a reduced mean holo-TC concentration, a lower percentage of total vitamin

B12 bound to TC, and a higher MMA concentration in individuals with the TC 776 GG genotype than in those with the TC 776 CC genotype (36). These results indicate that the TC 776 C→G polymorphism may alter the cellular availability of vitamin B12 and exacerbate the effects of low vitamin B12 status.

Dietary Sources of Folate and Vitamin B12 Intake Recommendations

Folic acid is the synthetic form of folate and is used for all enriched products in the US since January 1998 as mandated by the Food and Drug Administration (FDA). Enriched cereal and grain products are fortified with folic to a target level of 140 µg/100 g (39). In addition to cereal and grain products, folic acid fortified products are available throughout the US marketplace in the form of ready-to-eat breakfast cereals, snacks, meal replacements and many others. Naturally occurring dietary folate can be found mainly in orange juice, strawberries, peanuts, legumes, asparagus and dark green leafy vegetables.

The Dietary Reference Intakes (DRIs) represent the most current recommendations for each vitamin and mineral. It is the newest approach adopted by the Food and Nutrition Board to provide quantitative estimates of recommended nutrient intakes for different age and gender groups. The DRIs include the Estimated Average requirements (EAR), Recommended Dietary Allowances (RDA), Adequate Intake (AI), and Tolerable Upper Intake Level (UL). See Table 2-1 for definitions of each DRI (40).

The National Academy of Sciences Institute of Medicine (IOM) published the most recent DRI recommendations in 1998 (40). The goal of these DRI recommendations was to ensure optimum health rather than prevent clinical deficiencies. Folate recommendations based on the DRIs are presented in Table 2-2. The DRIs also include the new RDA for folate in dietary folate equivalents (DFEs). Dietary Folate Equivalents are units that account for differences in the absorption of naturally occurring food folate and the more bioavailable synthetic folic acid

(41). To calculate DFEs 1.7 is multiplied times the micrograms of folic acid and added to the micrograms of food folate. 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 during pregnancy and 500 μg DFE/d during lactation. The UL for folic acid is 1,000 $\mu\text{g}/\text{d}$. This intake level is established solely based on the fact folic acid supplementation can mask the diagnosis of vitamin B12 deficiency; as folate itself is not associated with any toxic side-effects. It is recommended that all women of childbearing age consume 400 $\mu\text{g}/\text{d}$ of folic acid from fortified foods and/or supplements. This is in addition to consuming food folate from varied dietary sources to reduce the risk of an NTD-affected pregnancy (41).

Dietary sources of vitamin B12 include animal-based products and fortified foods. Concentrated dietary sources of vitamin B12 include meat, dairy products, and eggs. It is not very common to develop a vitamin B12 deficiency due to short-term dietary restriction because liver stores of these vitamin can last for a few years. The most common reasons for developing a vitamin B12 deficiency are stomach and intestinal disorders that limit the release and/or absorption of vitamin B12 (42). The RDA is 2.4 $\mu\text{g}/\text{d}$ for adults, 2.6 $\mu\text{g}/\text{d}$ for pregnant women, and 2.8 $\mu\text{g}/\text{d}$ during lactation (40).

Congenital Heart Defects

Prevalence and Etiology

One million children per year are born with a CHD worldwide (43). Birth defects are the leading cause of death in new born infants (44), with CHD accounting for one death in every three defect-related infant deaths (45). Congenital heart defects occur as an isolated malformation due to abnormal organogenesis during embryonic development. The development of the cardiovascular system occurs between the third and eight weeks of embryonic growth after conception (46). During this period, both genetic and environmental factors play a critical

role in the development of the heart through proliferation and apoptosis, in which, inadequate proliferation or excess apoptosis can lead to congenital malformations such as CHD (47,48).

The interaction between environmental and genetic factors has been the focus of many research studies to identify the role of dietary and supplement intake in the prevention of CHD. Factors such as low folate and vitamin B12 status have been identified as risk factors for NTD. Neural tube defects are malformations occurring during the early embryonic stage (49-53). Both NTDs and CHDs share the same neural crest cells (NCCs) during the embryonic stage. Therefore, it is logical to assume a possible association between the status of both folate and vitamin B12 and the risk of CHD based on the similarity between NTD and CHD developmental stages.

These assumptions are supported by recent findings from investigations in which the role of both nutrients and CHD risk was evaluated (54-57). Recently, the American Heart Association (AHA) recommended that periconceptional use of multivitamins containing folic acid be taken by women of reproductive age to reduce the risk of CHD (58). The AHA recommendation was established in support of the possible protective effect of folate to reduce the risk of CHD, although the association could not be definitively confirmed due to the small number of studies available on which to base the recommendation (57,59).

Effect of Low Folate Status on CHD Risk

Low folate status alters 1-C metabolism resulting in impaired remethylation of Hcy to methionine, DNA methylation, and reduction of various polyglutamate forms of folate to THF. Consequently, this will lead to lower availability of THF which is required to form 5, 10 MTHF necessary for cell division reactions and nucleotide synthesis. In addition, it will lead to elevation of Hcy concentrations and alteration of the primary DNA methyl donor SAM (Figure 2-4) reactions 4,2, 6 and 8 (60).

It has long been recognized that there is an association between folate deficiency and increased incidence of congenital abnormalities (61). The relationship between folate status and CHD development is through impaired cardiac neural crest development. The neural crest cells develop into many organs and tissues during embryonic development, one of which is the cardiovascular system. Folate is required for cardiovascular development during the early stages through the migration of NCC, differentiation, dispersal and cell cycle programming. It has also been suggested that Hcy directly affects cardiac NCC function, however, the mechanism has not been elucidated (61). Both impaired DNA synthesis and elevation of Hcy concentration occur in response to low folate status. However, it is not clear whether it is the lower THF availability or the elevated Hcy concentrations associated with low folate status or both that may be associated with the impaired NCC development.

Periconceptional use of folic acid is well established in preventing NTDs, and emerging evidence suggests that multivitamins containing folic acid may also protect against CHD (57,62). An association between folate deficiency and CHD has been examined in both human epidemiological studies and animal experimentation (60,63-67). The first studies to show an association between B-vitamins and CHD were published in 1952 and 1954 (63,68). Congenital heart defects were associated with the consumption of folate-deficient diets in both studies using animal models. Developmental defects involving the heart and inhibition of myocardial proliferation were also associated with folate deficient diets in other investigations (69,70). Hobbs et al. reported that Hcy, SAH, and methionine concentrations are important biomarkers predictive of CHD case or control status (71).

The strongest evidence that folate status is involved in the CHD formation and prevention comes from a Hungarian randomized control intervention trial (RCT) in which multivitamins

containing folic acid reduced the risk of CHD up to 60% when taken during the periconceptional period (72). These results were confirmed in two population-based case-control studies (PCC) in the USA in which a 24% reduction in CHD risk was observed (55,57). In another study, the Baltimore Washington Infant Study (BWIS), an inverse relationship between daily maternal intake of folic acid and the incidence of cardiac outflow tract defects in offspring was found (56).

There are few possible links to how folate may be affecting the risk of CHD. As stated earlier, folate is a critical substrate for DNA methylation, as well as DNA and nucleotide synthesis, both of which are possibly associated. Research studies provide evidence that elevated Hcy concentration (hyperhomocysteinaemia) is associated with an increased CHD risk, which may be associated with low folate and vitamin B12 status (73). However, due to the small number of investigations, and the inconsistency of findings between some studies, further research assessing the effect of folic acid and the reduction of CHD risk is warranted.

Effect of Low Vitamin B12 Status on CHD Risk

The roles of folate and vitamin B12 are interrelated with regard to DNA and nucleotide synthesis as well DNA methylation. Folate and vitamin B12 participate in 1-C metabolism in which both are needed for the regeneration of THF, remethylation of Hcy, and production of SAM. As discussed earlier, folate is required to form the 5-MTHF through the action of the 5, 10 MTHFR. The methyl group is donated by 5-MTHF to form methionine via remethylation of Hcy, and to regenerate THF. This step cannot be completed without the cofactor vitamin B12, which is essential for the function of the MTR enzyme. Deficiencies of vitamin B12 and/or folic acid result in hyperhomocysteinaemia, which is associated with an increased risk of CHD (73-76). Similar to folate, vitamin B12 deficiency is linked to an increase risk of NTDs (77). Several studies linked vitamin B12 status with the risk of CHD through impaired methylation of

Hcy to methionine. This biochemical derangement leads to hyperhomocysteinaemia and low DNA methylation, which might be associated with the increase risk for CHD.

It is not clear whether vitamin B12 alone or in conjunction with low folate status is a risk factor for CHD. The association between specific polymorphisms in 1-C metabolism and nutrient status further complicate the relationship. More comprehensive studies that account for both folate and vitamin B12 and the polymorphisms related to their 1-C metabolism is required to determine risk associations with CHD.

Polymorphisms and CHD Risk

MTHFR (677 C→T and 1298 A→C) polymorphisms

In this section, the association of key folate and vitamin B12-related polymorphisms with CHD risk will be discussed. Polymorphisms in the MTHFR, MTRR, and TC genes are recognized to have some effect on CHD; whether this effect is dependent or independent of folate and vitamin B12 status is the subject of on-going investigations (78-81). Although folic acid containing supplements may have a protective effect in reducing CHD risk, the role that folate and vitamin B12-related polymorphisms may play in CHD risk is not clear enough to draw definitive conclusions and more research needs to be done in this area (82).

Studies examining the roles of these polymorphisms in affecting CHD risk have produced conflicting results. The MTHFR 677 TT genotype has been associated with reduced enzyme activity, decreased plasma and red blood cell folate, and mildly elevated Hcy concentrations especially when combined with the MTHFR 1298 A→C polymorphism (83-86). Data from clinical studies indicate that there is a positive association between the MTHFR 677 C→T polymorphism and CHD risk (79,80,87,88). In 2006, Beynum et al. reported that maternal MTHFR 677 C→T is a risk factor for CHD in offspring (80). Beynum group's conclusion was based on an observed increased risk of CHD in the offspring of mothers who did not use

supplements containing folic acid with either the MTHFR 677 CT or TT genotype. The risk increased 3-fold in mothers in the CT genotype group and 6-fold in the TT genotype group compared to women who had used supplements containing folic acid. However, there was no risk association between the MTHFR polymorphisms and CHD in the family-based transmission disequilibrium test (TDT). Briefly, the TDT is a family-based association test to detect the presence of linkage between a genetic marker and a trait by measuring the over-transmission of an allele from heterozygous parents to affected offspring.

Hobbs et al. reported that the highest estimated risk for having a CHD-affected pregnancy was among women who were in the highest quartile for Hcy with the MTHFR 677 CC genotype and were smokers (89). The group reported that maternal MTHFR 677 C → T polymorphism did not have an independent impact on the estimated risk of having a CHD-affected pregnancy. The sample size however, may have limited the power to detect an association between the MTHFR and CHD risk. Based on a 2007 meta-analysis, Van Beynum et al. concluded that there was no substantial evidence of increased CHD risk in individuals with either the MTHFR 677 CT or TT genotypes (90). The group suggested that heterogeneity regarding population background, study design and type of heart defects complicates the pooling and comparison of the studies (90).

Methyltetrahydrofolate reductase 1298 A → C polymorphism results in reduced enzyme activity (91). Combined heterozygosity for both MTHFR 677 C → T and 1298 A → C polymorphisms results in a lower MTHFR activity compared to heterozygosity of either polymorphism alone (92). The MTHFR 677 C → T polymorphism alone has a greater effect on Hcy than the MTHFR 1298 A → C polymorphism (93). In studies concerning NTD risk, an effect of the MTHFR 1298 A → C polymorphism was observed only when combined with the MTHFR 677 C → T polymorphism (93). In another study concerning cleft palate (CLP), the risk

increased when the MTHFR 677 CT and TT genotypes and the MTHFR 1298 AC were accompanied by low folate status during pregnancy (94). Due to the similarity in embryonic origin, evaluating other congenital conditions can be critical for the understanding of the etiology and risk factors associated with the condition.

The MTHFR 1298 and 677 AC and CT genotypes combined are more associated with elevation of Hcy when folate and vitamin B12 concentrations are low. The role of nutrient-gene interaction and gene-gene interaction in the development of CHD has not been fully determined. More research and well-defined phenotypic subcategory analyses as well as status assessment of both folate and vitamin B12 are needed to definitively determine whether the MTHFR 677 C→T and/or MTHFR 1298 A→C polymorphisms of the mothers are risk factors for the development of CHD.

MTRR 66 A→G and TC 776 C→G polymorphisms

Both MTRR and TC are essential proteins required for vitamin B12 function and in the remethylation of Hcy and DNA methylation. Unlike the MTHFR polymorphisms, MTRR and TC are two separate genes that directly involve vitamin B12 but not folate. The MTRR enzyme, officially known as 5-methyltetrahydrofolate homocysteine reductase, is required to activate the MTR through the reduction of vitamin B12. The later enzyme is important for the conversion of Hcy to methionine by accepting the methyl group from vitamin B12 and transferring it to Hcy, a reaction in which Hcy is remethylated to methionine. The importance of this enzyme in 1-C metabolism is based on its critical role in keeping Hcy and methionine concentrations in balance, which will result in normal DNA methylation and nucleotide synthesis.

The MTRR 66 A→G polymorphism is associated with an increased risk of NTD (26,95). In a case-control study to investigate the influence of the MTRR 66 A→G polymorphism on CHD risk and the possible interaction between the variant and MMA concentrations, Van

Beynum et al. reported that the MTRR 66 GG genotype in combination with a high MMA concentration was associated with a 3-fold increase in CHD risk (81). The increase risk was dependent on the elevation of MMA, which indicates that compromised vitamin B12 status may possibly be a risk factor for CHD risk.

A C→G substitution at base pair 776 in the gene that encodes transcobalamin (i.e. TC 776 C→G) may affect TC binding affinity for vitamin B12 which will lead to reduced cellular uptake (35,96,97). Von Castel-Dunwoody found that the presence of the TC 776 CG genotype negatively affected vitamin B12 metabolism and increased Hcy concentration in 359 non pregnant young women (38). These researchers found that the TC 776 GG genotype group had a significantly lower vitamin B12 concentration and slightly higher Hcy concentration when compared to CC and CG among CHD cases. However, folate, vitamin B12, and Hcy concentrations were not different in the case group with any of the MTRR genotypes. The group suggested that a larger sample size might provide a more definitive answer to whether these two polymorphisms are associated with CHD risk.

The limited number of studies, the complexity of gene-gene interactions, and nutrient-gene interactions limit the ability to conclude whether the MTRR and/or TC polymorphisms alone or in combination with low vitamin B12 has an effect on CHD risk. Further research investigating both MTRR 66 and TC 776 with the MTHFR 677 and 1298 polymorphisms and folate and vitamin B12 status is needed. This will help expand the understanding of the relationship among folate, vitamin B12 and polymorphisms occurring in genes that codes for key enzymes in folate and vitamin B12 metabolism and how these factors influence CHD risk.

Environmental and Sociodemographic Risk Factors

Environmental and sociodemographic factors such as maternal cigarette smoking, alcohol consumption, obesity, febrile illnesses, and use of medications, diabetes, ethnicity, and family history are important to consider when assessing the risk of CHD. There is relatively little research on the potential adverse effects of these non-inherited factors on the development of the fetal heart. However, a growing body of epidemiological studies investigating the critical involvement of such modifiable factors on the development of the fetal heart and risk for congenital malformations is emerging. It is estimated that up to 30% of congenital heart defect cases are attributable to identifiable and potentially modifiable factors (98).

Cigarette Smoking

Cigarette smoking during pregnancy has been documented over the years to be associated with various negative effects including severe impairment of embryonic development and infant mortality. A meta-analysis of studies published between 1971 and 1999 found no association with smoking for all types of heart defects combined and mixed outcomes for the analysis of specific groups or phenotypes (99). Some recent studies found an association between maternal smoking and various types of heart defects (100,101). Hobbs et al reported an association between smoking and CHD risk especially when combined with more elevated concentrations of Hcy (89). While these studies suggest an association, a study by Kallen et al., as well as a study by Correa-Villasenor et al. failed to corroborate these results (99,102). The difference in methods, classification, control of confounding factors, and sample size could be the cause of the inconclusive results.

Alcohol Consumption

It has been suggested that ethanol may produce fetal tissue edema and affect the turgor of the primitive cardiac loop (58). There are a large number of studies in which a wide range of

teratogenic effects of alcohol consumption during pregnancy were investigated. In a case-control study in Spain, the risk of congenital anomalies with different daily intakes of alcohol doses was investigated and an increase risk of CHD was only found with the highest level of maternal consumption of alcohol per day (ie, > 92 g/d) (103). In the study by Correa-Villasenor et al, alcohol consumption in early pregnancy was associated in a dose dependent manner (102). There is no conclusive evidence that moderate alcohol consumption is associated with increased risk of CHD; however, the studies provide more evidence associating heavy consumption with CHD risk (102,103).

Obesity

A number of studies have examined the association between maternal pre-pregnancy obesity and CHD. In two studies, no statistically significant increase in risk for any heart defect in relation to maternal obesity was observed (104,105). Walker et al reported a positive association between maternal obesity, defined as a body mass index (BMI) of > 26 kg/m², and defects of the great vessels (106). In a recent study, a 6.5-fold increased risk in aggregate cardiac defects was detected among black obese mothers (107). Careful assessment of an obesity effect on CHD risk when conducting observational studies is required to minimize the confounding complications by other factors such as diabetes.

Febrile Illnesses

There is strong evidence that febrile illnesses are associated with increased risk of congenital anomalies. Studies suggest that maternal febrile illnesses during the first trimester of pregnancy is associated with increased risk for certain heart defects (108-110). Mothers reporting any febrile illnesses during the first trimester had a 2-fold increase risk of having a child with heart defects (110).

Use of Medications

In a recently published paper by the AHA, research findings related to the use of different medications and the risk of congenital defects were reviewed (58). There was no conclusive evidence that the drugs that were investigated were associated with an increased risk of CHD. Maternal therapeutic drugs such as oral contraceptives, penicillin, ampicillin, and corticosteroids as well as maternal non-therapeutic caffeine were not associated with any variation of congenital anomalies. The exception was ibuprofen which was reported to be associated with some specific variations of congenital defects (98).

Diabetes

Studies have clearly documented a link between maternal pre-gestational diabetes and a range of congenital defects. This association is less consistent with gestational diabetes (111-114). The large body of evidence reviewed by the AHA recently suggested that diabetes is a well known prenatal maternal risk factor for CHD (58). As illustrated by the study of Ferencz et al., specific types of CHD associated with maternal pre-gestational diabetes include laterality and looping defects, transposition of the great vessels, hypoplastic left heart syndrome cardiomyopathy, and nonchromosomal atrioventricular septal defects (115).

Ethnicity

Ethnicity among different groups has been associated with various types of congenital defects. Increased risk prevalence of specific CHD has been reported among white infants compared to black infants (116,117). However, in a population-based study of variations in prevalence of birth defects comparing Hispanic and black mothers to non-Hispanic white mothers in California between 1987 and 1997, no differences in prevalence were detected (117,118).

Family History

The evidence for an association between family history and CHD is not strong. However, family history is critical in studies evaluating the risk of CHD. Family history may be a risk factor for CHD in association with other confounding factors. Although there is no direct evidence that CHD risk is associated with family history, careful assessment is required when evaluating related data in future studies.

Biochemical Markers: Folate and Vitamin B12 Status Indicators

Serum and Red Blood Cell Folate Concentrations

Serum folate concentration is a good indicator of current and recent folate intake. It is a sensitive measure that reflects the short-term folate status (119). Serum folate concentration decreases within one to three weeks after lower folate intake is maintained (86,120). Lower serum folate concentration is detected before any change occurs to RBC folate concentration. Defining inadequate serum folate status using the microbiological assay is based on the lower limit of the normal range ≤ 13.6 nmol/L (121). The radiobinding assay is also used as an alternative method to assess folate status, however, this method has been shown to yield lower blood folate values relative to the microbiological assay (122). The lower limit for the radiobinding assay is <7 nmol/L.

Red blood cell folate concentration is considered a better long-term indicator of folate status than serum folate concentration (120). While serum folate concentration reflects the early and short-term changes in folate status, RBC folate concentration reflects tissue storage. Folate uptake into the erythrocytes only occurs during early stages of erythropoiesis which takes place exclusively in the bone marrow. Folate therefore cannot permeate the membrane of mature RBC during its 120-day life span, thus RBC folate concentration is a good reflection of folate status four months prior to the time of blood sampling. Inadequate status defined using the lower limit

of normal of RBC folate concentrations using radiobinding assay is ≤ 317 nmol/L (120). Using the microbiological assay, deficient status is defined as a concentration of 362.6 nmol/L. The lower limit of normal or acceptable is 453.2 nmol/L (123).

Serum Vitamin B12

In the general US population, the mean serum vitamin B12 concentration for healthy individuals over four years of age is 381 pmol/L (124). In the clinical setting, serum vitamin B12 is the primary method for assessing vitamin B12 status; however other measures are necessary to avoid false diagnosis of impaired status (18). This is due to the nature by which vitamin B12 is metabolized as well as the way it is stored in the body. Vitamin B12 can be stored in some tissues such as the liver and kidney for longer periods of times, while some other tissues can be deficient. This can lead to inaccurate estimates as some individuals can have low normal serum vitamin B12 concentrations even though their body stores are deficient in vitamin B12. A plasma concentration >221 pmol/L is considered normal, concentrations between 148 and 221 pmol/L are considered marginally deficient or “low normal”, and a concentration <148 pmol/L is considered deficient.

Methylmalonic Acid

It is preferable to rely on status indicators that reflect vitamin B12 function. Specifically an elevation in MMA concentration is highly specific for a vitamin B12 deficiency.

Methylmalonic acid is a four carbon molecule related to valine, isoleucine, and propionic acid catabolism (17). Vitamin B12 is required for the conversion of methylmalonyl-CoA into succinyl-CoA, a reaction sensitive to vitamin B12 status in which it prevents elevation of MMA concentration. When vitamin B12 is low, the conversion is impaired leading to accumulation of methylmalonyl-CoA, which eventually is converted into MMA. For that reason, MMA is a better predictor of B12 status than serum B12 (17).

Elevated MMA concentrations can be detected in the early stages of vitamin B12 deficiency before a decrease in serum vitamin B12 can be measured (17). Van Beynum et al reported that maternal subjects with the MTRR 66 GG genotype in combination with high MMA levels above 80th percentile had a 3-fold increased risk for all types of CHD in offspring (81). Therefore, using MMA in conjunction with serum B12 to fully assess the risk of vitamin B12 and polymorphisms related to its function in 1-C metabolism is preferable. Normal serum MMA concentration is ≤ 271 nmol/L, with reported references ranges for serum MMA concentration of 50 to 400 nmol/L (125,126).

Homocysteine

Homocysteine is a dimer containing a sulfur group. Plasma Hcy concentration is inversely related to the concentration of plasma folate. This was evident from the implementation of folic acid fortification of cereal grains in 1998 by the US government with the aim of reducing the incidence of NTDs in pregnancy (127). This fortification strategy was successful in that it resulted in an improved folate status and lower plasma Hcy concentration (128). The mean Hcy concentration was significantly lower in the 1999-2000 post-fortification period compared to (1994-1998) pre-fortification period. In the 1994-1998 period, Hcy concentration was 9.5 $\mu\text{mol/L}$ while in the 1999-2000 period it was 7.9 $\mu\text{mol/L}$ (129).

Plasma Hcy concentration is maintained within narrow ranges through enzymes that metabolize methionine and Hcy. The cutoff for plasma Hcy concentration is ≥ 12 $\mu\text{mol/L}$. Values in this higher range have been associated with increased risks for adverse health effects (130). Kepusta et al. reported an association between mild maternal hyperhomocysteinemia and CHD risk, which was confirmed by Hobbs et al. (71,131). Bailey et al reported that vitamin B12 status < 221 pmol/L has a negative effect on Hcy concentration independent of the genotype (93). Homocysteine evokes oxidative stress through production of reactive oxygen species (ROS),

binds to nitric oxide (NO), or leads to accumulation of its precursor SAH which is a potent inhibitor of biological transmethylation (132). As discussed earlier, impaired folate and vitamin B12 status and polymorphisms related to both nutrients and the elevation of Hcy concentration were common factors for the risk of CHD in several investigations.

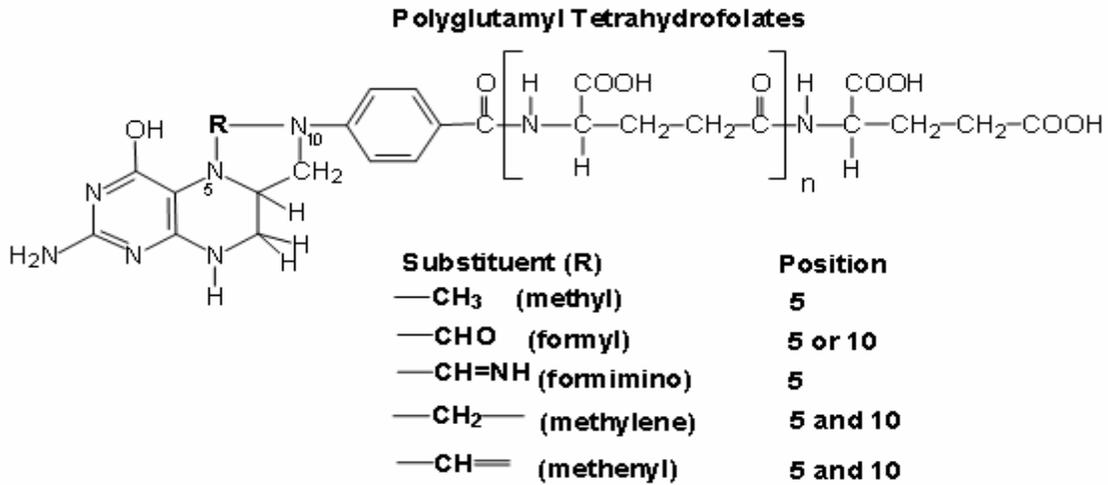


Figure 2-1. Structure of folate/folic acid. [Reprinted with permission from Baiely, L. 2006. Bailey, L. & Gregory, J. (2006) Folate. In: Present knowledge in nutrition, 9th ed. (Bowman, B. & Russell, R., eds.), pp. 278-301. ILSI Press, Washington, D.C.] Folic acid consists of a para-aminobenzoic acid 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 dihydrofolic 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.

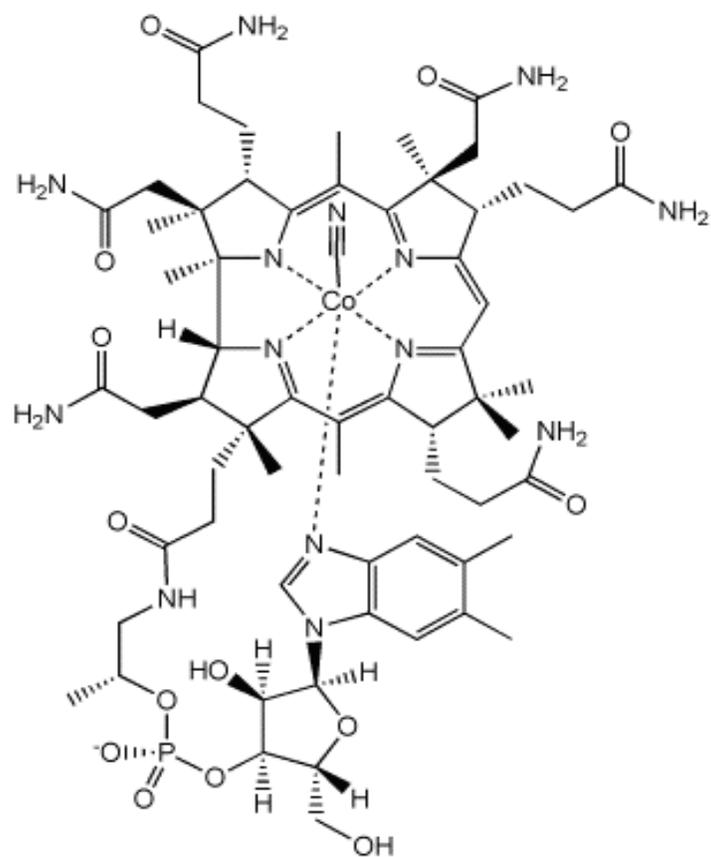


Figure 2-3. Vitamin B12 structure.

Nucleotide Biosynthesis

Methylation Reactions

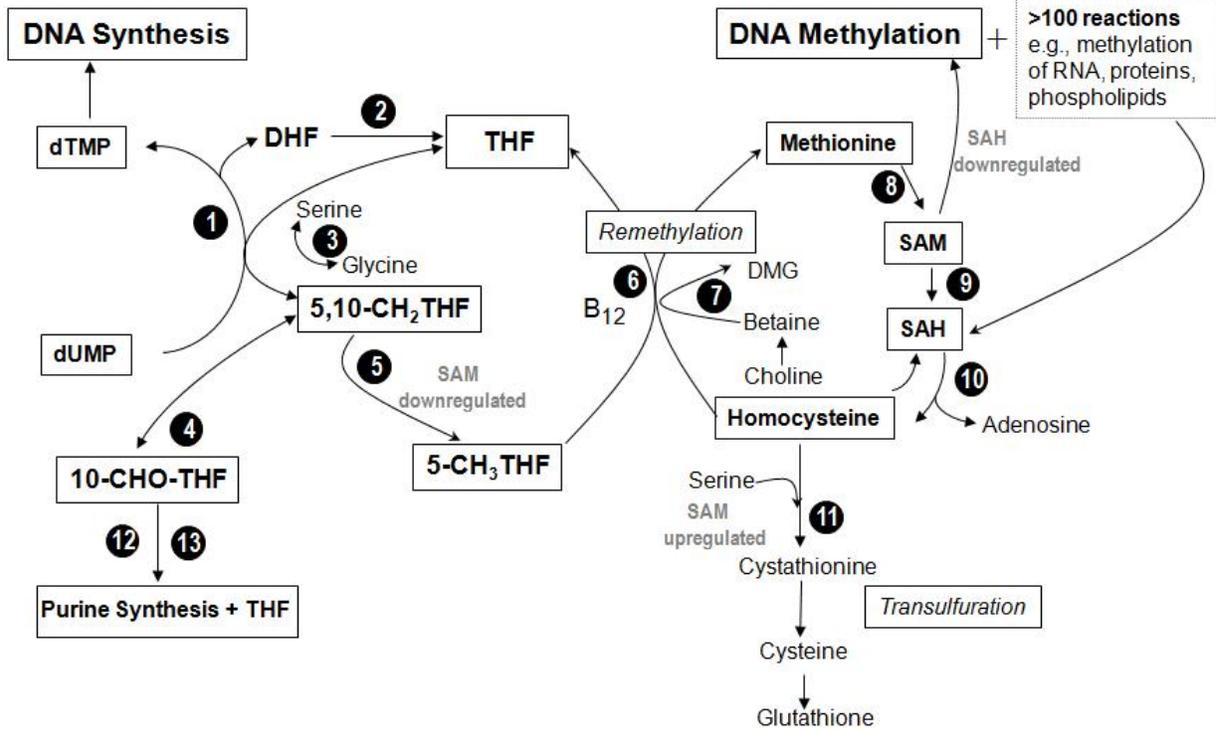


Figure 2-4. One-carbon metabolism folate-dependent pathway. [Reprinted with permission from Baiely, L. 2006. Bailey, L. & Gregory, J. (2006) Folate. In: Present knowledge in nutrition, 9th ed. (Bowman, B. & Russell, R., eds.), pp. 278-301. ILSI Press, Washington, D]

Table 2-1. Definitions of Dietary Reference Intake (DRI) recommendations.

DRI recommendation	Definition
Estimated Average Requirement (EAR)	A daily nutrient intake value that is estimated to meet the requirement of half the healthy individuals in a group.
Recommended Dietary Allowance (RDA)	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.
Adequate Intake (AI)	Individuals should aim for this intake level. 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.
Tolerable Upper Intake Level (UL)	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 increase.

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

DRI recommendation	
Estimated Average Requirement (EAR)	320 μg DEF/d
Recommended Dietary Allowance (RDA)	400 μg DEF/d
Adequate Intake (AI)	Not Applicable
Tolerable Upper Intake Level (UL)	1,000 μg synthetic folic acid/d

CHAPTER 3 MATERIALS AND METHODS

Study Design and Methods Overview

This study was designed by clinical investigators at the University of South Florida and All Children's Hospital in St. Petersburg, Florida as an initial pilot study to provide data for future grant proposals. The long-term goal of future studies would be to conduct large-scale well-designed investigations to evaluate the association between folate and vitamin B12 status and genetic polymorphisms and the etiology of CHDs. Following the initiation of the study, Drs. Bailey and Kauwell were invited to join the collaborative research team to analyze the folate and vitamin B12 status indicators, to do the data analysis, interpret the findings, and to provide advice regarding how the study design and data collection should be revised in future studies. This section provides a description of the design and methods used in the pilot study and the discussion section includes a critique and explanation of specific aspects of this pilot study that need revision for future investigations.

The study protocol was approved by the Institutional Review Board at the University of South Florida and All Children's Hospital where the study was conducted and the University of Florida, which received blood samples for analysis. Three hundred ninety (n= 390) non-pregnant volunteer subjects were enrolled in the pilot study. The subjects recruited were mothers of children and juvenile patients from the pediatric cardiology practice at All Children Hospital, St. Petersburg, FL, as well as staff of All Children's Hospital or the University of South Florida at St. Petersburg, FL. Inclusion criteria (a) "women who had previously delivered an infant"; (b) "previous pregnancy resulted in any variation of structural CHD-related complications (cases)"; (c) "previous pregnancy with no related structural CHD complications (controls)"; and (d) "not pregnant at time of data collection." Mothers were primarily recruited and informed of the study

at the time that they brought their children to All Children's Hospital for a clinical appointment. In addition, staff members were recruited who met the inclusion criteria. After determining eligibility, subjects were provided information regarding the study and were asked to sign an informed consent form. After obtaining the informed consent, blood samples were collected for the determination of polymorphisms in the MTHFR, MTRR and TC genes (MTHFR 677 C→T, MTHFR 1298 A→C, MTRR 66 A→G, and TC 776 C→G) and concentrations of serum folate, RBC folate, vitamin B12, Hcy, MMA, and hematological parameters. The categorization of the control (n=186) and the case group (n=204) was based on the diagnosis of the presence of CHD using prenatal and postnatal ultrasonography. Subjects were instructed to complete a self-administered questionnaire (Appendix A) designed to collect demographical data including information about their health status, supplement use, alcohol consumption, cigarette use, and prescription medication use during their index pregnancy.

Sample Collection and Processing

Blood samples were collected from each participant by a phlebotomist in vacutainer tubes (Vacutainer® Blood Collection Set; Becton Dickinson, Vacutainer® Systems; Franklin Lakes, NJ). A total of 30 mL of blood was collected for analysis of the following indices "RBC folate, plasma vitamin B12, serum MMA, serum Hcy, serum folate, and hematocrit." The vacutainer tubes were immediately shipped on dry ice to the University of Florida for processing and frozen storage prior to analysis.

Blood for serum folate 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 to prevent clotting (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 polymorphisms (MTHFR, TC, and MTRR). Aliquots were frozen at -30°C for subsequent analysis of serum and RBC folate concentrations.

Analytical Methods

Measurement of Serum Concentrations of Status Indicators

Serum folate and vitamin B12 concentrations

The serum folate concentrations of all subjects were determined using the MP Biomedicals, Inc. SimulTRAC-S® Radioassay Kit (Orangebury, New York). The radiobinding assay is conducted by adding dithiothreitol solution to the folate tracer (¹²⁵I, borate buffer with human serum albumin, dextran, potassium cyanide, dye and preservative). This mixture is added to serum folate 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 ¹²⁵I 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. Sample serum folate concentration was calculated using a standard curve on which the radioactivity was inversely related to serum folate concentration.

Plasma B12 was determined by RIA using a commercially available kit (Quantaphase II, Bio-Rad). Specifically, samples were incubated with a ^{57}Co labeled B12 tracer in a 100°C water bath to convert all forms of B12 to cyanocobalamin. Samples were brought to room temperature after boiling for 20 min, and then mixed with purified porcine IF bound to polymer beads and incubated for one hour. During incubation labeled and unlabeled B12 compete for binding to IF at rates that match their relative concentrations. Finally, samples were centrifuged, and supernatant containing unbound B12 was removed. Sample radioactivity was measured by gamma counter and B12 concentration was calculated using a standard curve on which the radioactivity was inversely related to B12 concentration. Folate concentrations were inversely related to the measured radioactivity. Serum folate and vitamin B12 concentrations $>12\text{ nmol/L}$ and $>148\text{ nmol/L}$, respectively, represented the lower limit of normal values for this study.

Red blood cell folate concentration

The red blood cell folate concentrations of blood specimens were determined using the *Lactobacillus casei* microbiological assay in a 96-well microplate system adapted from Tamura (133) and Horne and Patterson (134). The intra- and interassay CV for the microbiological assay were 8.7 and 7.1%.

Homocysteine and methylmalonic acid

Samples were shipped to the (Metabolite Laboratories, Inc. Denver, Colorado) for analysis to determine serum Hcy and MMA concentrations. Samples were analyzed using gas chromatography – mass spectrometry (135,136).

Genotype Identification

Genotypes of potential subjects were determined using Dynamic Allele Specific Hybridization (DASH) performed by DynaMetrix. Samples were sent to DynaMetrix Limited, University of Leicester (United Kingdom, where primers and probes were designed for the genotype polymorphisms and analyses were performed using the DASH™ method with DynaScore Software v. 0.7 (<http://www.dynametrix-ltd.com>).

Briefly, a short PCR product was created spanning the polymorphic position. One PCR primer was 5'-labeled with biotin for attachment of the amplified targets to streptavidin-coated 96-well microtiter plates. Following denaturation and a wash to remove the unbound strand, an allele-specific probe was hybridized to the bound target DNA strand at low temperature in the presence of the double-strand specific intercalating dye Sybr Green. Finally, the temperature was steadily increased while recording the probe-target duplex melting temperature, as monitored by diminution of Sybr Green fluorescence with a quantitative PCR analysis device.

Pregnancy Index

Pregnancy index is a term that indicates the elapsed time from the delivery of the affected infant for the case mothers or for the normal infant for the control mothers, and the time when the blood was drawn. The pregnancy index was calculated using the date the blood was drawn (transformed into numerical counts) minus the date of the delivery (transformed into numerical counts). The product was then divided by 365 which is the number of days in a year to obtain the number of years. The pregnancy index was then divided into three categories: < 3 years, 3-5 year, and > 5 years.

Selection of sub-sample population: Since the primary focus of the study was to determine if folate and vitamin B12 status during pregnancy was associated with risk for CHD, it was important to focus on women who had delivered their infants in a relatively short time from the

time the blood sample was drawn for nutrient and metabolite concentration determinations. For this reason, a subgroup of the study participants, those with a pregnancy index of less than three years were carefully evaluated. Since status indicators were more likely to reflect status during gestation.

Supplement Analysis

The results from the questionnaire regarding supplement use were modified to fit our model due to the low response rate. We combined all responses to either (Yes or No) categories instead of the actual categories (yes, no, infrequently, sometimes, or routinely). The following responses “yes”, “sometimes”, or “routinely” were counted as “yes” responses. A “no” or “infrequently” response was considered a “no” responses. Data on other supplements and herbal use were not assessed due to the high non-response rate.

Statistical Methods

The statistical analysis was performed using SAS, version 9.1, SAS Institute Inc. Cary, NC, USA. An initial analysis was conducted to determine basic statistics of the demographic and background variables and hematological profile: ethnicity, maternal age, pregnancy index, supplement use, cigarette and alcohol consumption, and hematological indices. One-way analysis of variance (ANOVA) test was used to determine if the continuous demographic and background variables means differed among the two groups (case and control). A Chi-square test was used to determine whether the proportion of responses in each of the categories differed between categorical variables. Status indicators were log-transformed so that the assumptions of normality are met, and the reported values were back-log transformed. Linear regression analysis was performed to check correlation between status indicators. An ANOVA test was used to compare the means of the transformed status indicators for both folate and vitamin B12 (Hcy, MMA, vitamin B12, serum folate, and RBC folate) and determine whether the status

indicators differed between cases and controls with regard to CHD risk. A Chi-square test was used to determine whether there was a relationship between subject status and genotype groups concerning the MTHFR, MTRR, and TC polymorphisms. Logistic regression was used to evaluate a complete model of the subject status (case/control) versus the genotype classification and status indicators. All first order interactions were included in the full model.

CHAPTER 4 RESULTS

Part I: Exploring the Data

Demographic Characteristics of the Study Sample Population

Subjects

Three hundred ninety (n= 390) non-pregnant subjects were enrolled in the pilot study. Subjects were primarily mothers of children and juvenile patients from the pediatric cardiology practice at All Children Hospital, St Petersburg, Florida. Subjects were both non-pregnant mothers of children with any variation of structural heart defects referred to as the case group (n=204) and non-pregnant mothers of non-CHD affected offspring referred to as controls (n=186).

Demographic Characteristics

Demographic characteristics of the sample population are presented in Table 4-1. No difference ($p > 0.05$) between case and control based on ethnicity, maternal age at birth, or pregnancy index was detected. Ethnicity was reported as African-American, Hispanic, white-American, or other, and there was no difference ($p > 0.05$) in the ethnic distribution between the case and control groups. When the subjects were categorized into different age groups (<18, 18-30, 30-45, and ≥ 45 years of age), no differences ($p > 0.05$) between the case and control groups were detected. Maternal age was not different ($p > 0.05$) between cases (28.5 ± 0.65 years) and controls (27.7 ± 0.74 years). Subjects were then categorized into three categories according to pregnancy index (< 3 years, 3-5, > 5 years). No significant difference ($p > 0.05$) was detected between cases and controls based on pregnancy index category.

Supplement Use

Low response rate for the supplement question in the questionnaire was recorded. Based on the response of one-third of the subjects in either the case or control group, use of either prenatal and/or folic acid supplements was determined not to differ ($p > 0.05$) between case and control groups as presented in (Table 4-2).

Cigarettes and Alcohol Consumption

Limited data were collected about cigarette and alcohol consumption among participants. Due to the limited data and response rate in the “yes” category (Table 4-3), a statistical analysis test to evaluate an association between smoking cigarettes and alcohol use and increased CHD risk was not conducted.

Medical Conditions

Data were collected for a small percentage of subjects related to diabetes, seizures, and anemia which developed during pregnancy (Table 4-4). No data on other medical conditions were available. Data were insufficient to conduct a statistically valid comparison.

Hematological Indices

Hematological indices are presented in (Table 4-5). No differences ($p > 0.05$) in any of the hematological indices (Hgb, MCV, MCH, MCHC, and Hct) were found between case and control groups.

Part II: Folate and Vitamin B12 and Status Indicators (Objective 1)

The first hypothesis was that low folate and/or vitamin B12 status are associated with increased risk for CHD. To test this hypothesis, association between maternal folate and vitamin B12 status biomarkers (serum and RBC folate, vitamin B12, Hcy, and MMA concentrations) on infant CHD risk was observed.

Status Indicators and CHD Risk

The association between maternal folate and vitamin B12 status and risk of having a child with CHD was evaluated. The specific status indicators for folate status that were compared included serum and RBC folate and Hcy concentrations and the vitamin B12 status indicators, that included serum vitamin B12, MMA and Hcy concentrations. There were no significant differences between any of the status indicators in the case versus control group (Table 4-6). One entry in the MMA concentrations reported for a subject was omitted from the MMA dataset because it was 10-fold higher than the maximum value for any of the remaining subjects, a value that is physiologically implausible.

Relationship of Status Indicator Variables in the Sample Population

The means for vitamin B12, RBC folate, and serum folate concentrations were inversely correlated with Hcy concentration ($p < 0.0001$), ($p < 0.002$) and ($p < 0.0001$), respectively. Mean concentrations for MMA and Hcy were positively correlated ($p < 0.0001$).

Status Indicators in the Sub-Sample Population

Subjects who delivered their babies in less than 3 years from the time the blood sample was drawn were selected as for this sub-sample. A total of 62 subjects had a pregnancy index of < 3 years, 35 of whom were cases and 27 were controls (Table 4-1). When folate and vitamin B12 status indicators were compared between the cases and controls in the sub-sample population groups (Table 4-7), a relationship ($p < 0.05$) between serum folate-concentration and CHD risk was detected. The mean serum vitamin B12 concentration between case and controls was associated with CHD risk in the case group ($p = 0.0016$) lower. No differences ($p > 0.05$) between case and control groups for the other status indicators (Hcy, MMA, RBC folate concentration).

Part III: Polymorphisms and CHD Risk (Objective 2)

The second hypothesis is that polymorphisms affecting genes encoding MTHFR, MTRR, and TC genes (i.e. MTHFR 677C→T and 1298 A→C, MTRR gene 66 A→G and TC 776 C → G) will exacerbate the effect of low folate and/or vitamin B12 status on CHD risk. To test this hypothesis, the association between maternal MTHFR 677 C→T and 1298 A→C, MTRR 66 A→G, and TC 776 C→G polymorphisms on infant CHD risk was associated. The distribution for all possible genotypes is presented in (Table 4-8).

MTHFR 677 C→T and MTHFR 1298 A→C Polymorphisms and CHD Risk

The relationship between both MTHFR 677 C→T and 1298 A→C polymorphism and the risk of CHD independent of the other two polymorphisms TC 776 C→G and MTRR 66 A→G was investigated using the Chi-square test. No association ($p > 0.05$) was detected between the MTHFR genotype groups and CHD (Table 4-9). More cases ($n=39$) have the double heterozygote (AC-CT) MTHFR polymorphism compared to the control group ($n=24$). When the double heterozygous AC-CT MTHFR genotype groups were compared between the case and control group, there was a trend ($P= 0609$) for a greater number of case mothers to have the AC-CT MTHFR genotype compared with all of the remaining genotypes (Table 4-9).

MTHFR 677 C→T Polymorphism

Comparison of the three genotypes (CC, CT, TT) between cases and controls resulted in no significant association ($P=0.1073$) (Table 4-10). Although not significant, the somewhat higher number of cases with the CT and TT genotypes compared to controls suggests a trend.

MTHFR 677 (CT/TT) Genotypes

Based on the higher number of cases observed having the CT and TT genotypes for the MTHFR gene, the MTHFR 677 CT and TT genotype groups were combined independently of

the MTHFR 1298 A→C polymorphism. This combination resulted in an association ($P=0.0356$) between CHD and the MTHFR 677 C→T polymorphism (Table 4-11).

MTHFR 1298 A→C Polymorphism

Similar to the MTHFR 677 C→T comparison between cases and controls based on the MTHFR 1298 A→C genotypes were carried out. No association ($p > 0.05$) between the MTHFR 1298 genotypes and CHD (independent of the MTHFR 677) was detected (Table 4-12).

MTHFR 677 (CT/TT) Compared to CC Genotype and Status Indicators in the sample population

Based on the findings from (Table 4-11), the data were further explored by comparing the status indicators between all subjects with either the CT or TT genotype for the MTHFR 677 C→T polymorphism to subjects with the CC genotype. There was no difference ($p > 0.05$) among status indicators between the two groups based on this method of comparison using the entire sample (Table 4-13).

MTRR 66 and TC 776 Genotypes and CHD Risk

The relationship of both MTRR 66 A→G polymorphism and the TC 776 C→G polymorphism and CHD risk was assessed and was not found to differ ($p > 0.05$). Number of subjects with the MTRR 66 GG genotype in the case group is lower than the control group (39, 48, respectively). To further characterize this relationship, the AA and AG genotype groups were compared to the double homozygous of the gene MTRR 66 GG genotype and a trend ($p = 0.0917$) for an association was detected.

For the TC 776 genotype groups, no association ($p > 0.05$) for any of the genotype groups and CHD risk was detected. Although not significant, the number of individuals with CG and GG genotypes tended to be higher in case compared to control groups (104, 92) and (47, 33),

respectively. The TC 776 CG and GG genotype groups were combined and case and control groups were compared, but no difference was detected ($p > 0.05$).

Part V: Relationship Between Polymorphisms Related to Folate and Vitamin B12 MTHFR 677 C→T, MTHFR 1298 A→C, MTRR 66 A→G, and TC 776 C→G and Status Indicators and CHD Risk (Objective 3)

The third hypothesis is that CHD risk associated with either folate and/or vitamin B12 related polymorphisms will be exacerbated by low folate and/or vitamin B12 status. Therefore, the third objective was to determine the interaction between maternal folate and vitamin B12 related polymorphisms and folate and vitamin B12 status indicators and infant CHD risk.

Status indicators and genotype relationship and CHD risk: The relationships among probable risk for CHD and status indicators for both folate and vitamin B12 and polymorphisms affecting the MTHFR, MTRR, and TC genes were assessed. The first model used was a stepwise procedure to evaluate all genotype groups and their first-order interactions. Only the MTHFR CT/TT combinations were associated with an increased risk of CHD ($p < 0.05$). No other polymorphism interactions were identified in the model. In the second model, a stepwise procedure was used to evaluate all status indicators and their first order interactions. At the ($\alpha = 0.05$) level of significance, no significant associations between all status indicators and the risk of CHD were detected. In the third model, both genotypes and status indicators were included. A step wise procedure was performed, and no association ($p > 0.05$) was ascertained for all status indicators and all genotypes which were removed from the model except for the MTHFR CT/TT genotypes group which was significant ($p < 0.05$).

Table 4-1. Demographic characteristics of sample population.

Demographic Variable	Case (n)	Control (n)	Total (n)	Missing	P-value
Ethnicity (%) ^b					0.2177
African American	10	9	19	NA	
Hispanic	17	21	38	NA	
White American	115	88	203	NA	
Other	5	10	15	NA	
Total (n)	147	128	275	115	
Maternal Age ^{a, c}	28.5 ± 0.65	27.7 ± 0.74			0.4166
Maternal Age Group ^b					0.4224
Less than 18	3	4	7	NA	
18-30	61	45	106	NA	
20-45	41	32	73	NA	
45 >	0	1	1	NA	
Total (n)	105	82	187	203	
Pregnancy Index (years) ^b					0.2852
< 3	35	27	62	NA	
3 – 5	13	6	19	NA	
>5	150	150	300	NA	
Total (n)	198	183	381	9	

^a One-way ANOVA was used for statistical comparisons between groups.

^b Chi-square test was used for statistical comparisons between groups.

^c Mean ± standard deviation (SD). NA: not applicable

Table 4-2. Distribution and response rates for prenatal use of folic acid in the sample population.

Supplement	Case	Control	Total (n)	P-value ^a
Prenatal				0.5579
Yes	117	98	215	
No	8	9	17	
NA ^b	-	-	104	
Folic Acid				0.2410
Yes	26	16	42	
No	114	105	219	
NA ^b	-	-	129	

^aChi-square test was used for statistical comparisons between groups. ^bData were missing (NA, not available).

Table 4-3. Distribution and response rates for cigarette and alcohol use in the sample population.

Factor	Case	Control	Total (n)
Cigarettes			
Yes	2	1	3
No	23	27	50
NA ^a	-	-	337
Alcohol			
Yes	1	0	1
No	24	27	51
NA ^a	-	-	338

^aData were missing (NA, not available).

Table 4-4. Distribution and response rates for diabetes, seizures, and anemia during pregnancy in the sample population.

Condition	Yes ^a	No ^b	Missing ^c	Total (n) ^d
Diabetes	6	48	336	390
Seizures	1	52	337	390
Anemia	12	41	337	390

^aNumber of subjects who answered with yes for the condition.

^bNumber of subjects who answered with no for the condition.

^cNumber of subjects who did not specify an answer for the condition.

^dTotal number of subjects enrolled in the study.

Table 4-5. Mean comparison of hematological indices between cases and controls in the sample population.^{a,b}

Index	Case	Control	Total (n)	P-value
Hgb (g/dl)	13.3 (13.1, 13.4)	13.3 (13.2, 13.5)	380	0.5643
MCV (fl)	87.9 (87.1, 88.7)	88.4 (87.6, 89.1)	380	0.4311
MCH (pg)	29.2 (28.9, 29.5)	29.4 (29.1, 29.7)	380	0.3674
MCHC (g/dl)	33.2 (33.0, 33.2)	33.3 (33.1, 33.4)	380	0.5003
Hct (%)	39.9 (39.5, 40.3)	40.7 (39.7, 40.5)	388	0.6325

^aMean (5%, 95% CI). ^bOne-way ANOVA was used for statistical comparisons between groups.

Table 4-6. Comparison of serum and RBC folate, Hcy, serum B12, and MMA concentrations by group (case/control) in the sample population.^a

Variable	Case	Control	Total (n)	P-value
Serum folate (nmol/L)	21.1 (19.3, 22.9)	21.1 (19.2, 23.0)	388	0.9384
RBC folate (nmol/L)	1447 (1328.2, 1565.8)	1447.3 (1324.5, 1570.1)	211	0.8233
Hcy (μM)	6.6 (5.9, 7.3)	7.3 (6.5, 8.1)	367	0.2555
Serum B12 (pmol/L)	367.7 (335.95, 399.45)	395.5 (362.3, 428.7)	390	0.2366
MMA (nM)	230.6 (213.5, 247.7)	222.5 (204.3, 240.7)	367	0.5255

^aMean (5%, 95% CI). One-way ANOVA was used for statistical comparisons between groups.

^bP-values were based on normalized (log transformed) data. The results have been back-transformed to original scale.

Table 4-7. Comparison of serum and RBC folate, Hcy, serum B12, and MMA concentrations by group (case/control) in the sub-sample population^{a, b, c}

Variable	Case (n = 35)	Control (n = 27)	P-value
Serum folate (nmol/L)	17.7 (14.8, 21.1)	24.2 (19.8, 29.8)	0.0258 ^d
RBC folate (nmol/L)	1254.6 (1044.1, 1507.6)	1418.3 (1123.0, 1791.1)	0.4246
Hcy (μM)	5.9 (5.4, 6.5)	5.4 (4.8, 6.0)	0.2060
Vitamin B12 (pmol/L)	307.3 (253.1, 372.9)	503.5 (403.8, 627.8)	0.0016 ^d
MMA (nM)	208.8 (185.2, 235.5)	189.8 (165.1, 218.1)	0.3113

^aSub-sample population is a category for all subjects who had pregnancy index of < 3 years.

^bMean (5%, 95% CI). One-way ANOVA was used for statistical comparisons between groups.

^cP-values were based on normalized (log transformed) data. The results have been back-transformed to original scale. ^dSignificantly lower than controls.

Table 4-8. Genotypes combination distribution in the sample population for specific polymorphisms.^a

TC 776/MTRR 66	MTHFR (677/1298)					
	CC/AA	CT/AA	TT/AA	CT/AC	CC/AC	CC/CC
CC/AA	8	10	6	3	7	5
CC/AG	7	10	8	9	3	2
CC/GG	2	7	1	5	7	2
CG/AA	8	16	8	10	8	5
CG/AG	14	28	14	11	28	3
CG/GG	10	5	1	12	12	2
GG/AA	4	8	1	5	5	3
GG/AG	3	8	5	3	7	4
GG/GG	None	9	4	5	3	1

^aNumbers indicate the occurrence of each genotype combination in the sample population according to their genetic makeup in the genes selected in this study (MTHFR, MTRR and TC).

Table 4-9. Frequency comparison of the MTHFR 677 C→T and 1298 A→C distribution in the sample population by group type (case/control).

MTHFR (677/1298)	Case (n = 204)	Control (n = 186)	Total (n = 387)	P-value ^a
CC-AA	25	33	58	0.3223
CT-AA	56	49	105	
TT-AA	28	20	48	
CC-AC	42	46	88	0.0609 ^b
CT-AC	39	24	63	
CC-CC	14	14	28	

^aChi-square test was used for statistical comparisons between genotype groups.

^bChi-square test was used to compare CT-AC genotypes between case and control.

Table 4-10. Frequency comparison of the MTHFR 677 genotype (CC, CT, TT) distribution by group type (case/control) in the sample population.

MTHFR 677	Case (n = 202)	Control (n = 185)	Total (n = 387)	P-value ^a
CC	79	92	171	0.1073
CT	95	78	168	
TT	28	20	48	

^aChi-square test was used for statistical comparisons between groups.

Table 4-11. Frequency comparison between MTHFR 677 CC genotype and the combined genotypes (CT/TT) for the MTHFR 677 C→T polymorphism by group type (case/control) in the sample population.

MTHFR 677	Case (n = 202)	Control (n = 185)	Total (n = 390)	P-value ^a
CC	79	92	171	0.0356
CT/ TT ^b	123	93	216	
Missing	-	-	3	

^aChi-square test was used for statistical comparisons between groups. Significantly higher frequency of CT/TT in cases compared to controls. ^bSubjects with either one of these genotypes were combined in one category.

Table 4-12. Frequency comparison of the MTHFR 1298 genotype (AA, AC, CC) distribution by group type (case/control) in the sample population.

MTHFR 1298	Case (n = 204)	Control (n = 186)	Total (n = 390)	P-value ^a
AA	109	101	210	0.8562
AC	81	70	151	
CC	14	15	29	

^aChi-square test was used for multiple statistical comparisons between groups.

Table 4-13. Comparison of serum and RBC folate, Hcy, serum B12, and MMA concentrations between the MTHFR 677 CC genotype and the combined (CT/TT) genotypes in the sample population.^{a, b}

MTHFR 677	Variable	Total (n)	P-value
CC CT/TT	Hcy (μM)	365	0.4781
	7.2 (6.4, 8.0) 6.7 (6.0, 7.4)		
CC CT/TT	MMA (nM)	362	0.4230
	232.7 (214.1, 251.3) 222.4 (205.5, 239.3)		
CC CT/TT	Serum B12 (pmol/L)	386	0.2113
	364.0 (329.3, 379.1) 394.0 (363.2, 424.8)		
CC CT/TT	Serum Folate (nmol/L)	384	0.6889
	20.7 (18.7, 22.7) 21.1 (19.3, 22.9)		
CC CT/TT	RBC Folate (nmol/L)	211	0.8526
	1466.6 (1347.0, 1585.6) 1450.3 (1326.6, 1574.0)		

^aMean (5%, 95% CI). One-way ANOVA was used for statistical comparisons between groups.

^bSubjects carrying either one of these two genotypes (CT/TT) were grouped together.

CHAPTER 5 DISCUSSION

The primary goal of this retrospective observational pilot study was to assess the roles of folate and vitamin B12 status and associated genetic polymorphisms on CHD risk and to provide a framework for future studies. The first objective of the retrospective pilot study was to assess the association between maternal folate and vitamin B12 status biomarkers on risk for having an infant with a CHD. An association between means of folate and vitamin B12 status indicators and CHD risk was not detected in this pilot study. A key issue to consider in evaluating this finding is whether the status at the time of the blood sampling for this study was similar to that when the fetal heart was developing. Since the average period of time that had elapsed from the time of delivery for both cases and controls was 11.8 years, it is likely that there may have been significant changes in folate and/or vitamin B12 status during this period of time due to changes in the diet or changes in the use of supplements. To address this issue, a small subset of women whose pregnancies occurred within three years of the time of the blood draw was evaluated. The case women in this subgroup were found to have significantly lower serum folate and B12 concentrations compared to that of control women. The sub-sample population used in this study, subjects with pregnancy index < 3 years proved more relevant to the study objectives related to the association of status indicators and CHD risk.

A more definitive answer related to this issue would be provided from a prospective study in which status indices were determined during the pregnancy in both case and controls. In a retrospective study conducted by Hobbs et al., folate and vitamin B12 status were evaluated using a shorter pregnancy index range. The median time between the end of pregnancy and the blood draw in the study was 24 months for controls and 14.9 months for cases, thus providing a narrower window of time for changes in folate and vitamin B12 status to occur (89,137).

Although, these investigators did not detect differences in folate and vitamin B12 status between case and control mothers, there were significant differences in related biomarkers including Hcy, SAH, methionine and SAM, which suggests that the metabolism of these two nutrients may be abnormal in case mothers (137). However, in the study by Hobbs et al., case subjects had a pregnancy index median of 14.9 months while control subjects had a pregnancy index median of 24 months which was addressed as a limitation in a study by Verkleij-Hagoort et al. (73). In the study by Verkleij-Hagoort et al., a range of ~17 months was used as a pregnancy index for both case and control subjects. Both studies, however, suggest a shorter pregnancy index to be more relevant to measure status indicators (73).

When evaluating the association between folic acid and vitamin B12 status and CHD risk, it is important to link potential differences in the use of supplements during gestation to birth outcomes so that conclusions can be drawn for public health recommendations. In a previous study conducted by Shaw et al., women who took multivitamins containing folic acid during critical periods of heart development were found to have a reduced risk of CHD (57). The strongest evidence that folate status is involved in CHD formation and prevention comes from a Hungarian RCT in which multivitamins containing folic acid reduced the risk of CHD up to 60% when taken during the periconceptional period (72). These results were confirmed in two population-based case-control studies in the US in which a 24% reduction in CHD risk was observed (55,57). In addition to the reduction in CHD risk associated with folic acid supplement use seen in these two studies, investigators from the Baltimore Washington Infant Study reported an inverse relationship between daily maternal intake of folic acid and the incidence of cardiac outflow tract defects in offspring (56).

In the present pilot study, information obtained from subjects regarding prenatal supplement use was limited in that information was missing from approximately one-third of the women which limited the power to detect a reduction in risk associated with folic acid and vitamin use during gestation. To address this issue in future investigations, detailed information regarding supplement use including specific brand name to obtain the exact nutrient composition and timing of the use of the supplement during pregnancy should be obtained. The ideal study design for an observational, non-intervention protocol is a prospective study in which information regarding supplement use prior to and during pregnancy is obtained and the outcome of pregnancy subsequently monitored. Another important source of folic acid for which information should be obtained in future studies are foods that are fortified with folic acid including ready-to-eat breakfast cereals that may provide daily folic acid doses that are comparable to supplements. No information was obtained regarding consumption of folic acid fortified products in the current pilot study and this limitation of the study design is shared by other previously conducted studies (138-141). Future investigations should incorporate the use of a detailed dietary intake questionnaire to allow for a quantitative estimate of daily folic acid intake during the index pregnancy.

The second objective of this pilot study was to assess the association between the MTHFR 677 C→T, 1298 A→C, MTRR 66 A→G and TC 776 C→G polymorphisms on maternal CHD risk. Detailed statistical analyses involving multiple comparisons were conducted to determine if there were any associations between specific genotypes associated with these polymorphisms and CHD risk. Initially, MTHFR 677 C→T genotypes proportions were compared to one another between cases and controls (CC, CT, and TT). There was a pattern for the T allele at the MTHFR 677 position in the case group but was not significant which warrants

further exploring. When the MTHFR 677 (CT/TT) genotype groups were combined in one group, a significantly higher number of cases with either of these genotypes were found compared to controls. Findings that are consistent with this observational study include those of Wenstrom et al. who reported that CHD risk was associated with the combined MTHFR 677 (CT/TT) genotypes compared to controls (79). Van Beynum et al. found that the MTHFR 677 (CT/TT) genotypes of the mother, when combined with no use of periconceptional folic acid supplements, increased the risk for CHDs in offspring (142).

Hobbs et al. reported that there was a higher risk for CHDs associated with the MTHFR 677 CC compared to the TT genotype, which is in contrast to the findings in the present pilot study and those of other investigators (89). A possible explanation for this inconsistency may be due to differences in folate status of the groups since a higher status is known to alleviate the metabolic abnormalities such as elevations in plasma homocysteine associated with this polymorphism (84). The results from Hobbs et al. have not been confirmed by other investigators and may be related to other risk factors that may not have been controlled for in that study. An elevation in plasma Hcy concentration is an established risk factor for CHD and may be associated with the MTHFR 677C →T polymorphism in women who delivered infants affected by CHD (79).

The findings in the present pilot study are consistent with the study by Junker et al. in which mothers carrying the MTHFR 677 TT genotype were found to be at significantly increased risk for the development of structural congenital heart malformations compared to mothers with the CC genotype (87). However, a meta-analysis by Verkleij-Hagoort et al. did not confirm that MTHFR 677 C→T polymorphism is independently associated with CHD risk (143). In addition, negative results were reported by other investigators concerning the association

between MTHFR 677 C→T polymorphism and CHD risk (71,88). The inconsistency of these findings regarding the MTHFR 677 C→T polymorphism might be due to the heterogeneity regarding population background, sample size, study design and the type of heart defects that complicates the pooling and comparison of the studies.

Only a few studies have investigated the combined effect of the MTHFR (677 C→T and 1298 A→C) polymorphism on CHD risk. The results of this study indicate that the MTHFR 1298 A→C polymorphism was not associated with an increased risk of CHD unless it was combined with the MTHFR 677 CT genotype group. Association between CHD and MTHFR 1298 AC was only observed when the MTHFR 1298 AC genotype group was combined with the MTHFR 677 CT genotype group. The frequency of MTHFR 1298 AC genotype was only higher in cases when it was combined with the MTHFR 677 CT genotype. The meta-analysis by Verkleij-Hagoort et al. concluded that the MTHFR polymorphisms 677 C→T and 1298 A→C in mothers are not independently associated with CHDs and further studies are needed (143).

To address the third objective, which was to determine the interaction between folate and vitamin B12 related polymorphisms and status indicators on maternal CHD risk, comparisons between all genotype groups for the polymorphisms and status indicators were compared and no differences were detected. Other investigations that have evaluated these associations include Van Beynum et al. who reported that the maternal MTRR 66 GG genotype in combination with high MMA concentration (above the 80th percentile) was associated with a 3-fold increased risk for all types of CHD in the offspring (81). Hobbs et al. did not find any of the biochemical indicators to be associated with increased CHD risk in conjunction with MTHFR C→T polymorphism except for Hcy, which was defined as an independent risk factor (89). Their results indicated that maternal MTHFR 677 C→T polymorphism did not have an independent

impact on the estimated risk of having a CHD-affected pregnancy. Their data also indicated that CHD risk in the group with the combined CT and TT genotypes increased only with elevated Hcy and smoking and that the CC genotype had a greater increase in risk compared to the CT and TT genotypes.

Vaughn et al. reported that MTRR 66 AA, AG and GG genotypes are associated with increased plasma Hcy concentration when combined with the MTHFR 677 TT genotype compared to other combinations of the MTHFR 677 C→T and 1298 A→G polymorphisms (93). Since the MTRR 66 GG genotype has previously been associated with elevated Hcy and elevation in Hcy has been shown to be associated with CHD in several studies, there is a plausible link between the presence of this polymorphism and CHD risk although an association was not observed in the present pilot study (93,144). Further analysis of the relationship between this polymorphism and the risk of CHD in this pilot study suggested a slightly lower incidence of MTRR 66 GG genotype in the cases (n=39) compared to the controls (n=45). When the genotype frequency distribution was evaluated, more individuals with the MTRR 66 AA and AG genotypes were observed in cases group and more controls with the GG genotype (p= 0.09) unlike what was observed and reported by Van Beynum et al. (81).

These findings suggest that MTRR 66 GG genotype may have a protective effect reducing CHD risk. A potential explanation for this observation is that the MTRR 66 GG genotype may impair the utilization of the methyl donor from the 5-MTHF resulting in less folate entering this irreversible pathway for making 5-MTHF, which would shunt this coenzyme toward the nucleotide and DNA synthesis part of the cycle. It is possible however, that these results are coincidental and that more controls by chance have the MTRR 66 GG genotype.

It is also important to consider that due to study design weaknesses it is not possible to thoroughly assess the interaction between genotype and folate/vitamin B12 status. Further studies need to be conducted to provide a careful assessment of status at the appropriate time during gestation or within a very short time following the index pregnancy to allow a more accurate measure of status indicators that are likely to interact with polymorphisms affecting folate and vitamin B12 status.

The primary purpose of this pilot study was to establish an infrastructure for future well-designed studies to determine the association between maternal folate and vitamin B12 status and related environmental and genetic factors on CHD risk. It is critical to identify the study limitations so that improvements can be made in the design of future large-scale studies to evaluate similar objectives.

One study limitation relates to pregnancy index, which is an important consideration when considering biochemical profile because status indicators can change over time in response to dietary and supplement intake. This retrospective study was designed to include women who had previous pregnancies that could be characterized as either case or control. No comprehensive exclusion criteria were used to select the subjects. As a result, subjects were recruited regardless of the period of time between the index pregnancy and the time the blood sample was drawn for analysis of folate, vitamin B12 and homocysteine concentrations. To determine the effect of folate and/or vitamin B12 status on risk of CHD during the index pregnancy, maternal blood samples must be collected in a timely manner following delivery of the affected infant (case) or normal infant (control) since changes in blood indices may change significantly following delivery of the infant due to factors including alterations in diet, supplement use, and drug use. For the majority of study participants, the index pregnancy

occurred more than five years prior to the time that the blood sample was drawn. Previous studies designed to assess the association between status indicators and CHD risk used pregnancy indexes of ~17 and 24 months from the time of delivery to the time of blood collection and ascertainment of information related to supplement use and environmental exposure (73,137).

Another limitation of not excluding subjects whose index pregnancies were > 3 years is the inability for participants to recall significant details associated with the pregnancy. In this study, the index pregnancy occurred > 5 years (average 11.8 years) from the time that the blood sample was drawn and questionnaire information was obtained. Since the data collected by questionnaire were highly dependent on memory recall, the long period of time between the index pregnancy and time of study introduced probable error and bias in the subjects' responses. For example, some of the questions included in the questionnaire requested at least one of the following: supplement brand, frequency of use, and dosages, questions that require very exact recall, which becomes increasingly difficult over time. For this reason the data obtained in this study may have been biased, inaccurate, and incomplete due to issues related to poor recall

Selection of the type of questions to ask is a critical part of any study. The questionnaire used in the study (Appendix A) was not developed to address the objectives of the study. The primary problems associated with the questionnaire include the fact that the questions were insufficient to obtain essential information required to address the study objectives and the fact that the data collection was incomplete. For example, questions related to medical conditions prior to pregnancy, drugs use, and supplement use had low response rates. In addition, some questions were inaccurately answered (e.g. a question was answered with a "yes" while the response requested was the supplement dose). The fact that the questionnaire was self-

conducted could have led to the low and inaccurate response rate. To obtain detailed and more accurate and detailed information, the answers to study questions should be obtained by trained personnel in an interview situation. Reviewing medical files is another way to ensure completeness and accuracy of answers, which is critical to obtaining valid results.

The collection of demographic and background information was a weakness of this study as the data collected for each subject were very limited. For example, no data were collected on marital status, educational level, employment status, weight and height (BMI), family and/or offspring history of congenital malformations, and number of prior pregnancies. The lack of these data limits the ability to describe the sample population and to either control for or determine the association of these factors with risk for CHD. Even though the questionnaire included questions about employment and father's ethnicity, data were not collected. Reviewing medical records and having trained personal ask the questions could have improved the response rate.

The data on supplement use both during the index pregnancy and at the time of blood collection were poorly asked and collected. Questions on supplement use are very critical in this type of study since supplements may have had a significant effect on folate and vitamin B12 status indicators. Large amounts of data regarding the prenatal and folic acid intake from supplements were missing. One factor that may have reduced the amount of data obtained related to prenatal supplement use was the length of time that had elapsed from the index pregnancy to the time of the study reducing the ability of the subjects to recall this information. The supplement questions requested information related to specific brands, the exact time of use during pregnancy, whether the subject switched the type of supplement used or not, and the name of the later brand. In addition, the categories provided as an answer to specify the

frequency of supplement use were very confusing (yes, no, infrequently, sometimes and routinely). This could be improved by limiting the number of categorical answers to yes, no, and sometimes and/or providing numerical values for each category for example: routinely can be described as (2-3 times a week).

Another weakness of this study was the fact that no data were obtained related to consumption of folate or vitamin B12 and folic acid or B12-fortified foods since dietary intake may have significantly impacted status. A primary objective of this study was to determine whether there was a relationship between low concentrations of blood folate and/or vitamin B12 and an increased risk for CHD, which makes it important to assess the intake of these nutrients from both supplements and dietary sources. Medical conditions are critical when assessing biochemical status and risk for congenital malformations since medical conditions and/or medications used to treat their conditions associations might explain and/or indicate unusual concentrations of biomarkers in the blood. However, in this study no data were collected on medical conditions prior to pregnancy, and only limited data were available during pregnancy

Cigarette and alcohol consumption are risk factors for various malformations (145), however, there were very low response rates regarding the use of substances. This is a limitation in the study because assessing risk of CHD requires comprehensive analysis of all possible elements, especially the ones with known risk associations.

Summary of Pilot Study Design Limitation

This pilot study presented an opportunity to identify key study design issues that can be addressed in future studies. Limitations included (1) "restricted exclusion criteria"; (2) "large pregnancy index range"; (3) "low response rate"; (4) "large number of questions that depended heavily on memory recall"; (5) "critical responses were missing"; (6) "self-administrated test"; (7) "inconclusive questionnaire"; and (8) "small sample size especially after adjusting for

pregnancy index.” Improvement for future studies should include an exclusion criterion for pregnancy index that limits the number of years between delivery and sample collection. A more detailed and comprehensive questionnaire that would include dietary intake, and drug and supplement use related questions should be administered to participants, which would minimize the number of unknown variables that may affect the outcomes of the study, thus enabling researchers to draw better conclusions related to the effects of both folate and vitamin B12 status on CHD risk. Trained personnel to supervise the administration of the questionnaire and collection of data would minimize errors and increase response rate in future studies. Last but not least, sample size must be considered especially in observational studies due to their nature and design complexity. In this study, the initial sample size was similar to other studies, but after correcting for pregnancy index, it became very small (73,138).

Public Health Significance of Research

The primary goal of this retrospective observational pilot study was to provide a framework for future studies in the area of folate and vitamin B12 and related genetic polymorphisms and CHD risk. Congenital heart defect-related studies that involve modifiable factors are critical to further minimize the limits surrounding the intervention efforts to reduce the occurrence of CHD. This initial pilot study provides data for future studies and preliminary findings to support future grant requests for funding large scale well-designed studies to investigate the association between folate and vitamin B12 in the etiology of CHDs. Future studies should correct for the limitations inherent in this pilot study to allow for more definitive results and conclusions to be drawn. Future studies are needed to advance our understanding of CHD etiology and the risk factors associated with it. Effective intervention studies designed to reduce the risk of CHD will depend on the outcome of future well-designed studies.

APPENDIX
QUESTIONNAIRE

Folic acid study: mother's questionnaire

(All answers will be kept strictly confidential)

Name: _____

Date of Birth: _____

Height: _____ Weight: _____

Child's name: _____ Date of Birth: _____

Reason child is seen by pediatric cardiologist: _____

Child's birth weight: _____ Full Term or Preterm

How many weeks: _____

Gestational age when born: _____

Any malformations or syndromes for this child? Y N if so, what?

Your ethnicity: African-American Asian Hispanic Native-American White

Other: _____

Father's occupation: _____

Please answer the following questions about your pregnancy with this child:

Any significant health problems prior to your pregnancy? Y N if so, what?

Did you take supplements prior to your pregnancy? Y N

Which ones? _____

How long did you take them prior to your pregnancy? _____

Did you take a prenatal vitamin? Y N Infrequently Sometimes Routinely

How far along into the pregnancy were you when you started taking the prenatal vitamins?

Brand name: _____

Did you take the same ones throughout the whole pregnancy? Y N _____

Did you switch to a different pre-natal vitamin during the pregnancy and which one:

Did you take additional folic acid? Y N Infrequently Sometimes Routinely

What was the dose? _____

Did you take other nutritional supplement or herbs? Y N

Which ones? _____

Did you develop diabetes during the pregnancy? Y N Was insulin used? Y N

Any seizures before or during your pregnancy? Y N if so, what were you treated with:

Did you develop anemia during your pregnancy? Y N if so, what were you treated with:

Please check if you took any of the following medications during your pregnancy:

__Aspirin

__Ibuprofen (Advil, Motrin)

__Anti-depressants: _____

__Anti-seizure medication: _____

__Diabetes medicine: _____

__Other: _____

How many cigarettes did you average per day during pregnancy? _____ packs/day

How much alcohol did you drink during pregnancy? _____

How much coffee/tea/chocolate/caffeinated soft drinks did you drink during your pregnancy? _____

Did you drink or eat products containing Aspartame (Nutrasweet, no sugar added or diet on the label)? Y N Infrequently Sometimes Routinely

Did you use any other artificial sweeteners? Y N Type: _____

Were you on a special diet during your pregnancy? Y N Type: _____

Family History:

Any family history of a heart defect from birth? Y N if so, what?

How many pregnancies have you had? _____

Number of miscarriages? _____

How many other children do you have? _____ Ages: _____

Congenital heart defects in your other children? _____

Any other malformations or syndromes for your other children? Y N if so, what?

Any health problems for your other children? _____

Are you planning to have more children? Y N Undecided

May we contact you in the future? Y N

Do you want to be contacted with your lab results? Y N

Phone no: _____

Thank you for your assistance!

LIST OF REFERENCES

1. Bailey, L. & Gregory, J. (2006) Folate. In: Present knowledge in nutrition, 9th ed. (Bowman, B. & Russell, R., eds.), pp. 278-301. ILSI Press, Washington, D.C.
2. Stokstad, E. L. R. (1990) Historical perspective on key advances in the biochemistry and physiology of folates. In: Folic acid metabolism in health and disease (Picciano, M. F., Stokstad, E. L. R., Gregory, J. F. & eds., eds.), pp. 1-15. Wiley-Liss, New York.
3. Carmel, R. & Jacobson, D. (2001) Homocysteine in Health and Disease. Cambridge University Press, Cambridge, UK.
4. Gregory, J. F., 3rd (1997) Bioavailability of folate. *Eur J Clin Nutr* 51 Suppl 1: S54-59.
5. Sauberlich, H. E., Kretsch, M. J., Skala, J. H., Johnson, H. L. & Taylor, P. C. (1987) Folate requirement and metabolism in nonpregnant women. *Am J Clin Nutr* 46: 1016-1028.
6. Pfeiffer, C. M., Rogers, L. M., Bailey, L. B. & Gregory, J. F., 3rd (1997) Absorption of folate from fortified cereal-grain products and of supplemental folate consumed with or without food determined by using a dual-label stable-isotope protocol. *Am J Clin Nutr* 66: 1388-1397.
7. Persad, V. L., Van den Hof, M. C., Dube, J. M. & Zimmer, P. (2002) Incidence of open neural tube defects in Nova Scotia after folic acid fortification. *Can Med Assoc J* 167: 241-245.
8. Lopez-Camelo, J. S., Orioli, I. M., da Graca Dutra, M., Nazer-Herrera, J., Rivera, N., Ojeda, M. E., Canessa, A., Wettig, E., Fontannaz, A. M. et al. (2005) Reduction of birth prevalence rates of neural tube defects after folic acid fortification in Chile. *Am J Med Genet A* 135: 120-125.
9. Botto, L. D. & Correa, A. (2003) Decreasing the burden of congenital heart anomalies: an epidemiologic evaluation of risk factors and survival. *Progress in Pediatric Cardiology* 18: 111-121.
10. Botto, L. D., Olney, R. S. & Erickson, J. D. (2004) Vitamin supplements and the risk for congenital anomalies other than neural tube defects. *Am J Med Genet C Semin Med Genet* 125C: 12-21.
11. Bailey, L. B. & Berry, R. J. (2005) Folic acid supplementation and the occurrence of congenital heart defects, orofacial clefts, multiple births, and miscarriage. *Am J Clin Nutr* 81: 1213S-1217.
12. Henderson, G. B. (1990) Folate-binding proteins. *Annu Rev Nutr* 10: 319-335.
13. Shane, B. (1989) Folylpolyglutamate synthesis and role in the regulation of one-carbon metabolism. *Vitam Horm* 45: 263-335.

14. Hoppner, K. & Lampi, B. (1980) Folate levels in human liver from autopsies in Canada. *Am J Clin Nutr* 33: 862-864.
15. Scott, J. M. (1986) Catabolism of folates. In: *Folates and pterins* (Blakley, R. L., Whitehead, V. M. & eds., eds.), pp. 307-327. John Wiley & Sons, New York.
16. Whitehead, V. M. (1986) Pharmacokinetics and physiological disposition of folate and its derivatives. In: *Folates and pterins* (Blakley, R. L., Whitehead, V. M. & eds., eds.), pp. 177-205. John Wiley & Sons, New York.
17. Klee, G. G. (2000) Cobalamin and Folate Evaluation: Measurement of Methylmalonic Acid and Homocysteine vs Vitamin B12 and Folate. *Clin Chem* 46: 1277-1283.
18. Stabler, S. P. (2001) Vitamin B12. In: *Present Knowledge in Nutrition* (Bowman, B. A., Russell, R. M. & eds., eds.), pp. 230-240. ILSI Press, Washington, D.C.
19. Watanabe F, T. S., Kittaka-Katsura H, Ebara S, Miyamoto E (2002) Characterization and bioavailability of vitamin B12-compounds from edible algae. *J Nutr Sci Vitaminol (Tokyo)* 48: 325-331.
20. Watanabe, F. (2007) Vitamin B12 Sources and Bioavailability. *Experimental Biology and Medicine* 232: 1266-1274.
21. Festen, H. P. (1991) Intrinsic factor secretion and cobalamin absorption. *Physiology and pathophysiology in the gastrointestinal tract. Scand J Gastroenterol Suppl* 188: 1-7.
22. Quadros, E. V., Regec, A. L., Khan, K. M., Quadros, E. & Rothenberg, S. P. (1999) Transcobalamin II synthesized in the intestinal villi facilitates transfer of cobalamin to the portal blood. *Am J Physiol* 277: G161-166.
23. Allen, R. H. (1976) The plasma transport of vitamin B12. *Br J Haematol* 33: 161-171.
24. Hase, Y. (1998) [Disorders of transsulfuration; disorders of sulfur-aminoacids metabolism]. *Ryoikibetsu Shokogun Shirizu*: 205-218.
25. Leclerc, D., Wilson, A., Dumas, R., Gafuik, C., Song, D., Watkins, D., Heng, H. H., Rommens, J. M., Scherer, S. W. et al. (1998) Cloning and mapping of a cDNA for methionine synthase reductase, a flavoprotein defective in patients with homocystinuria. *Proc Natl Acad Sci U S A* 95: 3059-3064.
26. Wilson, A., Platt, R., Wu, Q., Leclerc, D., Christensen, B., Yang, H., Gravel, R. A. & Rozen, R. (1999) A common variant in methionine synthase reductase combined with low cobalamin (vitamin B12) increases risk for spina bifida. *Mol Genet Metab* 67: 317-323.
27. Stabler, S., Lindenbaum, J., Savage, D. & Allen, R. (1993) Elevation of serum cystathionine levels in patients with cobalamin and folate deficiency. *Blood* 81: 3404-3413.

28. Lissak, A., Sharon, A., Fruchter, O., Kassel, A., Sanderovitz, J. & Abramovici, H. (1999) Polymorphism for mutation of cytosine to thymine at location 677 in the methylenetetrahydrofolate reductase gene is associated with recurrent early fetal loss. *Am J Obstet Gynecol* 181: 126-130.
29. Frosst, P., Blom, H. J., Milos, R., Goyette, P., Sheppard, C. A., Matthews, R. G., Boers, G. J., den Heijer, M., Kluijtmans, L. A. et al. (1995) A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet* 10: 111-113.
30. van der Put, N. M., Gabreels, F., Stevens, E. M., Smeitink, J. A., Trijbels, F. J., Eskes, T. K., van den Heuvel, L. P. & Blom, H. J. (1998) A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube defects? *Am J Hum Genet* 62: 1044-1051.
31. Weisberg, I., Tran, P., Christensen, B., Sibani, S. & Rozen, R. (1998) A Second Genetic Polymorphism in Methylenetetrahydrofolate Reductase (MTHFR) Associated with Decreased Enzyme Activity. *Molecular Genetics and Metabolism* 64: 169-172.
32. Gaughan, D. J., Kluijtmans, L. A. J., Barbaux, S., McMaster, D., Young, I. S., Yarnell, J. W. G., Evans, A. & Whitehead, A. S. (2001) The methionine synthase reductase (MTRR) A66G polymorphism is a novel genetic determinant of plasma homocysteine concentrations. *Atherosclerosis* 157: 451-456.
33. Carmel, R. (1985) The distribution of endogenous cobalamin among cobalamin-binding proteins in the blood in normal and abnormal states. *Am J Clin Nutr* 41: 713-719.
34. von Castel-Dunwoody, K. M., Kauwell, G. P., Shelnutt, K. P., Vaughn, J. D., Griffin, E. R., Maneval, D. R., Theriaque, D. W. & Bailey, L. B. (2005) Transcobalamin 776C->G polymorphism negatively affects vitamin B-12 metabolism. *Am J Clin Nutr* 81: 1436-1441.
35. Afman, L. A., Van Der Put, N. M. J., Thomas, C. M. G., Trijbels, J. M. F. & Blom, H. J. (2001) Reduced vitamin B12 binding by transcobalamin II increases the risk of neural tube defects. *QJM* 94: 159-166.
36. Miller, J. W., Ramos, M. I., Garrod, M. G., Flynn, M. A. & Green, R. (2002) Transcobalamin II 775G>C polymorphism and indices of vitamin B12 status in healthy older adults. *Blood* 100: 718-720.
37. McCaddon, A., Blennow, K., Hudson, P., Regland, B., Hill, D., Namour, F. & Gueant, J.-L. (2001) Transcobalamin polymorphism and homocysteine. *Blood* 98: 3497-3500.
38. von Castel-Dunwoody, K. M., Kauwell, G. P., Shelnutt, K. P., Vaughn, J. D., Griffin, E. R., Maneval, D. R., Theriaque, D. W. & Bailey, L. B. (2005) Transcobalamin 776C->G polymorphism negatively affects vitamin B-12 metabolism. *Am J Clin Nutr* 81: 1436-1441.

39. Ulrich, C. M. & Potter, J. D. (2006) Folate Supplementation: Too Much of a Good Thing? *Cancer Epidemiol Biomarkers Prev* 15: 189-193.
40. Yates, A. A., Schlicker, S. A. & Sutor, C. W. (1998) Dietary Reference Intakes: The New Basis for Recommendations for Calcium and Related Nutrients, B Vitamins, and Choline. *Journal of the American Dietetic Association* 98: 699-706.
41. Sutor, C. W. & Bailey, L. B. (2000) Dietary folate equivalents: interpretation and application. *J Am Diet Assoc* 100: 88-94.
42. Carmel, R. (1997) Cobalamin, the stomach, and aging. *Am J Clin Nutr* 66: 750-759.
43. Verkleij-Hagoort, A. C., de Vries, J. H., Ursem, N. T., de Jonge, R., Hop, W. C. & Steegers-Theunissen, R. P. (2006) Dietary intake of B-vitamins in mothers born a child with a congenital heart defect. *Eur J Nutr* 45: 478-486.
44. Anderson, R. N. (2002) Deaths: leading causes for 2000. *Natl Vital Stat Rep* 50: 1-85.
45. Rosano, A., Botto, L. D., Botting, B. & Mastroiacovo, P. (2000) Infant mortality and congenital anomalies from 1950 to 1994: an international perspective. *J Epidemiol Community Health* 54: 660-666.
46. Bekker, M. N., van den Akker, N. M., Bartelings, M. M., Arkesteijn, J. B., Fischer, S. G., Polman, J. A., Haak, M. C., Webb, S., Poelmann, R. E. et al. (2006) Nuchal edema and venous-lymphatic phenotype disturbance in human fetuses and mouse embryos with aneuploidy. *J Soc Gynecol Investig* 13: 209-216.
47. Fisher, S. A., Langille, B. L. & Srivastava, D. (2000) Apoptosis during cardiovascular development. *Circ Res* 87: 856-864.
48. Gittenberger-de Groot, A. C., Bartelings, M. M., Deruiter, M. C. & Poelmann, R. E. (2005) Basics of cardiac development for the understanding of congenital heart malformations. *Pediatr Res* 57: 169-176.
49. van der Linden, I. J., Afman, L. A., Heil, S. G. & Blom, H. J. (2006) Genetic variation in genes of folate metabolism and neural-tube defect risk. *Proc Nutr Soc* 65: 204-215.
50. Pulikkunnel, S. T. & Thomas, S. V. (2005) Neural tube defects: pathogenesis and folate metabolism. *J Assoc Physicians India* 53: 127-135.
51. Liu, S., West, R., Randell, E., Longerich, L., O'Connor K, S., Scott, H., Crowley, M., Lam, A., Prabhakaran, V. & McCourt, C. (2004) A comprehensive evaluation of food fortification with folic acid for the primary prevention of neural tube defects. *BMC Pregnancy Childbirth* 4: 20.

52. Ray, J. G. & Blom, H. J. (2003) Vitamin B12 insufficiency and the risk of fetal neural tube defects. *QJM* 96: 289-295.
53. Steen, M. T., Boddie, A. M., Fisher, A. J., Macmahon, W., Saxe, D., Sullivan, K. M., Dembure, P. P. & Elsas, L. J. (1998) Neural-tube defects are associated with low concentrations of cobalamin (vitamin B12) in amniotic fluid. *Prenat Diagn* 18: 545-555.
54. Czeizel, A. E. (1998) Periconceptional folic acid containing multivitamin supplementation. *Eur J Obstet Gynecol Reprod Biol* 78: 151-161.
55. Botto, L. D., Mulinare, J. & Erickson, J. D. (2000) Occurrence of congenital heart defects in relation to maternal multivitamin use. *Am J Epidemiol* 151: 878-884.
56. Scanlon, K. S., Ferencz, C., Loffredo, C. A., Wilson, P. D., Correa-Villasenor, A., Khoury, M. J. & Willett, W. C. (1998) Preconceptional folate intake and malformations of the cardiac outflow tract. Baltimore-Washington Infant Study Group. *Epidemiology* 9: 95-98.
57. Shaw, G. M., O'Malley, C. D., Wasserman, C. R., Tolarova, M. M. & Lammer, E. J. (1995) Maternal periconceptional use of multivitamins and reduced risk for conotruncal heart defects and limb deficiencies among offspring. *Am J Med Genet* 59: 536-545.
58. Jenkins, K. J., Correa, A., Feinstein, J. A., Botto, L., Britt, A. E., Daniels, S. R., Elixson, M., Warnes, C. A. & Webb, C. L. (2007) Noninherited risk factors and congenital cardiovascular defects: current knowledge: a scientific statement from the American Heart Association Council on Cardiovascular Disease in the Young: endorsed by the American Academy of Pediatrics. *Circulation* 115: 2995-3014.
59. Werler, M. M., Hayes, C., Louik, C., Shapiro, S. & Mitchell, A. A. (1999) Multivitamin supplementation and risk of birth defects. *Am J Epidemiol* 150: 675-682.
60. Scott, J. M., Weir, D. G., Molloy, A., McPartlin, J., Daly, L. & Kirke, P. (1994) Folic acid metabolism and mechanisms of neural tube defects. *Ciba Found Symp* 181: 180-187; discussion 187-191.
61. Stoller, J. Z. & Epstein, J. A. (2005) Cardiac neural crest. *Seminars in Cell & Developmental Biology* 16: 704-715.
62. Botto, L. D., Mulinare, J. & Erickson, J. D. (2003) Do multivitamin or folic acid supplements reduce the risk for congenital heart defects? Evidence and gaps. *Am J Med Genet* 121A: 95-101.
63. Baird, C. D., Nelson, M. M., Monie, I. W. & Evans, H. M. (1954) Congenital cardiovascular anomalies induced by pteroylglutamic acid deficiency during gestation in the rat. *Circ Res* 2: 544-554.
64. Eskes, T. K. & Steegers-Theunissen, R. P. (1994) Primary prevention of neural-tube defects with folic acid. *Eur J Obstet Gynecol Reprod Biol* 53: 147-152.

65. Lucock, M. D., Wild, J., Schorah, C. J., Levene, M. I. & Hartley, R. (1994) The methylfolate axis in neural tube defects: in vitro characterisation and clinical investigation. *Biochem Med Metab Biol* 52: 101-114.
66. Milunsky, A., Jick, H., Jick, S. S., Bruell, C. L., MacLaughlin, D. S., Rothman, K. J. & Willett, W. (1989) Multivitamin/folic acid supplementation in early pregnancy reduces the prevalence of neural tube defects. *Jama* 262: 2847-2852.
67. Gunther, T., Struwe, M., Aguzzi, A. & Schughart, K. (1994) Open brain, a new mouse mutant with severe neural tube defects, shows altered gene expression patterns in the developing spinal cord. *Development* 120: 3119-3130.
68. Nelson, M. M., Asling, C. W. & Evans, H. M. (1952) Production of multiple congenital abnormalities in young by maternal pteroylglutamic acid deficiency during gestation. *J Nutr* 48: 61-79.
69. Li, D. & Rozen, R. (2006) Maternal folate deficiency affects proliferation, but not apoptosis, in embryonic mouse heart. *J Nutr* 136: 1774-1778.
70. Burgoon, J. M., Selhub, J., Nadeau, M. & Sadler, T. W. (2002) Investigation of the effects of folate deficiency on embryonic development through the establishment of a folate deficient mouse model. *Teratology* 65: 219-227.
71. Hobbs, C. A., Cleves, M. A., Melnyk, S., Zhao, W. & James, S. J. (2005) Congenital heart defects and abnormal maternal biomarkers of methionine and homocysteine metabolism. *Am J Clin Nutr* 81: 147-153.
72. Czeizel, A. E. & Dudas, I. (1992) Prevention of the first occurrence of neural-tube defects by periconceptional vitamin supplementation. *N Engl J Med* 327: 1832-1835.
73. Verkleij-Hagoort, A. C., Verlinde, M., Ursem, N. T., Lindemans, J., Helbing, W. A., Ottenkamp, J., Siebel, F. M., Gittenberger-de Groot, A. C., de Jonge, R. et al. (2006) Maternal hyperhomocysteinaemia is a risk factor for congenital heart disease. *Bjog* 113: 1412-1418.
74. Stabler, S. P., Marcell, P. D., Podell, E. R., Allen, R. H., Savage, D. G. & Lindenbaum, J. (1988) Elevation of total homocysteine in the serum of patients with cobalamin or folate deficiency detected by capillary gas chromatography-mass spectrometry. *J Clin Invest* 81: 466-474.
75. Brattstrom, L., Israelsson, B., Lindgarde, F. & Hultberg, B. (1988) Higher total plasma homocysteine in vitamin B12 deficiency than in heterozygosity for homocystinuria due to cystathionine beta-synthase deficiency. *Metabolism* 37: 175-178.

76. Savage, D. G., Lindenbaum, J., Stabler, S. P. & Allen, R. H. (1994) Sensitivity of serum methylmalonic acid and total homocysteine determinations for diagnosing cobalamin and folate deficiencies. *Am J Med* 96: 239-246.
77. Groenen, P. M. W., van Rooij, I. A. L. M., Peer, P. G. M., Gooskens, R. H., Zielhuis, G. A. & Steegers-Theunissen, R. P. M. (2004) Marginal maternal vitamin B12 status increases the risk of offspring with spina bifida. *American Journal of Obstetrics and Gynecology* 191: 11-17.
78. Verkleij-Hagoort, A. C., Driel, L. M. J. W. v., Lindemans, J., Isaacs, A., Steegers, E. A. P., Helbing, W. A., Uitterlinden, A. G. & Steegers-Theunissen, R. P. M. Genetic and lifestyle risk factors related to the periconceptional vitamin B12 status and congenital heart defects: a Dutch case-control study.
79. Wenstrom, K. D., Johanning, G. L., Johnston, K. E. & DuBard, M. (2001) Association of the C677T methylenetetrahydrofolate reductase mutation and elevated homocysteine levels with congenital cardiac malformations. *Am J Obstet Gynecol* 184: 806-812; discussion 812-807.
80. van Beynum, I. M., Kapusta, L., den Heijer, M., Vermeulen, S. H., Kouwenberg, M., Daniels, O. & Blom, H. J. (2006) Maternal MTHFR 677C>T is a risk factor for congenital heart defects: effect modification by periconceptional folate supplementation. *Eur Heart J* 27: 981-987.
81. van Beynum, I. M., Kouwenberg, M., Kapusta, L., den Heijer, M., van der Linden, I. J., Daniels, O. & Blom, H. J. (2006) MTRR 66A>G polymorphism in relation to congenital heart defects. *Clin Chem Lab Med* 44: 1317-1323.
82. Pierpont, M. E., Basson, C. T., Benson, D. W., Jr., Gelb, B. D., Giglia, T. M., Goldmuntz, E., McGee, G., Sable, C. A., Srivastava, D. & Webb, C. L. (2007) Genetic basis for congenital heart defects: current knowledge: a scientific statement from the American Heart Association Congenital Cardiac Defects Committee, Council on Cardiovascular Disease in the Young: endorsed by the American Academy of Pediatrics. *Circulation* 115: 3015-3038.
83. Frosst, P., Blom, H. J., Milos, R., Goyette, P., Sheppard, C. A., Matthews, R. G., Boers, G. J., den Heijer, M., Kluijtmans, L. A. et al. (1995) A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet* 10: 111-113.
84. Jacques, P. F., Bostom, A. G., Williams, R. R., Ellison, R. C., Eckfeldt, J. H., Rosenberg, I. H., Selhub, J. & Rozen, R. (1996) Relation between folate status, a common mutation in methylenetetrahydrofolate reductase, and plasma homocysteine concentrations. *Circulation* 93: 7-9.
85. Anonymous (1996) Relation between folate status, a common mutation in methylenetetrahydrofolate reductase, and plasma homocysteine concentrations. *Circulation* 93: 7-9.

86. Kauwell, G. P., Wilsky, C. E., Cerda, J. J., Herrlinger-Garcia, K., Hutson, A. D., Theriaque, D. W., Boddie, A., Rampersaud, G. C. & Bailey, L. B. (2000) Methylenetetrahydrofolate reductase mutation (677C-->T) negatively influences plasma homocysteine response to marginal folate intake in elderly women. *Metabolism* 49: 1440-1443.
87. Junker, R., Kotthoff, S., Vielhaber, H., Halimeh, S., Kosch, A., Koch, H.-G., Kassenbohmer, R., Heineking, B. & Nowak-Gottl, U. (2001) Infant methylenetetrahydrofolate reductase 677TT genotype is a risk factor for congenital heart disease. *Cardiovascular Research* 51: 251-254.
88. Storti, S., Vittorini, S., Lascone, M. R., Sacchelli, M., Collavoli, A., Ripoli, A., Cocchi, G., Biagini, A. & Clerico, A. (2003) Association between 5,10-methylenetetrahydrofolate reductase C677T and A1298C polymorphisms and conotruncal heart defects. *Clin Chem Lab Med* 41: 276-280.
89. Hobbs, C. A., James, S. J., Jernigan, S., Melnyk, S., Lu, Y., Malik, S. & Cleves, M. A. (2006) Congenital heart defects, maternal homocysteine, smoking, and the 677 C>T polymorphism in the methylenetetrahydrofolate reductase gene: Evaluating gene-environment interactions. *American Journal of Obstetrics and Gynecology* 194: 218-224.
90. van Beynum, I. M., den Heijer, M., Blom, H. J. & Kapusta, L. (2007) The MTHFR 677C->T polymorphism and the risk of congenital heart defects: a literature review and meta-analysis. *QJM: hcm094*.
91. van der Put, N. M., Gabreels, F., Stevens, E. M., Smeitink, J. A., Trijbels, F. J., Eskes, T. K., van den Heuvel, L. P. & Blom, H. J. (1998) A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube defects? *Am J Hum Genet* 62: 1044-1051.
92. Engbersen, A. M., Franken, D. G., Boers, G. H., Stevens, E. M., Trijbels, F. J. & Blom, H. J. (1995) Thermolabile 5,10-methylenetetrahydrofolate reductase as a cause of mild hyperhomocysteinemia. *Am J Hum Genet* 56: 142-150.
93. Vaughn, J. D., Bailey, L. B., Shelnut, K. P., Dunwoody, K. M., Maneval, D. R., Davis, S. R., Quinlivan, E. P., Gregory, J. F., 3rd, Theriaque, D. W. & Kauwell, G. P. (2004) Methionine synthase reductase 66A->G polymorphism is associated with increased plasma homocysteine concentration when combined with the homozygous methylenetetrahydrofolate reductase 677C->T variant. *J Nutr* 134: 2985-2990.
94. Shotelersuk, V., Ittiwut, C., Siriwan, P. & Angspatt, A. (2003) Maternal 677CT/1298AC genotype of the MTHFR gene as a risk factor for cleft lip. *J Med Genet* 40: e64.
95. Zhu, H., Wicker, N. J., Shaw, G. M., Lammer, E. J., Hendricks, K., Suarez, L., Canfield, M. & Finnell, R. H. (2003) Homocysteine remethylation enzyme polymorphisms and increased risks for neural tube defects. *Molecular Genetics and Metabolism* 78: 216-221.

96. Miller, J. W., Ramos, M. I., Garrod, M. G., Flynn, M. A. & Green, R. (2002) Transcobalamin II 775G>C polymorphism and indices of vitamin B12 status in healthy older adults. *Blood* 100: 718-720.
97. Afman, L. A., Lievers, K. J., van der Put, N. M., Trijbels, F. J. & Blom, H. J. (2002) Single nucleotide polymorphisms in the transcobalamin gene: relationship with transcobalamin concentrations and risk for neural tube defects. *Eur J Hum Genet* 10: 433-438.
98. Wilson, P. D., Loffredo, C. A., Correa-Villasenor, A. & Ferencz, C. (1998) Attributable fraction for cardiac malformations. *Am J Epidemiol* 148: 414-423.
99. Kallen, K. (1999) Maternal smoking and congenital heart defects. *Eur J Epidemiol* 15: 731-737.
100. Torfs, C. P. & Christianson, R. E. (1999) Maternal risk factors and major associated defects in infants with Down syndrome. *Epidemiology* 10: 264-270.
101. Woods, S. E. & Raju, U. (2001) Maternal smoking and the risk of congenital birth defects: a cohort study. *J Am Board Fam Pract* 14: 330-334.
102. Correa-Villasenor, A., Ferencz, C., Loffredo, C. & Magee, C. (1993) Paternal exposures and cardiovascular malformations. The Baltimore-Washington Infant Study Group. *J Expo Anal Environ Epidemiol* 3 Suppl 1: 173-185.
103. Martinez-Frias, M. L., Bermejo, E., Rodriguez-Pinilla, E. & Frias, J. L. (2004) Risk for congenital anomalies associated with different sporadic and daily doses of alcohol consumption during pregnancy: a case-control study. *Birth Defects Res A Clin Mol Teratol* 70: 194-200.
104. Watkins, M. L. & Botto, L. D. (2001) Maternal prepregnancy weight and congenital heart defects in offspring. *Epidemiology* 12: 439-446.
105. Shaw, G. M., Todoroff, K., Schaffer, D. M. & Selvin, S. (2000) Maternal height and prepregnancy body mass index as risk factors for selected congenital anomalies. *Paediatr Perinat Epidemiol* 14: 234-239.
106. Waller, D. K., Mills, J. L., Simpson, J. L., Cunningham, G. C., Conley, M. R., Lassman, M. R. & Rhoads, G. G. (1994) Are obese women at higher risk for producing malformed offspring? *Am J Obstet Gynecol* 170: 541-548.
107. Mikhail, L. N., Walker, C. K. & Mittendorf, R. (2002) Association between maternal obesity and fetal cardiac malformations in African Americans. *J Natl Med Assoc* 94: 695-700.
108. Tikkanen, J. & Heinonen, O. P. (1991) Maternal hyperthermia during pregnancy and cardiovascular malformations in the offspring. *Eur J Epidemiol* 7: 628-635.

109. Zhang, J. & Cai, W. W. (1993) Association of the common cold in the first trimester of pregnancy with birth defects. *Pediatrics* 92: 559-563.
110. Botto, L. D., Lynberg, M. C. & Erickson, J. D. (2001) Congenital heart defects, maternal febrile illness, and multivitamin use: a population-based study. *Epidemiology* 12: 485-490.
111. Janssen, P. A., Rothman, I. & Schwartz, S. M. (1996) Congenital malformations in newborns of women with established and gestational diabetes in Washington State, 1984-91. *Paediatr Perinat Epidemiol* 10: 52-63.
112. Wren, C., Birrell, G. & Hawthorne, G. (2003) Cardiovascular malformations in infants of diabetic mothers. *Heart* 89: 1217-1220.
113. Sharpe, P. B., Chan, A., Haan, E. A. & Hiller, J. E. (2005) Maternal diabetes and congenital anomalies in South Australia 1986-2000: a population-based cohort study. *Birth Defects Res A Clin Mol Teratol* 73: 605-611.
114. Bower, C., Stanley, F., Connell, A. F., Gent, C. R. & Massey, M. S. (1992) Birth defects in the infants of aboriginal and non-aboriginal mothers with diabetes in Western Australia. *Med J Aust* 156: 520-524.
115. Ferencz, C., Correa-Villasenor A Loffredo CA, eds. (1997) Genetic and Enviromental Risk Factors of Major Cardiovascular MalformationsL The Baltimore-Washington Infant Study: 1981-1989. Futura Publishing Co, Armonk, NY.
116. Correa-Villasenor, A., McCarter, R., Downing, J. & Ferencz, C. (1991) White-black differences in cardiovascular malformations in infancy and socioeconomic factors. The Baltimore-Washington Infant Study Group. *Am J Epidemiol* 134: 393-402.
117. Hernandez, F. A., Miller, R. H. & Schiebler, G. L. (1969) Rarity of coarctation of the aorta in the American Negro. *J Pediatr* 74: 623-625.
118. Carmichael, S. L., Nelson, V., Shaw, G. M., Wasserman, C. R. & Croen, L. A. (2003) Socio-economic status and risk of conotruncal heart defects and orofacial clefts. *Paediatr Perinat Epidemiol* 17: 264-271.
119. Herbert, J., Wilcox, J. N., Pham, K. T., Fremeau, R. T., Jr., Zeviani, M., Dwork, A., Soprano, D. R., Makover, A., Goodman, D. S. et al. (1986) Transthyretin: a choroid plexus-specific transport protein in human brain. The 1986 S. Weir Mitchell award. *Neurology* 36: 900-911.
120. Herbert, V. & Zalusky, R. (1962) Interrelations of vitamin B12 and folic acid metabolism: folic acid clearance studies. *J Clin Invest* 41: 1263-1276.
121. Sauberlich, H. E., Dowdy, R. P. & Skala, J. H. (1973) Laboratory tests for the assessment of nutritional status. *CRC Crit Rev Clin Lab Sci* 4: 215-340.

122. Fazili, Z., Pfeiffer, C. M. & Zhang, M. (2007) Comparison of serum folate species analyzed by LC-MS/MS with total folate measured by microbiologic assay and Bio-Rad radioassay. *Clin Chem* 53: 781-784.
123. Choumenkovitch, S. F., Jacques, P. F., Nadeau, M. R., Wilson, P. W. F., Rosenberg, I. H. & Selhub, J. (2001) Folic Acid Fortification Increases Red Blood Cell Folate Concentrations in the Framingham Study. *J. Nutr.* 131: 3277-3280.
124. Wright, J. D., Bialostosky, K., Gunter, E. W., Carroll, M. D., Najjar, M. F., Bowman, B. A. & Johnson, C. L. (1998) Blood folate and vitamin B12: United States, 1988-94. *Vital Health Stat* 11: 1-78.
125. Rasmussen, K. & Nathan, E. (1990) The clinical evaluation of cobalamin deficiency by determination of methylmalonic acid in serum or urine is not invalidated by the presence of heterozygous methylmalonic-acidaemia. *J Clin Chem Clin Biochem* 28: 419-421.
126. Allen, R. H., Stabler, S. P., Savage, D. G. & Lindenbaum, J. (1990) Diagnosis of cobalamin deficiency I: usefulness of serum methylmalonic acid and total homocysteine concentrations. *Am J Hematol* 34: 90-98.
127. Honein, M. A., Paulozzi, L. J., Mathews, T. J., Erickson, J. D. & Wong, L. Y. (2001) Impact of folic acid fortification of the US food supply on the occurrence of neural tube defects. *JAMA* 285: 2981-2986.
128. Hoey, L., McNulty, H., Askin, N., Dunne, A., Ward, M., Pentieva, K., Strain, J. J., Molloy, A. M., Flynn, C. A. & Scott, J. M. (2007) Effect of a voluntary food fortification policy on folate, related B vitamin status, and homocysteine in healthy adults. *Am J Clin Nutr* 86: 1405-1413.
129. Ganji, V. & Kafai, M. R. (2006) Trends in Serum Folate, RBC Folate, and Circulating Total Homocysteine Concentrations in the United States: Analysis of Data from National Health and Nutrition Examination Surveys, 1988-1994, 1999-2000, and 2001-2002. *J. Nutr.* 136: 153-158.
130. Jacques, P. F., Selhub, J., Bostom, A. G., Wilson, P. W. & Rosenberg, I. H. (1999) The effect of folic acid fortification on plasma folate and total homocysteine concentrations. *N Engl J Med* 340: 1449-1454.
131. Kapusta, L., Haagmans, M. L., Steegers, E. A., Cuyper, M. H., Blom, H. J. & Eskes, T. K. (1999) Congenital heart defects and maternal derangement of homocysteine metabolism. *J Pediatr* 135: 773-774.
132. Perna, A. F., Ingrosso, D. & De Santo, N. G. (2003) Homocysteine and oxidative stress. *Amino Acids* 25: 409-417.

133. Tamura, T., Freeberg, L. E. & Cornwell, P. E. (1990) Inhibition of EDTA of growth of *Lactobacillus casei* in the folate microbiological assay and its reversal by added manganese or iron. *Clin Chem* 36: 1993.
134. Horne, D. W. & Patterson, D. (1988) *Lactobacillus casei* microbiological assay of folic acid derivatives in 96-well microtiter plates. *Clin Chem* 34: 2357-2359.
135. Stabler, S. P., Marcell, P. D., Podell, E. R. & Allen, R. H. (1987) Quantitation of total homocysteine, total cysteine, and methionine in normal serum and urine using capillary gas chromatography-mass spectrometry. *Analytical Biochemistry* 162: 185-196.
136. Marcell, P. D., Stabler, S. P., Podell, E. R. & Allen, R. H. (1985) Quantitation of methylmalonic acid and other dicarboxylic acids in normal serum and urine using capillary gas chromatography-mass spectrometry. *Anal Biochem* 150: 58-66.
137. Hobbs, C. A., Cleves, M. A., Melnyk, S., Zhao, W. & James, S. J. (2005) Congenital heart defects and abnormal maternal biomarkers of methionine and homocysteine metabolism. *Am J Clin Nutr* 81: 147-153.
138. Storti, S., Vittorini, S., Iascone, M. R., Sacchelli, M., Collavoli, A., Ripoli, A., Cocchi, G., Biagini, A. & Clerico, A. (2003) Association between 5,10-Methylenetetrahydrofolate Reductase C677T and A1298C Polymorphisms and Conotruncal Heart Defects. *Clinical Chemistry & Laboratory Medicine* 41: 276-280.
139. Lee, C. N., Su, Y. N., Cheng, W. F., Lin, M. T., Wang, J. K., Wu, M. H. & Hsieh, F. J. (2005) Association of the C677T methylenetetrahydrofolate reductase mutation with congenital heart diseases. *Acta Obstet Gynecol Scand* 84: 1134-1140.
140. Junker, R., Kotthoff, S., Vielhaber, H., Halimeh, S., Kosch, A., Koch, H. G., Kassenbohmer, R., Heineking, B. & Nowak-Gottl, U. (2001) Infant methylenetetrahydrofolate reductase 677TT genotype is a risk factor for congenital heart disease. *Cardiovasc Res* 51: 251-254.
141. Galdieri, L. C., Arrieta, S. R., Silva, C. M., Pedra, C. A. & D'Almeida, V. (2007) Homocysteine concentrations and molecular analysis in patients with congenital heart defects. *Arch Med Res* 38: 212-218.
142. van Beynum, I. M., Kapusta, L., den Heijer, M., Vermeulen, S. H. H. M., Kouwenberg, M., Daniels, O. & Blom, H. J. (2006) Maternal MTHFR 677C>T is a risk factor for congenital heart defects: effect modification by periconceptional folate supplementation. *Eur Heart J* 27: 981-987.
143. Verkleij-Hagoort, A., Blik, J., Sayed-Tabatabaei, F., Ursem, N., Steegers, E. & Steegers-Theunissen, R. (2007) Hyperhomocysteinemia and MTHFR polymorphisms in association with orofacial clefts and congenital heart defects: a meta-analysis. *Am J Med Genet A* 143: 952-960.

144. Barbosa, P. R., Stabler, S. P., Machado, A. L., Braga, R. C., Hirata, R. D., Hirata, M. H., Sampaio-Neto, L. F., Allen, R. H. & Guerra-Shinohara, E. M. (2007) Association between decreased vitamin levels and MTHFR, MTR and MTRR gene polymorphisms as determinants for elevated total homocysteine concentrations in pregnant women. *Eur J Clin Nutr.*

145. Shaw, G. M., Nelson, V., Carmichael, S. L., Lammer, E. J., Finnell, R. H. & Rosenquist, T. H. (2002) Maternal periconceptional vitamins: interactions with selected factors and congenital anomalies? *Epidemiology* 13: 625-630.

BIOGRAPHICAL SKETCH

Younis was born in Kuwait. After completing high school in Kuwait, he went to the US to earn his Bachelor of Science in food science and human nutrition from California State University Fresno in 2005. After graduating in 2005, Younis went back to Kuwait and worked for the Kuwait health system. Younis worked as a weight and disease management consultant. He was also granted a part time job at Kuwait University to work as a teaching assistant in the Nutrition Department before he was awarded a scholarship to pursue graduate studies. In 2006, Younis was accepted into the University of Florida's nutritional sciences' Master of Science degree program. Younis plans to pursue his academic career and earn his PhD in nutritional sciences to later become a faculty member in the Nutrition Department at Kuwait University.