THE EFFECTS OF METHOTREXATE AND GENETIC POLYMORPHISMS ON THE FOLATE/HOMOCYSTEINE PATHWAY

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A Dissertation in Pharmacology

Presented to the Faculties of the University of Pennsylvania

In Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

2011

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DEDICATION

I would like to dedicate this thesis to my loving husband, Jared, and his family for their support, encouragement, and willingness to make sacrifices. I would also like to dedicate this thesis to my parents for their understanding and unwavering support.

ACKNOWLEGEMENTS

I would like to thank my advisor, Alexander S. Whitehead, for mentoring me over these past few years. He has trained me in planning and executing experiments, and provided valuable guidance in developing my writing skills. I would also like to thank all of my professors, in particular, Andrew Cucchiara, for spending long hours teaching me SAS programming. I would like to thank all of the past and present members of the Whitehead lab for their support and advice. In particular I would like to thank Karen Brown, Carmel Kealey, Anna Stanislawska-Sachadyn, Liselotte Jensen, Zhi-Yong Lu, and Andrea Hammons. They have given me advice and helped me to learn techniques. I would like to thank Stefanie Khartulyari, Arnaldo Diaz, Suhong Zhang, and Jasbir Arora from the Blair lab and Megan Morales from the Von Feldt lab for their collaboration. I would also like to thank the Assoian lab for their help and sharing of their equipment. I would like to thank members of my thesis committee, Ian Blair, Wade Berrettini, Caryn Lerman, and Liselotte Jensen for their guidance. I would like to thank UPenn Pharmacological Sciences, in particular Linda Leroy, Mary Scott, Sarah Squire, Lise Miller, Gail Vogel, Rosalyn Rucker, and Camie Minieri for taking care of practical issues.

The Industrial Workers study was supported by National Institutes of Health grants AR47663, CA108862, ES013508, HD039195; PA Department of Health grant 4100038714; the Wellcome Trust; and the British Heart Foundation. Initial funding was obtained through the Institute of Vitamin Research in Berne, Switzerland, courtesy of Professor Fred Gey.

The Premenopausal Women study was supported by National Institutes of Health grants AR47663, HD039195, and ES013508.

The Neural Tube Defect study was supported by National Institutes of Health grants HD039195 and HD039081; Ethel Brown Foerderer Fund for Excellence; and General Clinical Research Center of the Children's Hospital of Philadelphia grant M01-RR00240.

The Systemic Lupus Erythematosus study was supported by National Institutes of Health grants AR47663 and ES013508; Lupus Research Institute grants; and Clinical and Translational Research Center grant UL1RR024134 from the National Center for Research Resources.

The Rheumatoid Arthritis study was supported by National Institutes of Health grant AR47663.

The cell culture studies were supported by National Institutes of Health grants AR47663 and ES013508; and Pennsylvania Department of Health grant 4100038714.

ABSTRACT

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High homocysteine (Hcy) and low folate status are associated with many clinical conditions ranging from cardiovascular disease to neural tube defects. Hcy and folate levels are affected by diet as well as lifestyle and genetic factors. Associations between genetic polymorphisms of the enzymes involved in folate/Hcy metabolism and Hcy levels and folate phenotypes were examined. Genetic polymorphisms were studied in a range of populations, which included healthy individuals, systemic lupus erythematosus (SLE) patients, rheumatoid arthritis (RA) patients, and families with a child affected by neural tube defects (NTDs). Chronic low folate is associated with development of a "proatherosclerotic" phenotype in the endothelial cell line, EA.hy 926. The effect of the anti-folate, methotrexate (MTX), on the expression of inflammatory genes was studied in EA.hy 926 cells in the context of folate status and activation by TNF-α.

Genotyping was performed by TaqMan allelic discrimination assays or by size difference PCR. Total Hcy (tHcy) concentrations and levels of plasma and red blood cell (RBC) folate derivatives were measured by stable isotope dilution liquid chromatography multiple reaction monitoring mass spectrometry. Affymetrix microarrays were used to

assess changes in gene expression *in vitro*. Candidate inflammatory genes were then queried using qRT-PCR. ELISAs were performed to confirm changes in protein levels.

Several polymorphisms had effects on tHcy levels and not only on total RBC folate but on individual RBC folate derivatives. Specifically effects were observed within the studies in healthy men, healthy women, and RA patients, but not in SLE patients. Also none of the polymorphisms studied showed an association with increased risk for NTDs using Transmission Disequilibrium Test analyses. Genetic polymorphisms of the enzymes of the folate/Hcy pathway impact levels of tHcy and folate, which may then impact risk for various clinical conditions.

MTX increased mRNA expression and protein levels of several inflammatory genes, which included C3 and IL-8. Activation of endothelial cells by TNF-α did not seem to be affected by treatment with MTX, with exception of the up regulation of C3. MTX lowered intracellular folate and altered the distribution of folate derivatives, which had an effect on inflammatory gene expression in endothelial cells.

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Chapter 1: Introduction

1.1 The Folate/Homocysteine Metabolic Pathway

The folate/homocysteine pathway supports two biologically important functions, methylation and nucleotide biosynthesis. Low folate and high homocysteine (Hcy), hyperhomocysteinemia, is associated with a wide range of pathologies including spina bifida (van der Put et al. 2001), cardiovascular disease (Refsum et al. 1998), Alzheimer's (Mattson and Shea 2003), cancer (Stover 2004), and many others. It remains to be proven whether it is the high Hcy or low folate levels which present more risk. This is further complicated by their intrinsic inverse relationship. Dietary intakes of folate and folic acid, known as vitamin B_9, as well as intake of other B vitamins have an inverse relationship with Hcy levels. B vitamins, such as B_6 and B_{12}, are cofactors for several enzymes in the pathway and thus deficient levels lead to increased Hcy concentrations. Elevated Hcy levels are also associated with older age, male gender, and lifestyle factors such as smoking, alcohol intake, coffee consumption, and lack of exercise (Schneede et al. 2000).

Folate is important in methylation because the methyl group from 5-methyltetrahydrofolate (5-MTHF) is used for the remethylation of Hcy, which becomes methionine (Figure 1-1). Methionine then becomes S-adenosylmethionine (SAM) which is the methyl donor for hundreds of methylation reactions, which includes methylation of DNA and proteins. Once SAM loses its methyl group it becomes S-adenosylhomocysteine (SAH), which is then converted back to Hcy. Hcy can also be methylated to become methionine by betaine-homocysteine S-methyltransferase (BHMT), but this enzyme is found mostly in the liver (Pajares and Perez-Sala 2006) and uses the methyl group from betaine. The transsulfuration pathway metabolizes Hcy into

cystathionine, which is then converted into cysteine and can be used to produce glutathione. The transsulfuration pathway is present in only four tissues, liver, kidney, small intestine, and pancreas (Finkelstein 1998). There are two folate derivatives which are important for nucleotide synthesis. 5,10-methyleneTHF is used for the production of the pyrimidine, deoxythymidine monophosphate (dTMP), while 10-formylTHF is used for production of purines.

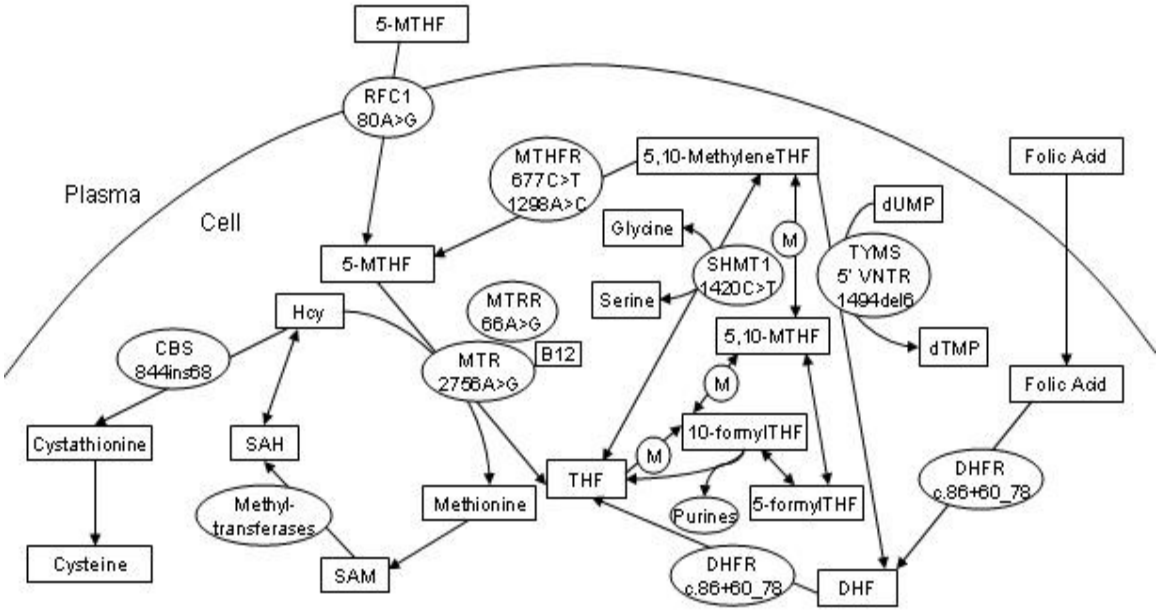


Figure 1-1. The folate/homocysteine metabolic pathway with common functional polymorphisms. 5-MTHF, 5-methyltetrahydrofolate, 5,10-MTHF, 5,10-methenyltetrahydrofolate, CBS, cystathionine beta-synthase, DHF, dihydrofolate, DHFR, dihydrofolate reductase, dTMP, deoxythymidine monophosphate, dUMP, deoxyuridine monophosphate, Hcy, homocysteine, M, methylenetetrahydrofolate dehydrogenase 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase 1958G>A, MTHFR, methylenetetrahydrofolate reductase, MTR, methionine synthase, MTRR, methionine synthase reductase, RFC1, reduced folate carrier 1, SAH, S-adenosylhomocysteine, SAM, S-adenosylmethionine, SHMT1, serine hydroxymethyltransferase 1, THF, tetrahydrofolate, TYMS, thymidylate synthase. (published in (Summers et al. 2010))

1.2 Folate Transport

Naturally occurring forms of folate are found mostly in vegetables and some fruits and are polyglutamated. During digestion folates are hydrolyzed into monoglutamates by two enzymes, gamma-glutamyl hydrolase (GGH) and folate hydrolase 1 (FOLH1). In the small intestine reduced folate carrier 1 (RFC1) transports monoglutamated folates and folic acid. The circulating form of folate is 5-methyltetrahydrofolate (5-MTHF) monoglutamate and once it enters the cell it becomes polyglutamated by folylpolyglutamate synthase (FPGS) in order to trap folate within the cell as well as increasing their affinity for intracellular enzymes (Stover 2004). RFC1 is ubiquitously expressed and transports folate in many tissues as diverse as the kidneys and the central nervous system. The folate receptor (FR) family of genes are only expressed in specific tissues, during certain developmental times, and tend to be overexpressed in certain cancers (Matherly and Goldman 2003). Red blood cell (RBC) folate levels are a reflection of folate status during the last 3-4 months while plasma folate levels are a reflection of recent folate intake.

1.3 Common Functional Polymorphisms

The hereditability of homocysteine was found to be 57% in a twin study (Siva et al. 2007) and therefore genetic polymorphisms are likely to contribute to the variation in Hcy levels. Common functional polymorphisms within the enzymes of the folate/Hcy metabolic pathway affect folate/Hcy phenotype and may therefore increase risk for clinical conditions that are associated with dysfunction of folate/Hcy metabolism.

1.3.1 MTHFR

5,10-methylenetetrahydrofolate reductase (MTHFR) irreversibly reduces 5,10-methyleneTHF into 5-MTHF, which is the methyl donor for the remethylation of Hcy into methionine (Figure 1). The cofactor for this enzyme is flavin adenine dinucleotide (FAD), which is derived from vitamin B_2, riboflavin. This enzyme is important because it directs whether folate is used for methylation or for nucleotide synthesis (Ueland et al. 2001). Frosst et al. (1995) was the first to identify the single nucleotide polymorphism (SNP) in *MTHFR* 677C>T (rs1801133), which causes an alanine to valine substitution at position 222 of the protein and the result is a thermolabile form of the enzyme with reduced activity. *MTHFR* 677TT homozygous individuals have higher fasting homocysteine levels especially when folate levels are low (Harmon et al. 1996; Jacques et al. 1996). From the study by Guenter et al. (1999) on the homologous mutation in MTHFR from *E. coli* the thermolabile properties of the human 677T enzyme are due to the decreased binding of FAD. When folate levels are low the thermolabile enzyme loses its FAD cofactor more easily than the wildtype enzyme. Conversely, when folate levels are high the variant containing enzyme becomes stabilized and less likely to lose its cofactor.

Bagley and Selhub (1998) were the first to discover that *MTHFR* 677TT homozygotes had an altered distribution of folate derivatives, specifically that formylated folates were present in red blood cells (RBCs), whereas 677CC homozygotes had predominantly 5-MTHF. Not only does this polymorphism affect Hcy levels and the distribution of folate derivatives but it also carries increased and decreased risks of clinical conditions. For example, the 677TT genotype in infants carries a pooled odds

ratio of 1.8 for risk of spina bifida in a meta-analysis by Botto and Yang (2000) and in mothers the pooled odds ratio is 2.0 for risk of carrying a child with spina bifida. Also 677TT homozygotes are at increased risk of cardiovascular disease (Kluijtmans and Whitehead 2001) but carry an increased protection from colon cancer (Ma et al. 1997), the rationale for the latter being that the 677T enzyme is inefficient and folates other than 5-MTHF accumulate and are more readily available for nucleotide synthesis. A study by DeVos et al. (2008) showed that the *MTHFR* 677TT genotype was associated with 34% lower DNA uracil content, and the misincorporation of uracil into DNA is believed to be one of the first steps of colon cancer (Ma et al. 1997).

A second *MTHFR* SNP, 1298A>C (rs1801131), is in linkage disequilibrium with the 677C>T SNP, such that the two variant alleles rarely occur together on the same chromosome (van der Put et al. 1998). The 1298A>C transition encodes a substitution of glutamic acid to alanine at position 429 of the protein. The 1298A>C polymorphism has contradictory associations with folate levels in the literature. Parle-McDermott et al. (Parle-McDermott et al. 2006b) found that within the *MTHFR* 677CC genotype those with the 1298CC genotype had higher RBC folate than those with the 1298AA genotype, while a large-scale study by Ulvik et al. (2007) found that within the *MTHFR* 677CC genotype those with the 1298CC genotype had lower serum folate and higher Hcy than those with the 1298AA genotype. Van der Put et al. (1998) have reported that the double homozygote 677CT/1298AC is associated with elevated Hcy and lower plasma folate.

1.3.2 MTR

Methionine synthase (MTR) uses the methyl group from 5-MTHF to remethylate homocysteine. The products of this reaction are methionine and THF. Vitamin B_{12} is a

cofactor for this enzyme. The most studied SNP in this gene is 2756A>G (rs1805087), which mandates an aspartic acid to glycine change at position 919 in the protein. Harmon et al. (1999) found that 2756AA homozygotes had higher Hcy levels in a study in Irish middle-aged men called the Industrial Workers study. Tsai et al. (2009) confirmed this finding in African Americans in the US, but in Caucasians Hcy levels were not associated with this polymorphism. A large scale study in Norway also found that 2756AA homozygotes had higher Hcy levels (Fredriksen et al. 2007).

1.3.3 MTRR

Methionine synthase reductase (MTRR) keeps MTR in its active form via reductive methylation of B_{12}. There is a polymorphism in this gene at position 66 that has an A>G transition (rs1801394). This polymorphism leads to an isoleucine to methionine change at position 22 in the protein. It was found that 66GG homozygotes have high Hcy levels in the Industrial Workers study in Irish men (Gaughan et al. 2001; Gaughan et al. 2002).

1.3.4 CBS

Cystathionine beta-synthase (CBS) irreversibly metabolizes Hcy as part of the transsulfuration pathway, and requires vitamin B_6 as a cofactor. Within *CBS* there is a 68 base pair (bp) insertion found in exon 8 referred to as 844ins68. This insertion has been shown to carry a mutation 833T>C which should cause a premature stop codon but *in vitro* studies showed that the inserted sequence is skipped, allowing a full length functional CBS enzyme (Tsai et al. 1996). Only two studies (Dekou et al. 2001; Fredriksen et al. 2007) have found that the 844ins68 carriers have lower Hcy levels than

noncarriers, while several other studies did not find any association (Bowron et al. 2005; Kluijtmans et al. 2003; Wang et al. 1999 Sep).

Individuals homozygous for loss of function mutations in *CBS*, *MTR*, or *MTHFR* have homocystinuria, which is characterized by extremely high levels of Hcy above 100 μM. This rare disease manifests as mental retardation, dislocation of the optic lens, osteoporosis, and increased cardiovascular events which leads to early mortality (Mudd et al. 1985). Hyperhomocysteinemia is defined as an intermediate level of Hcy between 15 and 100 μM. High levels of Hcy have been proven to be an independent risk factor for cardiovascular disease (Refsum et al. 1998).

1.3.5 DHFR

Dihydrofolate reductase (DHFR) metabolizes synthetic folic acid, which is found in supplements and fortified foods, into dihydrofolate (DHF) and DHFR also metabolizes this into THF. There is a 19bp insertion/deletion polymorphism within the first intron of *DHFR*, which is referred to as c.86 + 60_78. The deletion removes a possible Sp1 transcription factor binding site, which may affect transcriptional regulation (Johnson et al. 2004). The effect of this polymorphism on folate and Hcy levels was consistent in two studies in healthy individuals. A study in young reproductive aged adults from Northern Ireland found that women with the del/del genotype had increased serum and RBC folate concentrations compared to insertion carriers (Stanislawska-Sachadyn et al. 2008a). A study from the Netherlands found that del/del homozygotes had decreased Hcy levels compared to ins/ins homozygotes (Gellekink et al. 2007).

1.3.6 TYMS

Thymidylate synthase (TYMS) competes with MTHFR for one of its substrates 5,10-methyleneTHF, which is used to convert deoxyuridine monophosphate (dUMP) into deoxythymidine monophosphate (dTMP). Nucleotide synthesis is important for DNA replication and repair. There are two common polymorphisms within this gene, one in the 5' untranslated region (UTR) and one in the 3' UTR. The polymorphism in the 5' UTR is a variable number of tandem repeats (VNTR) consisting of a 28bp repeat with either 2 or 3 repeats being the most common and 4 and 5 repeats being more rare (Luo et al. 2002). Mandola et al. (2003) suggested that the repeat contains a USF1 transcription factor binding site, so the higher number of repeats would lead to increased transcription. The 3R/3R homozygotes had lower plasma folate in a study in Singapore Chinese subjects (Trinh et al. 2002).

The polymorphism in the 3' UTR of *TYMS* is a 6bp insertion/deletion polymorphism referred to as 1494del6 (rs16430). A study by Mandola et al. (2004) suggests that this polymorphism affects mRNA stability and translation. TYMS del/del homozygotes were associated with less mRNA. Del/del homozygotes had higher RBC folate and lower Hcy levels compared to ins carriers in young adults from Northern Ireland (Kealey et al. 2005). There is linkage disequilibrium between the polymorphisms in the 5' and 3' UTRs such that the 2R allele rarely occurs on the same chromosome with the del allele (Mandola et al. 2004).

1.3.7 SHMT1

Serine hydroxymethyltransferase 1 (SHMT1) encodes an enzyme which localizes to the cytoplasm while SHMT2 encodes an enzyme which localizes to the mitochondria.

SHMT1 and SHMT2 both catalyze the same reaction, the reversible conversion of serine and THF to glycine and 5,10-methyleneTHF. *SHMT1* has a polymorphism 1420C>T (rs1979277), which substitutes a leucine for a phenylalanine at position 474 of the protein. In a study in mothers of children with neural tube defects 1420CC homozygotes had increased Hcy levels compared to 1420T carriers. In the combined group of mothers, children, and controls 1420CC homozygotes had decreased plasma and RBC folate levels (Heil et al. 2001).

1.3.8 MTHFD1

Methylenetetrahydrofolate dehydrogenase 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase (MTHFD1) is a trifunctional enzyme which catalyzes three different reactions. This enzyme provides precursors for nucleotide synthesis and converts THF into 10-formylTHF and then into 5,10-methyleneTHF, which are used in the synthesis of purines and thymidylate, respectively. The 1958G>A polymorphism (rs2236225) present in *MTHFD1* substitutes an arginine for a glutamine at position 653 in the protein, which is in the formyltetrahydrofolate synthetase region of the enzyme. Although one study has not identified any effect of this polymorphism on plasma folate, RBC folate, or Hcy concentrations (Brody et al. 2002) two separate sets of samples from the Irish population have revealed that the 1958AA genotype is associated with an increased risk for mothers to carry a child with spina bifida (Brody et al. 2002; Parle-McDermott et al. 2006a).

1.3.9 RFC1

Reduced folate carrier 1 (RFC1), officially known as solute carrier family 19 member 1 (SLC19A1), transports 5-MTHF bi-directionally with high affinity and also

facilitates the transport of folic acid but with lower affinity (Matherly and Goldman 2003). *RFC1* has a polymorphism 80G>A (rs1051266), which substitutes an arginine for a histidine at position 27 of the protein. The effect of this polymorphism on folate and homocysteine levels is varied in the literature. A few studies did not find any association between *RFC1* 80G>A genotype and folate or homocysteine levels (Chango et al. 2000; Devlin et al. 2006; Vesela et al. 2005). A study in young reproductive aged adults from Northern Ireland found that women with the 80GG genotype had decreased RBC folate levels compared to 80GA and 80AA genotypes (Stanislawska-Sachadyn et al. 2009). A study by Morin et al. (2003) found that 80GG homozygous mothers of children with or without spina bifida had a borderline significant decrease in RBC folate. In contrast, a large-scale study observed the 80A allele was associated with decreased serum folate but with only borderline significance (Fredriksen et al. 2007).

1.4 Summary and Specific Aims

Folate/Hcy metabolism is important for nucleotide biosynthesis and methylation. Dysregulation of folate/Hcy metabolism characterized by low folate and high Hcy levels is associated with many clinical conditions, ranging from developmental abnormalities to diseases with an inflammatory component. Polymorphisms within the enzymes of the folate/Hcy metabolic pathway may have an effect on Hcy and folate levels that could affect risk for various clinical conditions.

Specific Aims:

1) Examine the effects of the above mentioned polymorphisms on tHcy and folate levels in healthy men and women.

2) Examine the risk associated with the above mentioned polymorphisms in families with children affected by spina bifida.
3) Examine the effects of the above mentioned polymorphisms in patients with inflammatory diseases such as systemic lupus erythematosus and rheumatoid arthritis patients taking the anti-folate drug, methotrexate.
4) Examine the effects of methotrexate, alone and when used prior to treatment with TNF-α, on inflammatory gene expression in an endothelial cell culture model under high and low folate conditions.

Chapter 2: Effect of Polymorphisms on Homocysteine and Folate Levels in Healthy Individuals

2.1 Abstract

High Hcy and low folate levels are associated with many clinical conditions ranging from cardiovascular disease to spina bifida. Genetic polymorphisms within the enzymes of the folate/Hcy metabolic pathway may have independent effects or these effects may synergize or counteract one another. In the Industrial Workers study, which is composed of healthy middle-aged Irish males, the 844ins68 polymorphism within *cystathionine β-synthase* (*CBS*) was examined alone and in the context of *5,10-methylenetetrahydrofolate reductase* (*MTHFR*) 677C>T genotypes for an effect on tHcy and folate levels. *MTHFR* 677TT homozygotes had higher tHcy and lower folate concentrations relative to individuals with 677CC and 677CT genotypes. *CBS* 844ins68 carriers did not have significantly different tHcy or folate levels, but after stratifying by *MTHFR* 677 genotypes, carriers of the 844ins68 allele who were *MTHFR* 677TT homozygotes had normalized levels of tHcy and folate compared to 677TT/844ins68 noncarriers.

In the Premenopausal Women study, which was composed of 26 Caucasian and 23 African American healthy premenopausal women, there were 11 polymorphisms within 9 genes of enzymes of the folate/Hcy metabolic pathway which were examined for effects on tHcy, plasma folate, and red blood cell (RBC) folate derivative levels. In African Americans tHcy levels were associated with polymorphisms in *methionine synthase (MTR)* and *thymidylate synthase (TYMS)*. RBC folate derivative levels were associated with polymorphisms in *MTR*, *TYMS*, *methionine synthase reductase (MTRR)*, and *reduced folate carrier 1 (RFC1)*. In Caucasians tHcy levels were associated with

polymorphisms in *5,10-methylenetetrahydrofolate reductase (MTHFR)* and *MTR*. RBC folate derivative levels were associated with polymorphisms in *MTHFR*, *TYMS*, and *RFC1*.

2.2 Introduction

2.2.1 Factors Affecting Hcy Levels

Hcy concentrations are impacted by dietary intake of folate and B vitamins, by lifestyle factors such as smoking, and by genetic factors such as common functional polymorphisms in the enzymes of the folate/Hcy metabolic pathway. B_{12} levels are inversely related to Hcy levels because B_{12} is a cofactor for MTR, the enzyme responsible for the remethylation of Hcy to methionine. The methylfolate trap hypothesis states that deficient B_{12} levels lower the activity of MTR such that 5-MTHF is trapped resulting in the build up of Hcy and was demonstrated in a single case of B_{12} deficiency (Smulders et al. 2006). Smoking is associated with increased Hcy, decreased folate, B_{12}, and B_6 levels (Brown et al. 2004b; Gabriel et al. 2006; Nygard et al. 1998). Smoking is also associated with poor pregnancy outcome, which includes oral clefts, preterm birth, and low birth weight infants (Meyer et al. 1976; Wyszynski et al. 1997).

2.2.2 Interaction of MTHFR 677C>T and CBS 844ins68 genotypes

Destefano et al. (1998) found that *MTHFR* 677TT individuals carrying the *CBS* 844ins68 allele had lower homocysteine levels than noncarriers in a large study in European men. A large study in British men by Dekou et al. (2001) confirmed the finding that carriers of the *CBS* 844ins68 allele who were *MTHFR* 677TT homozygotes had lower Hcy levels than 677TT/844ins68 noncarrier individuals. Folate levels were not reported for either of these studies, and the aim of the Industrial Workers Study described

here was to examine whether the *CBS* 844ins68 allele is associated with significantly different levels of folate and Hcy, either alone or within classes of *MTHFR* 677C>T genotypes.

2.2.3 Racial Distributions of Polymorphisms

The population in which the polymorphisms are studied is very important because the frequencies of the genotypes vary by race. *MTHFR* 677C>T genotypes vary significantly in frequency among the different racial groups. The percent of *MTHFR* 677TT homozygotes ranges from 8-18% in Caucasians, 1% of African Americans, and 12% of Asians (Botto and Yang 2000). *MTHFR* 677TT homozygotes are more prevalent in Caucasian than African American populations and the implication maybe that risk of neural tube defects is higher in Caucasians. The frequency of *CBS* 844ins68 allele carriers also varies by race. Carriers of 844ins68 make up 14% of Caucasians, 41% of African Americans, and are absent in Asians (Franco et al. 1998; Tsai et al. 2009).

2.2.4 Association of High Hcy and Low Folate with Cardiovascular Disease

Homocystinuria, extremely high levels of Hcy, due to an inborn error of metabolism was first discovered in 1962 (Gerritsen et al. 1962) and was associated with early onset cardiovascular disease (Mudd et al. 1985). Since then it has been concluded that Hcy contributes to formation of atherosclerotic plaques (McCully 2007). Specifically Hcy is capable of damaging arterial tissues by inducing the release of cytokines and other mediators of inflammation. Hcy also contributes to fibrosis, calcification, and elastic tissue damage. Hcy can cause oxidative stress which leads to oxidation of LDL, uptake of which leads macrophages to become foam cells (Loscalzo 1996). Many clinical studies have established that elevated Hcy is an independent risk factor for cardiovascular

disease (McCully 2007). A meta-analysis found that a 5 μmol/L increase in Hcy is associated with a 27% increased risk of venous thrombosis in prospective studies and a 60% increase in retrospective studies (Den Heijer et al. 2005). The best evidence that adequate folate is protective against cardiovascular disease is in the early 1960s in the US cardiovascular disease reached a peak and subsequently death rates declined by 60% (CDC 1999). This time period coincides with the introduction of synthetic folic acid to the food supply, before it was mandated by the FDA in 1998.

2.2.5 Association of High Hcy and Low Folate with Pregnancy Complications

High Hcy and low folate concentrations are associated with pregnancy complications such as preeclampsia, premature birth, low birth weight infants, stillbirth, and recurrent pregnancy loss (D'Uva et al. 2007; Vollset et al. 2000). A high Hcy and low folate phenotype has also been associated with congenital abnormalities such as NTDs, clubfoot, heart defects, limb deficiencies, cleft lip/palate, and Down syndrome (Czeizel 1998; James et al. 1999; Little et al. 2008; Vollset et al. 2000). Suboptimal folate status in the mother is associated with an eight-fold increased risk of carrying a child with an NTD (Molloy et al. 1998a). The adverse pregnancy outcomes listed above have been shown to be reduced by maternal periconceptional folic acid supplementation (Bukowski et al. 2009; Czeizel and Dudas 1992; Timmermans et al. 2009; Yang et al. 1997).

2.2.6 Methods of Folate Measurement

There were two common methods by which RBC folate was typically measured. Microbiological assays were based on the growth of a specific strain of folate-dependent bacteria which was directly related to folate concentration (Molloy and Scott 1997). Radiometric competitive binding assays (radioassays) were then developed to measure

RBC folate via competition of radiolabeled tracer folate and unlabeled folate from the sample for protein binding sites. The problem that arose was the conflicting association of RBC folate levels with the *MTHFR* 677TT genotype. One study had shown that 677TT individuals had higher RBC folate than 677CT or 677CC individuals when using a radioassay to measure RBC folate (van der Put et al. 1995). Another study measured RBC folate by microbiological assay and revealed that *MTHFR* 677TT homozygotes had lower RBC folate than 677CT heterozygotes or 677CC homozygotes (Molloy et al. 1997). A study by Molloy et al. (1998b) addressed this problem by using both methods of RBC folate measurement on the same samples of various *MTHFR* 677C>T genotypes. The results confirmed that the radioassay measured lower folates in *MTHFR* 677C carriers compared to 677TT homozygotes, while the microbiological assay showed the opposite. The results also showed that the radioassay measured less folate in *MTHFR* 677CT and 677CC individuals than the microbiological assay while the radioassay measured more folate in *MTHFR* 677TT individuals than the microbiological assay.

A possible explanation lies in the difference in the distribution of folate derivatives between the *MTHFR* 677C>T genotypes. *MTHFR* 677TT homozygotes have an altered distribution of RBC folate derivatives, which favors formylated folates, while 677CC homozygotes have mostly 5-MTHF (Bagley and Selhub 1998). It is possible that a specific folate derivative may be more readily measurable in the radioassay but not utilized efficiently by the bacteria in the microbiological assay. The recent development of stable isotope dilution liquid chromatography multiple reaction monitoring mass spectrometry (LC/MRM/MS) provides higher specificity than previous methods through the use of [$^{13}C_5$]-labeled internal standards. Also the capacity to measure three distinct

folate derivatives (THF, 5-MTHF, and 5,10-MTHF) instead of just total RBC folate gives this method an advantage. Specifically individual RBC folate derivatives will used to characterize in detail the effects of polymorphisms in enzymes of folate/Hcy metabolic pathway. While Smulders et al. (2007) using a similar method has shown that non-methylfolate accumulation in RBCs is associated with the MTHFR 677TT genotype, it remains to be explored what effects the other polymorphisms in the enzymes of the folate/Hcy metabolic pathway have on specific RBC folate derivatives.

Certain genetic variants or folate/Hcy phenotypes may be associated with an individual woman's risk of pregnancy complications. The Premenopausal Women Study was undertaken to examine the relationships of 11 polymorphisms in 9 genes to folate/Hcy phenotype using stable isotope dilution LC/MRM/MS to measure total Hcy and folate derivatives in plasma and RBCs. The analyses were divided into two racial groups, African Americans and Caucasians, because these groups have different prevalences of certain pregnancy complications.

2.3 Methods

2.3.1 Industrial Workers Study

Subjects

Subjects from the Industrial Workers study (n=614) were recruited from an industrial company in Belfast, Northern Ireland as previously described (Harmon et al. 1996; Summers et al. 2008b). Subjects were men that were 29-53 years old. The study was approved by the Research Ethics Committee of the Faculty of Medicine, The Queen's University of Belfast. All subjects provided written informed consent. Fasting subjects gave blood samples for biochemical and genetic analysis.

Biochemical Assays

THcy concentrations were determined previously using high-performance liquid chromatography and serum folate concentrations were determined previously using a commercial kit (ICN Pharmaceuticals) (Harmon et al. 1996).

Genotyping

MTHFR 677C>T genotyping was RFLP based and genotypes for the study population have previously been reported (Harmon et al. 1996). *CBS* 844ins68 genotypes were obtained using a PCR-based size difference method. PCR amplifications were performed in a total volume of 25 μl containing 50 ng genomic DNA, 0.4 μM of each forward and reverse primer (sequences of which were previously published (Barbaux et al. 2000)), 0.8 μM dNTPs, 10x PCR Buffer (Applied Biosystems, Foster City, CA), 1.5 mM $MgCl_2$, and 1U AmpliTaq DNA polymerase (Applied Biosystems). Cycling conditions were as follows: 94℃ 5 min, 35 cycles of 94℃ 1 min, 55℃ 1 min, 72℃ 1 min. PCR products were separated on 3% agarose gels, run for 45 min at 140V, and stained with ethidium bromide.

Statistics

Statistical analyses were performed with SAS version 9.1 (SAS Institute, Cary, NC). Even after logarithmic transformation, distributions of total homocysteine and serum folate were positively skewed, so all analyses were performed using untransformed data. Hardy–Weinberg equilibrium was assessed for the *MTHFR* 677C>T and *CBS* 844ins68 genotypes. Differences between genotype groups for homocysteine and serum folate were assessed using the Wilcoxon rank-sum or Kruskal–Wallis test.

2.3.2 Premenopausal Women Study

Subjects

Premenopausal female staff and students were recruited at the University of Pennsylvania School of Medicine from January 9, 2007 to July 26, 2007. A similar number of subjects who self-reported as Caucasians and African Americans were recruited. Study subjects who had a major medical condition, such as an autoimmune disease, or who were currently taking an anti-folate drug, or were pregnant were excluded. This study was approved by the Institutional Review Board of the University of Pennsylvania School of Medicine. All subjects gave written informed consent. Subjects provided fasting blood samples and completed an in-person interview. This study was previously described (Summers et al. 2010).

Biochemical Assays

THcy levels and plasma and RBC folate derivatives were measured using stable isotope dilution liquid chromatography multiple reaction monitoring mass spectrometry (LC/MRM/MS) as previously described (Huang et al. 2007; Huang et al. 2008). The measured folate derivatives include 5-MTHF, THF, and 5,10-MTHF. The measurement of 5,10-MTHF is representative of the sum of 5-formylTHF and 10-formylTHF, which under acidic conditions convert to 5,10-MTHF.

Vitamin B_{12} levels were measured with Immulite 2000 Vitamin B_{12} Assays (Diagnostic Products Corp., Los Angeles, CA).

Genotyping

QIAamp DNA Mini Kits (Qiagen, Santa Clarita, CA) were used to extract DNA from whole blood. TaqMan genotyping methods were previously described for *MTHFR*

677C>T, *MTHFR* 1298A>C, *MTR* 2756A>G, *MTRR* 66A>G (Summers et al. 2008a), *MTHFD1* 1958G>A, *RFC1* 80A>G, and *SHMT1* 1420C>T (Summers et al. 2010). Briefly, real-time polymerase chain reaction (PCR) assays were performed on a DNA Engine Opticon 2 continuous fluorescence detection system (Bio-Rad, Hercules, CA). PCR amplifications took place in 20 µl volumes containing 20 ng genomic DNA, forward and reverse primers, Fam and Vic labeled allele-specific probes, and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Cycling conditions consisted of an initial incubation at 50℃ for 2 min, 95℃ for 10 min, and 50 cycles of assay specific temperatures for denaturation and extension. Dual fluorescence was measured at the end of each cycle. Genotype interpretations were performed with Opticon Monitor Analysis software, version 2.02 (Bio-Rad).

Size difference PCR methods were used to genotype *CBS* 844ins68, *TYMS* 1494del6, *TYMS* 5' VNTR, and *DHFR* c.86+60_78 as previously described (Summers et al. 2010). Briefly, PCR amplifications were performed using 50 ng genomic DNA, forward and reverse primers, dNTPs, 10x PCR buffer (Applied Biosystems), and AmpliTaq DNA polymerase (Applied Biosystems) in 25 µl volumes. PCR products were separated on 3% agarose gels run for 45 min at 140V then stained with ethidium bromide.

Statistics

Statistical analyses were performed using SAS version 9.1 (SAS Institute, Cary, NC). Discrete variables were presented as counts and proportions and continuous variables were presented as means and standard deviations. Total RBC folate is the sum of RBC 5-MTHF, THF, and 5,10-MTHF. THF values which were not detectable (n=4) were given a value corresponding to the lower limit of quantitation (4.5 nmol/L) with

adjustment for individual hematocrit values, allowing both absolute THF levels and the ratio of RBC 5-MTHF:THF to be analyzed as continuous variables. RBC 5,10-MTHF levels were not detectable in a large portion of the samples and therefore this variable was treated as dichotomous (detectable/not detectable).

Hardy-Weinberg equilibrium was calculated for each polymorphism within each race (Caucasian and African American). Fisher's Exact test was used to check for significant differences in genotype distributions between the two groups. Simple linear regression analyses estimated the coefficient of determination (R^2), which measures the proportion of variation in tHcy and various folates (dependent variable) that is explained by each predictor variable. Significance in each model was assessed using either the t-statistic or Fisher's exact test. Unadjusted p values <0.10 were considered significant because of the small sample size of this study. The large number of comparisons made are exploratory in nature and the results should be interpreted in that context.

2.4 Results

2.4.1 Industrial Workers Study

MTHFR 677C>T and *CBS* 844ins68 genotypes were each in Hardy-Weinberg equilibrium. Frequencies of *MTHFR* 677C>T genotypes were 45% CC, 43.6% CT, 11.4% TT and frequencies of *CBS* 844ins68 genotypes were 82.2% W/W, 17.6% W/I, 0.2% I/I. A previous report from the Industrial Workers Study found that *MTHFR* 677TT homozygotes had increased homocysteine and decreased serum folate relative to 677CT and 677CC individuals (Harmon et al. 1996). *CBS* 844ins68 genotypes did not have significantly different tHcy and serum folate levels (Table 2-1).

Table 2-1. Associations between *CBS* 844ins68 genotype and biochemical parameters.

		CBS 844ins68		
Genotype	All	W/W	W/I + I/I[1]	P value
Frequency %(n)	100 (614)	82.2 (505)	17.8 (109)	-
Homocysteine (μmol/L)	7.13 [5.87-8.62]	7.13 [5.86-8.72]	6.95 [6.02-8.23]	
(n)	(614)	(505)	(109)	0.45
Serum Folate (nmol/L)	11.04 [8.51-14.03]	10.81 [8.51-14.03]	11.16 [9.20-13.80]	
(n)	(599)	(493)	(106)	0.23

Plasma tHcy and serum folate concentrations are expressed as median [interquartile range]. Statistical significance for *CBS* genotypes was assessed by Wilcoxon Rank Sum. [1]Combined genotype class comprises 108 W/I and one I/I. (Summers et al. 2008b)

When *CBS* 844ins68 genotypes were stratified by *MTHFR* 677 genotypes, *CBS* 844ins68 carriers had significantly different tHcy levels than noncarriers only within *MTHFR* 677TTs. *CBS* 844ins68 carrier status had no significant impact on tHcy or serum folate concentrations within either *MTHFR* 677CT or 677CC genotypes. *MTHFR* 677TT/*CBS* 844ins68 carriers had 24.1% lower tHcy levels compared to 677TT/844ins68 noncarriers (6.66 vs 8.77 umol/L, respectively p=0.045) (Figure 2-1).

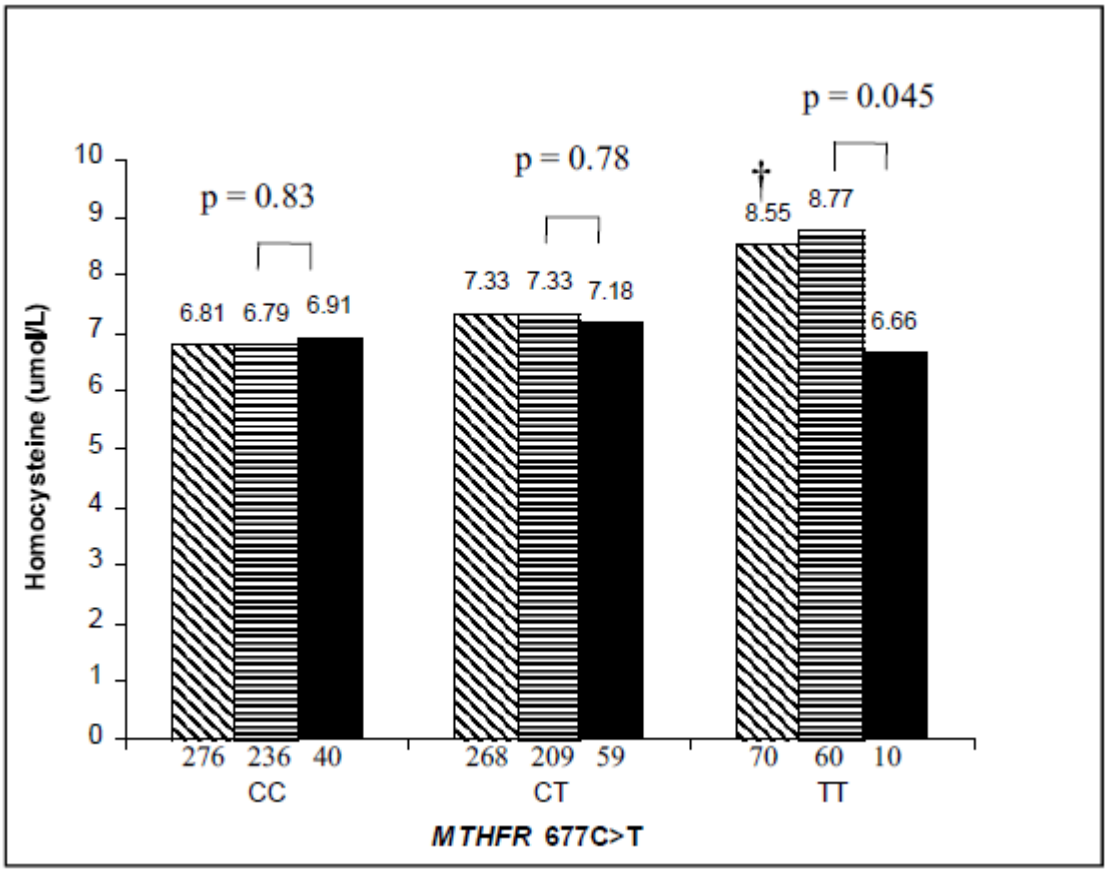


Figure 2-1. Median total homocysteine levels by *CBS* 844ins68 genotype within each *MTHFR* 677C>T genotype class. Bars represent total homocysteine levels in each *MTHFR* 677C>T genotype class: ▨ regardless of *CBS* 844ins68 genotype, ▤ in *CBS* 844ins68 non-carriers, and ■ in *CBS* 844ins68 carriers. The number of subjects in each group is given. † *MTHFR* 677TT homozygotes have significantly higher total homocysteine concentrations than CT heterozygotes and CC homozygotes ($p<0.0001$ by Kruskal-Wallis). (Summers et al. 2008b)

When *CBS* genotypes were stratified by *MTHFR* 677 genotypes, *CBS* 844ins68 carriers had significantly different serum folate concentrations than noncarriers only within *MTHFR* 677TTs. *MTHFR* 677TT/*CBS* 844ins68 carriers had 27.7% higher serum folate concentrations compared to 677TT/844ins68 noncarriers (11.16 vs 8.74 nmol/L, respectively $p=0.034$) (Figure 2-2). The tHcy raising and folate lowering effect of the *MTHFR* 677TT genotype appears to be 'counterbalanced' by the *CBS* 844ins68 allele.

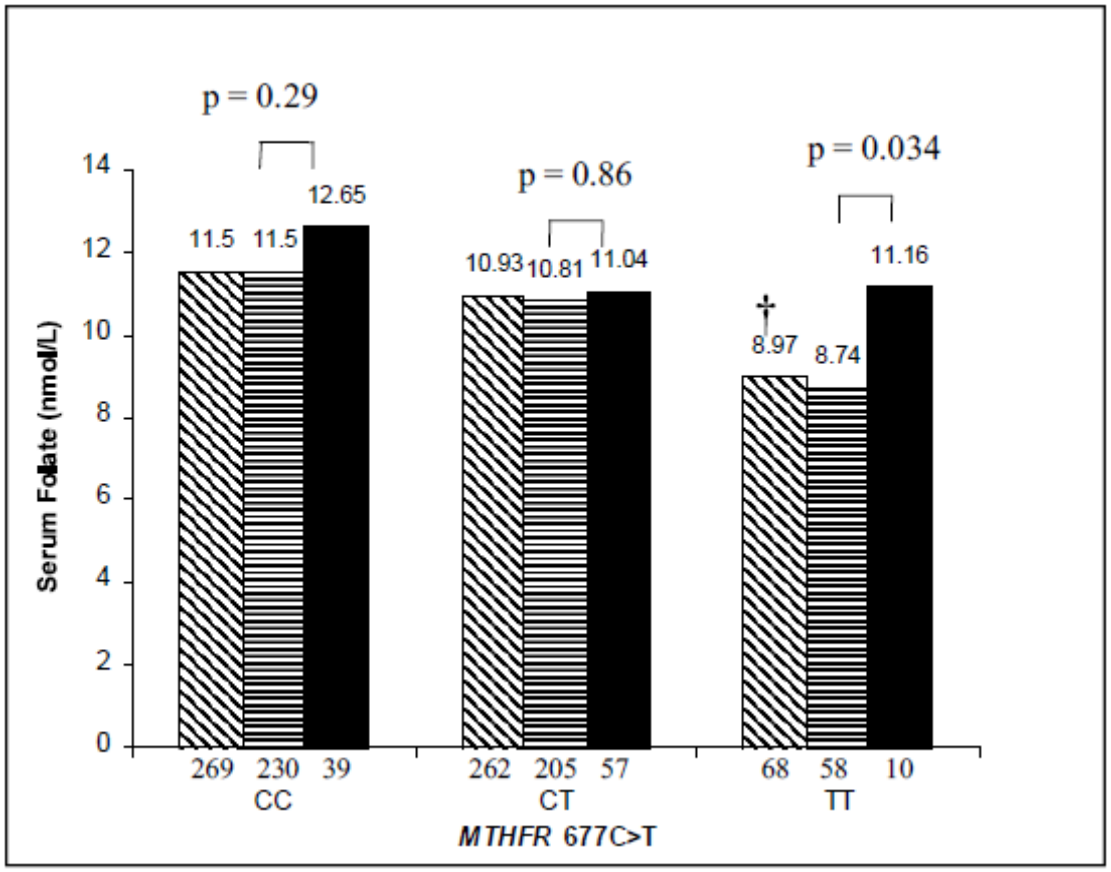


Figure 2-2. Median serum folate levels by *CBS* 844ins68 genotype within each *MTHFR* 677C>T genotype class. Bars represent serum folate levels in each *MTHFR* 677C>T genotype class: ▨ regardless of *CBS* 844ins68 genotype, ▤ in *CBS* 844ins68 non-carriers, and ■ in *CBS* 844ins68 carriers. The number of subjects in each group is given. † *MTHFR* 677TT homozygotes have significantly lower serum folate concentrations than CT heterozygotes and CC homozygotes ($p<0.0001$ by Kruskal-Wallis). (Summers et al. 2008b)

2.4.2 Premenopausal Women study

Subject Characteristics

In the study 53% (n=26) self-reported as Caucasian and 47% (n=23) as African American. Subject characteristics are presented in Table 2-2. Several of the biochemical variables were significantly different between African Americans and Caucasians. Total RBC folate was higher in Caucasians (1186.0 vs 939.1 nmol/L, p=0.013). Out of the individual RBC folate derivatives THF levels were higher (118.2 vs 19.1 nmol/L,

p=0.030) and 5,10-MTHF was detectable in more Caucasians than African Americans (61.5% vs 8.7%, p<0.0001). Additionally, plasma 5-MTHF levels were higher in Caucasians (48.4 vs 33.5 nmol/L, p=0.009), while B_{12} levels were lower in Caucasians (390.5 vs 542.4 pmol/L, p=0.013). Since many biochemical variables were different by race all analyses were performed separately for each group. A Caucasian subset was created in order to perform analyses without *MTHFR* 677TT homozygotes, since this genotype is known to have a profound impact on folate/Hcy phenotype. Removal of these subjects also ensured that analyses of other variables would not contain 677TT individuals. Since the Premenopausal Women study is relatively small the 677TT individuals could be inadvertently grouped together during the analysis of other variables leading to false associations. All analyses in Caucasians were done in the subset which excluded 677TT individuals except those analyses designed to examine the effect of the *MTHFR* 677TT genotype.

Table 2-2. Subject characteristics and biochemical phenotypes (Mean±SD or n%).

Subject Characteristics	African American (N = 23)	Caucasian (N = 26)	Caucasian Subset (N=21)[1]
Age (years)	31.6 ± 6.0	33.3 ± 6.5	32.7 ± 6.1
Body mass index (kg/m^2)	28.3 ± 5.9	23.5 ± 3.4	23.6 ± 3.8
Smoking			
Yes	4 (17.4)	5 (19.2)	4 (19.0)
No	19 (82.7)	21 (80.8)	17 (81.0)
Alcohol use			
Yes	16 (69.6)	22 (84.6)	18 (85.7)
No	7 (30.4)	4 (15.4)	3 (14.3)
Vitamin use[2]			
Yes	15 (65.2)	17 (65.4)	15 (71.4)
No	8 (34.8)	9 (34.6)	6 (28.6)
Biochemical Phenotypes			
tHcy (µmol/L)	8.9 ± 2.5	9.6 ± 2.7	8.9 ± 1.9
Total RBC folate (nmol/L)[3]	939.1 ± 339.0	1186.0 ± 328.5	1165.3 ± 282.6
RBC 5-MTHF (nmol/L)	919.3 ± 334.1	1040.3 ± 333.0	1122.3 ± 278.8
RBC THF (nmol/L)	19.1 ± 9.1	118.2 ± 214.3	37.5 ± 31.2
RBC 5,10-MTHF			
Not Detectable	21 (91.3)	10 (38.5)	9 (42.9)
Detectable	2 (8.7)	16 (61.5)	12 (57.1)
Ratio RBC 5-MTHF: THF	51.5 ± 15.3	35.7 ± 23.6	42.9 ± 19.9
Plasma 5-MTHF (nmol/L)	33.5 ± 17.2	48.4 ± 20.5	50.2 ± 22.0
B_{12} (pmol/L)	542.4 ± 231.6	390.5 ± 176.6	393.0 ± 173.0

[1]After exclusion of *MTHFR* 677TT individuals.
[2]Includes multivitamins, B vitamins, and folic acid.
[3]Total RBC folate = (RBC 5-MTHF) + (RBC THF) + (RBC 5, 10-MTHF) (Summers et al. 2010)

Genotype Distributions

African American and Caucasian genotype distributions were significantly different for 5 out of the 11 polymorphisms and included: *MTHFR* 677C>T, *MTRR* 66A>G, *CBS* 844ins68, *TYMS* 1494del6, and *MTHFD1* 1958G>A (Table 2-3). *MTHFD1* 1958G>A was the only polymorphism that was not in Hardy-Weinberg equilibrium and this was only in Caucasians (data not shown).

Table 2-3. Genotype distributions (n%).

SNP (dbSNP rs no.)	Genotypes	African American (N = 23)	Caucasian (N = 26)	Caucasian Subset (N=21)[1]	P Value[2]
MTHFR 677C>T	CC	16 (69.6)	8 (30.8)	8 (38.1)	0.009
rs1801133	CT	7 (30.4)	13 (50.0)	13 (61.9)	
	TT	0	5 (19.2)	0	
MTHFR 1298A>C	AA	13 (56.5)	14 (53.8)	9 (42.9)	0.61
rs1801131	AC	10 (43.5)	10 (38.5)	10 (47.6)	
	CC	0	2 (7.7)	2 (9.5)	
MTR 2756A>G	AA	10 (43.5)	15 (57.7)	14 (66.7)	0.17
rs1805087	AG	10 (43.5)	11 (42.3)	7 (33.3)	
	GG	3 (13.0)	0	0	
MTRR 66A>G	AA	13 (56.5)	6 (23.1)	5 (23.8)	0.039
rs1801394	AG	7 (30.4)	17 (65.4)	13 (61.9)	
	GG	3 (13.0)	3 (11.5)	3 (14.3)	
CBS 844ins68	WW	12 (52.2)	21 (80.8)	17 (80.9)	0.040
	WI	8 (34.8)	5 (19.2)	4 (19.1)	
	II	3 (13.0)	0	0	
TYMS 5' VNTR	2R/2R	3 (13.0)	2 (7.7)	1 (4.8)	0.14
	2R/3R	8 (34.8)	17 (65.4)	13 (61.9)	
	3R/3R	9 (39.1)	7 (26.9)	7 (33.3)	
	2R/4R	2 (8.7)	0	0	
	3R/4R	1 (4.4)	0	0	
TYMS 1494del6	ins/ins	2 (8.7)	9 (34.6)	7 (33.3)	0.006
rs16430	ins/del	11 (47.8)	15 (57.7)	12 (57.1)	
	del/del	10 (43.5)	2 (7.7)	2 (9.5)	
DHFR c.86+60_78	ins/ins	5 (21.7)	6 (23.1)	6 (28.6)	1
	ins/del	13 (56.5)	14 (53.8)	12 (57.1)	
	del/del	5 (21.7)	6 (23.1)	3 (14.3)	
RFC1 80A>G	AA	8 (34.8)	7 (26.9)	6 (28.6)	0.59
rs1051266	AG	12 (52.2)	16 (61.5)	13 (61.9)	
	GG	3 (13.0)	3 (11.5)	2 (9.5)	
*MTHFD1 1*958G>A	GG	13 (56.5)	12 (46.2)	8 (38.1)	0.082
rs2236225	GA	8 (34.8)	5 (19.2)	3 (14.3)	
	AA	2 (8.7)	9 (34.6)	10 (47.6)	
SHMT1 1420C>T	CC	10 (43.5)	14 (53.8)	13 (61.9)	0.59
rs1979277	CT	8 (34.8)	9 (34.6)	8 (38.1)	
	TT	5 (21.7)	3 (11.5)	0	

[1]After exclusion of *MTHFR* 677TT individuals.

[2]P values by Fisher's exact test are comparison of genotype distributions in African Americans (n=23) and Caucasians (n=26). (Summers et al. 2010)

Associations with Biochemical Phenotypes

Since this study included a small number of subjects in each group p values <0.10 were considered to be significant.

Lifestyle Factors

Alcohol Use. In African Americans alcohol users had tHcy concentrations that were 2.0 μmol/L higher than nonusers (9.5 vs 7.5 μmol/L, p=0.068, Table 2-4). In Caucasians alcohol users had total RBC folate that was 316.5 nmol/L higher than nonusers (1210.0 vs 893.5 nmol/L, p=0.072) and this was due to RBC 5-MTHF levels being 288.5 nmol/L higher in alcohol users (1163.5 vs 875.0 nmol/L, p=0.098).

Smoking. Total RBC folate concentrations were 380.1 nmol/L lower in African American smokers than nonsmokers (625.1 vs 1005.2 nmol/L, p=0.038, Table 2-4). This difference was largely attributable to the difference in RBC 5-MTHF levels which were 375.4 nmol/L lower in smokers than nonsmokers (609.2 vs 984.6 nmol/L, p=0.038). Also plasma 5-MTHF levels were 16.4 nmol/L lower in smokers (20.0 vs 36.4 nmol/L, p=0.083) and B_{12} levels were 239.6 pmol/L lower in smokers (344.5 vs 584.1 pmol/L, p=0.058). In Caucasians similar relationships were observed but were not significant. For example, total RBC folate concentrations were 189.7 nmol/L lower in smokers than nonsmokers (p=0.24).

Vitamin Use. In Caucasians vitamin users had total RBC folate concentrations that were 326.5 nmol/L higher than nonusers (1398.6 vs 1072.1 nmol/L, p=0.013) and this was mostly due to RBC 5-MTHF levels being 326.9 nmol/L higher than nonusers (1355.8 vs 1028.9 nmol/L, p=0.011). Plasma 5-MTHF levels were 23.7 nmol/L higher in

users than nonusers (67.2 vs 43.5 nmol/L, p=0.022). Similar associations were not observed in African Americans.

Table 2-4. Proportion of variation (R^2) in biochemical variables explained by selected subject characteristics and biochemical phenotypes.

	Subject Characteristics		African-American (N=23)		Caucasian Subset (N=21)[1]	
Dependent Variables	**Explan-atory Variables**		**Parameter Estimate (SE)**	**R^2 (P-value)**	**Parameter Estimate (SE)**	**R^2 (P-value)**
tHcy (µmol/L)	Alcohol	Intercept-No	7.5 (0.9)	0.15	8.3 (1.1)	0.02
		Yes	2.0 (1.1)	**(0.068)**	0.8 (1.2)	(0.52)
Total RBC folate (nmol/L) [2]	Smoking	Intercept-No	1005.2 (71.7)	0.19	1200.9 (68.0)	0.07
		Yes	-380.1 (171.9)	**(0.038)**	-189.7 (155.7)	(0.24)
	Alcohol	Intercept-No	1009.7 (130.6)	0.02	897.4 (153.7)	0.16
		Yes	-103.7 (156.5)	(0.51)	312.6 (166.0)	**(0.075)**
	Vitamin use	Intercept-No	926.4 (90.0)	0.002	1072.1 (63.3)	0.29
		Yes	31.8 (152.6)	(0.84)	326.5 (118.4)	**(0.013)**
RBC 5-MTHF (nmol/L)	Smoking	Intercept-No	984.6 (70.6)	0.19	1155.8 (67.1)	0.06
		Yes	-375.4 (169.4)	**(0.038)**	-175.5 (153.8)	(0.27)
	Alcohol	Intercept-No	987.5 (128.0)	0.02	875.0 (153.4)	0.14
		Yes	-98.0 (153.5)	(0.53)	288.5 (165.7)	**(0.098)**
	Vitamin use	Intercept-No	910.5 (88.3)	0.001	1028.9 (62.0)	0.29
		Yes	25.2 (149.6)	(0.87)	326.9 (116.1)	**(0.011)**
RBC THF (nmol/L)	RBC 5,10-MTHF	Intercept-ND[3]	17.9 (1.8)	0.19	24.1 (9.9)	0.14
		D	13.8 (6.2)	**(0.037)**	23.3 (13.0)	**(0.089)**
Ratio RBC 5-MTHF: THF	RBC 5,10-MTHF	Intercept-ND	52.0 (3.4)	0.02	52.3 (6.2)	0.18
		D	-6.7 (11.5)	(0.57)	-16.4 (8.2)	**(0.059)**
Plasma 5-MTHF (nmol/L)	Smoking	Intercept-No	36.4 (3.8)	0.14	51.1 (5.5)	0.01
		Yes	-16.4 (9.0)	**(0.083)**	-4.4 (12.5)	(0.73)
	Vitamin use	Intercept-No	32.1 (4.5)	0.013	43.5 (5.1)	0.25
		Yes	4.0 (7.7)	(0.60)	23.7 (9.5)	**(0.022)**
B_{12} (pmol/L)	Smoking	Intercept-No	584.1 (49.8)	0.16	414.1 (41.6)	0.07
		Yes	-239.6 (119.5)	**(0.058)**	-110.8 (95.3)	(0.26)

The parameter estimate for the intercept refers to the mean, and in the second line the parameter estimate is the difference between the two categories. Comparisons with P-values <0.10 in at least one group are listed.

[1] *MTHFR* 677TT individuals were removed for this analysis.

[2] Total RBC folate = (RBC 5-MTHF) + (RBC THF) + (RBC 5, 10-MTHF)

[3] ND = Not Detectable, D = Detectable (Summers et al. 2010)

Biochemical Variables

Relationships between tHcy and folate derivatives. In African Americans tHcy concentrations were inversely associated with total RBC folate (p=0.022, Table 2-5) and RBC 5-MTHF levels (p=0.022) and trended towards being inversely associated with

RBC THF (p=0.12) while being positively associated with plasma 5-MTHF levels (p=0.028). In Caucasians the ratio of RBC 5-MTHF:THF was inversely related to tHcy concentrations (p=0.009) and accordingly tHcy was inversely associated with RBC 5-MTHF but only as a trend (p=0.12). In contrast to the inverse trend in African Americans, RBC THF levels were positively associated with tHcy concentrations in Caucasians (p=0.010).

Relationships among the folate derivatives. African Americans with detectable levels of RBC 5,10-MTHF had RBC THF levels that were 13.8 nmol/L higher than those with undetectable levels (31.7 vs 17.9 nmol/L, p=0.037, Table 2-4). In Caucasians a similar relationship was observed where detectable RBC 5,10-MTHF was associated with RBC THF levels that were 23.3 nmol/L higher than those with undetectable levels (47.4 vs 24.1 nmol/L, p=0.089). Only in Caucasians was detectable RBC 5,10-MTHF associated with a lower ratio of RBC 5-MTHF:THF (35.9 vs 52.3, p=0.059). This suggests that these two folate derivatives, THF and 5,10-MTHF, share some degree of metabolic control.

In African Americans, RBC 5-MTHF levels were positively associated with RBC THF levels (p=0.017, Table 2-5). Plasma 5-MTHF levels were positively associated with total RBC folate (p<0.0001), specifically with RBC 5-MTHF levels (p<0.0001) as well as the ratio of 5-MTHF:THF (p=0.001). In Caucasians, plasma 5-MTHF levels were positively associated with total RBC folate concentrations (p=0.017), specifically with RBC 5-MTHF levels (p=0.015). This relationship was similar to that observed in African Americans.

Relationships of tHcy and folate derivatives to B_{12} levels. In African Americans B_{12} levels were inversely associated with tHcy levels (p=0.049) and positively associated with total RBC folate (p=0.063), in particular with the folate derivatives 5-MTHF (p=0.069) and THF (p=0.028). In Caucasians, B_{12} levels were positively associated with total RBC folate only as a trend (p=0.13), in particular B_{12} was significantly associated with THF levels (p=0.018).

Table 2-5. Proportion of variation (R^2) in biochemical variables explained by selected biochemical phenotypes.

	Biochemical Phenotypes	**African-American (N=23)**		**Caucasian (N=21)[1]**	
Dependent Variables	**Explanatory Variables**	**Parameter Estimate (SE)**	**R^2 (P-value)**	**Parameter Estimate (SE)**	**R^2 (P-value)**
tHcy (µmol/L)	Total RBC folate (nmol/L) [2]	-0.003 (0.001)	0.23 **(0.022)**	-0.002 (0.001)	0.08 (0.22)
	RBC 5-MTHF (nmol/L)	-0.004 (0.001)	0.23 **(0.022)**	-0.002 (0.001)	0.12 (0.12)
	RBC THF (nmol/L)	-0.090 (0.056)	0.11 (0.12)	0.034 (0.012)	0.30 **(0.010)**
	Ratio RBC 5-MTHF: THF	-0.006 (0.035)	0.002 (0.86)	-0.054 (0.018)	0.31 **(0.009)**
	Plasma 5-MTHF (nmol/L)	0.065 (0.028)	0.21 **(0.028)**	-0.026 (0.019)	0.09 (0.19)
	B_{12} (pmol/L)	-0.004 (0.002)	0.17 **(0.049)**	0.003 (0.002)	0.07 (0.24)
Total RBC folate (nmol/L) [2]	Plasma 5-MTHF (nmol/L)	16.12 (2.46)	0.67 **(<0.0001)**	6.63 (2.52)	0.27 **(0.017)**
	B_{12} (pmol/L)	0.58 (0.29)	0.16 **(0.063)**	0.56 (0.35)	0.12 (0.13)
RBC 5-MTHF (nmol/L)	RBC THF (nmol/L)	18.06 (6.95)	0.24 **(0.017)**	0.46 (2.01)	0.003 (0.82)
	Plasma 5-MTHF (nmol/L)	16.05 (2.37)	0.69 **(<0.0001)**	6.65 (2.47)	0.28 **(0.015)**
	B_{12} (pmol/L)	0.56 (0.29)	0.15 **(0.069)**	0.46 (0.35)	0.08 (0.21)
RBC THF (nmol/L)	B_{12} (pmol/L)	0.018 (0.008)	0.21 **(0.028)**	0.09 (0.04)	0.26 **(0.018)**
Ratio RBC 5-MTHF: THF	Plasma 5-MTHF (nmol/L)	0.57 (0.15)	0.41 **(0.001)**	0.09 (0.21)	0.01 (0.68)

Parameter estimate refers to the change in units of the dependent variable for every 1 unit increase in the explanatory variable. Comparisons with P-values <0.10 in at least one group are listed. (Summers et al. 2010) [1]*MTHFR* 677TT individuals were removed for this analysis. [2]Total RBC folate = (RBC 5-MTHF) + (RBC THF) + (RBC 5, 10-MTHF)

Genetic associations with tHcy concentrations

In African Americans tHcy levels were 2.3 μmol/L higher in *TYMS* 1494del6 del/del homozygotes than insertion carriers (10.2 vs 7.9 μmol/L, p=0.023, Table 2-6). *MTR* 2756AA homozygotes had 2.7 μmol/L higher tHcy than 2756G carriers (10.4 vs 7.7 μmol/L, p=0.006). In Caucasians there were no *MTR* 2756GG homozygotes, however there was a significant difference between *MTR* 2756AA and 2756AG genotypes, specifically 2756AA homozygotes had 2.0 μmol/L higher tHcy than 2756AG heterozygotes (9.6 vs 7.6 μmol/L, p=0.017, Table 2-7). Caucasian *MTHFR* 677TT homozygotes had 3.2 μmol/L higher tHcy than 677C carriers (12.2 vs 9.0 μmol/L, p=0.012). *MTHFR* 1298C carriers had tHcy concentrations that were 1.8 μmol/L higher than 1298AA homozygotes (9.7 vs 7.9, p=0.031).

Genetic associations with folate derivatives

***MTR* 2756A>G.** African American *MTR* 2756AA homozygotes had total RBC folate concentrations that were 286.4 nmol/L lower than 2756G carriers (777.2 vs 1063.6 nmol/L, p=0.042, Table 2-6). In particular RBC 5-MTHF was 280.6 nmol/L lower in 2756AA homozygotes (760.7 vs 1041.3 nmol/L, p=0.043). *MTR* 2756AA homozygotes also had 14.1 nmol/L lower plasma 5-MTHF (25.5 vs 39.6 nmol/L, p=0.049). In Caucasians *MTR* 2756AA homozygotes had a lower ratio of 5-MTHF:THF (37.2 vs 54.2, p=0.061, Table 2-7), which indicated that this polymorphism was associated with the proportional distribution of these two key forms of folate.

***MTRR* 66A>G.** African American *MTRR* 66G carriers had higher levels of 5-MTHF and lower levels of THF than 66AA homozygotes, but neither of these differences were statistically significant. These differences were substantial enough for 66G carriers

Table 2-6. Proportion of variation (R^2) in biochemical variables explained by selected genotypes in African Americans.

			African-American (N=23)	
Dependent Variables	**Genotype**		**Parameter Estimate (SE)**	**R^2 (P-value)**
tHcy (µmol/L)	*MTR* 2756A>G	Intercept-AA	10.4 (0.7)	0.31
		G Carrier	-2.7 (0.9)	**(0.006)**
	TYMS 1494del6	Intercept-del/del	10.2 (0.7)	0.22
		Ins Carrier	-2.3 (0.9)	**(0.023)**
Total RBC folate (nmol/L) [1]	*MTR* 2756A>G	Intercept-AA	777.2 (99.2)	0.18
		G Carrier	286.4 (131.9)	**(0.042)**
RBC 5-MTHF (nmol/L)	*MTR* 2756A>G	Intercept-AA	760.7 (97.9)	0.18
		G Carrier	280.6 (130.2)	**(0.043)**
RBC THF (nmol/L)	*RFC1* 80A>G	Intercept-AA	25.7 (2.8)	0.30
		G Carrier	-10.2 (3.4)	**(0.007)**
Ratio RBC 5-MTHF: THF	*MTRR* 66A>G	Intercept-AA	45.4 (3.9)	0.21
		G Carrier	13.8 (5.9)	**(0.028)**
	TYMS 5' VNTR[2]	Intercept-3R/3R	41.3 (4.4)	0.32
		2R Carrier	17.2 (5.9)	**(0.010)**
	RFC1 80A>G	Intercept-AA	39.3 (4. 5)	0.35
		G Carrier	18.7 (5.5)	**(0.003)**
Plasma 5-MTHF (nmol/L)	*MTR* 2756A>G	Intercept-AA	25.5 (5.1)	0.17
		G Carrier	14.1 (6.8)	**(0.049)**
	TYMS 5' VNTR[2]	Intercept-3R/3R	26.1 (5.7)	0.16
		2R Carrier	14.4 (7.7)	**(0.077)**

P-values <0.10 are listed.
[1]Total RBC folate = (RBC 5-MTHF) + (RBC THF) + (RBC 5, 10-MTHF)
[2]Genotypes containing 4R were removed from the analysis. (Summers et al. 2010)

to have a higher ratio of 5-MTHF:THF (59.3 vs 45.5, p=0.028). In Caucasians there was no such relationship observed between this polymorphism and any of the folate derivatives.

***MTHFR* 677C>T.** In Caucasians, although total RBC folate concentrations were not significantly different between *MTHFR* 677C carriers and 677TT homozygotes, the individual folate derivatives were drastically different. *MTHFR* 677TT homozygotes had 426.3 nmol/L lower RBC 5-MTHF levels (696.0 vs 1122.3 nmol/L, p=0.007). RBC THF was 419.7 nmol/L higher in 677TT homozygotes (457.2 vs 37.5 nmol/L, p<0.0001).

Hence the ratio of RBC 5-MTHF:THF was drastically lower in 677TT homozygotes (5.4 vs 42.9, p=0.0004). RBC 5,10-MTHF was detectable in 4 out of 5 677TT homozygotes, in 10 out of 13 677CT heterozygotes, and in only 2 out of 8 677CC homozygotes (p=0.043, Table 2-8). The mean of the detectable concentrations was much higher in 677TT homozygotes than the means of the detectable concentrations in 677CC and CT genotypes (149.4, 9.8, and 9.7 nmol/L, respectively).

***MTHFR* 1298A>C.** Caucasian *MTHFR* 1298C carriers had 27.0 nmol/L higher RBC THF levels than 1298AA homozygotes (49.1 vs 22.1 nmol/L, p=0.046). Although RBC 5-MTHF levels were not significantly different, the ratio of 5-MTHF:THF was lower in 1298C carriers (33.4 vs 55.6, p=0.008).

***TYMS* 5' VNTR and 1494del6.** In African Americans *TYMS* 5' VNTR 2R carriers had higher ratios of 5-MTHF:THF than 3R/3R homozygotes (58.5 vs 41.3, p=0.010). 2R carriers also had 14.4 nmol/L higher plasma 5-MTHF (40.5 vs 26.1 nmol/L, p=0.077). In Caucasians *TYMS* 1494del6 deletion carriers had total RBC folate levels that were 218.1 nmol/L higher than ins/ins homozygotes (1238.0 vs 1019.9 nmol/L, p=0.096). Specifically RBC 5-MTHF levels were 217.4 nmol/L higher in 1494del6 deletion carriers (1194.8 vs 977.4 nmol/L, p=0.092). Plasma 5-MTHF levels were 21.7 nmol/L higher in 1494del6 deletion carriers (57.5 vs 35.8 nmol/L, p=0.030).

***RFC1* 80A>G.** African American *RFC1* 80G carriers had 10.2 nmol/L lower RBC THF levels than 80AA homozygotes (15.5 vs 25.7 nmol/L, p=0.007). Even though RBC 5-MTHF levels were not statistically different, 80G carriers had ratios of 5-MTHF:THF that were higher than 80AA homozygotes (58.0 vs 39.3, p=0.003). A similar

relationship was observed in Caucasians, 80G carriers had higher ratios of 5-MTHF:THF (49.5 vs 26.5, p=0.012).

Table 2-7. Proportion of variation (R^2) in biochemical variables explained by selected genotypes in Caucasians.

			Caucasian (N=26)	
Dependent Variables	**Genotype**		**Parameter Estimate (SE)**	**R^2 (P-value)**
tHcy (µmol/L)	*MTHFR* 677C>T	Intercept-C Carrier TT	8.9 (0.5) 3.2 (1.2)	0.24 **(0.012)**
RBC 5-MTHF (nmol/L)	*MTHFR* 677C>T	Intercept-C Carrier TT	1122.3 (63.6) -426.3 (145.0)	0.26 **(0.007)**
RBC THF (nmol/L)	*MTHFR* 677C>T	Intercept-C Carrier TT	37.5 (29.4) 419.7 (67.1)	0.62 **(<0.0001)**
Ratio RBC 5-MTHF: THF	*MTHFR* 677C>T	Intercept-C Carrier TT	42.9 (4.0) -37.6 (9.2)	0.41 **(0.0004)**
			Caucasian (N=21)[1]	
Homocysteine (µmol/L)	*MTHFR* 1298A>C	Intercept-AA C Carrier	7.9 (0.6) 1.8 (0.8)	0.22 **(0.031)**
	MTR 2756A>G	Intercept-AA AG	9.6 (0.5) -2.0 (0.8)	0.27 **(0.017)**
Total RBC folate (nmol/L) [2]	*TYMS* 1494del6	Intercept-ins/ins Del Carrier	1019.9 (101.7) 218.1 (124.6)	0.14 **(0.096)**
RBC 5-MTHF (nmol/L)	*TYMS* 1494del6	Intercept-ins/ins Del Carrier	977.4 (100.2) 217.4 (122.7)	0.14 **(0.092)**
RBC THF (nmol/L)	*MTHFR* 1298A>C	Intercept-AA C Carrier	22.1 (9.6) 27.0 (12.7)	0.19 **(0.046)**
Ratio RBC 5-MTHF: THF	*MTHFR* 1298A>C	Intercept-AA C Carrier	55.6 (5.6) -22.1 (7.4)	0.32 **(0.008)**
	MTR 2756A>G	Intercept-AA AG	37.2 (5.0) 17.0 (8.6)	0.17 **(0.061)**
	RFC1 80A>G	Intercept-AA G Carrier	26.5 (7.0) 23.0 (8.3)	0.29 **(0.012)**
Plasma 5-MTHF (nmol/L)	*TYMS* 1494del6	Intercept-ins/ins Del Carrier	35.8 (7.5) 21.7 (9.2)	0.23 **(0.030)**

P-values <0.10 are listed. (Summers et al. 2010)
[1]*MTHFR* 677TT individuals were removed for this analysis.
[2]Total RBC folate = (RBC 5-MTH) + (RBC THF) + (RBC 5, 10-MT)

Table 2-8. Distribution of *MTHFR* 677 genotypes in Caucasians by detection of RBC 5,10-MTHF.

Genotype		RBC 5,10-MTHF			
		Not Detectable (n)	Detectable (n)	P value	Detectable Mean (nmol/L) [Range][1]
MTHFR 677	CC	6	2	0.043	9.8 [6.8-12.7]
	CT	3	10		9.7 [4.9-20.0]
	TT	1	4		149.4 [74.6-224.7]

[1]Mean of individuals having detectable levels. (Summers et al. 2010)

Folate Phenotypes

Type I-IV folate phenotypes were defined in the publication of the LC/MRM/MS method of RBC folate derivative measurements (Huang et al. 2008). A Type I distribution of RBC folate derivatives was considered to be 5-MTHF >95% of total folate with THF and 5,10-MTHF each <5%. A Type II was considered to be 5-MTHF <95% with THF 5-20% and 5,10-MTHF still <5%. A Type III was 5-MTHF >55% with THF >20% and 5,10-MTHF >5%. A Type IV was 5-MTHF <55% with THF >20% and 5,10-MTHF >5%. A depiction of Types I-IV with the Caucasians in this study is presented in Figure 2-3. When the number of individuals in each folate phenotype was broken down by MTHFR 677/1298 combined genotypes, it was obvious that the double heterozygotes (677CT/1298AC) and the 677TT/1298AA homozygotes were associated with Types II-IV, while the four other combined genotypes were strictly Type I (Table 2-9).

Figure 2-3. Relative amounts of RBC folate derivatives in each folate phenotype in Caucasians.

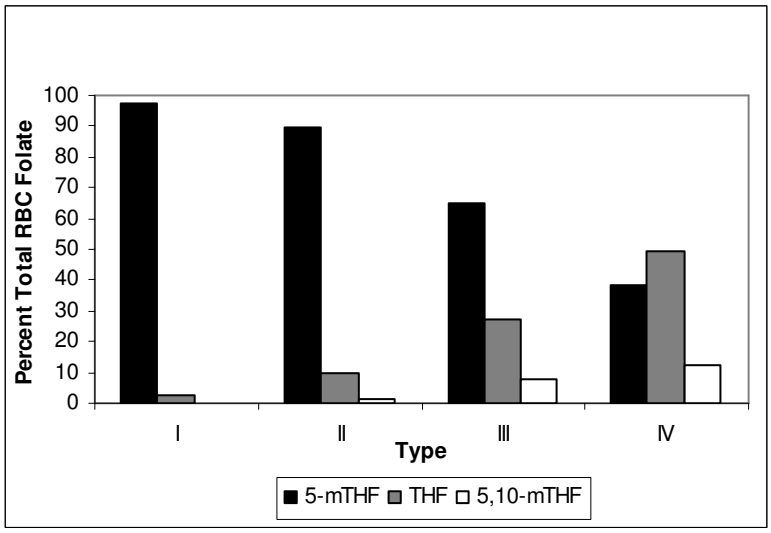


Table 2-9. Number of Caucasians in each folate phenotype by MTHFR 677/1298 combined genotypes.

MTHFR 677/1298	Type I	II	III	IV
CC/AA	4	0	0	0
CC/AC	2	0	0	0
CC/CC	2	0	0	0
CT/AA	5	0	0	0
CT/AC	5	3	0	0
TT/AA	1	0	1	3

(n)

2.5 Discussion

High Hcy and low folate are associated with cardiovascular disease, which is relevant to the male population in the Industrial Workers study. Therefore, polymorphisms or interactions of polymorphisms which affect Hcy and folate levels could alter an individual's risk of developing cardiovascular disease. *MTHFR* 677TT individuals have high tHcy levels and low folate levels in the Industrial Workers Study and other populations (Harmon et al. 1996; Jacques et al. 1996). *MTHFR* 677 TT individuals not only have altered folate/Hcy phenotypes but also they have altered risk of

diseases, whish are associated with high Hcy and low folate. A meta-analysis has established that *MTHFR* 677TT individuals are at 20% increased risk of venous thrombosis compared to 677CC individuals (Den Heijer et al. 2005).

Large-scale studies by Dekou et al. (2001) and Fredriksen et al. (2007) found that *CBS* 844ins68 carriers had Hcy levels significantly lower than noncarriers. Other studies have not been able to find a statistically significant difference in fasting Hcy levels between *CBS* 844ins68 genotypes (De Stefano et al. 1998; Kluijtmans et al. 2003; Tsai et al. 2000; Tsai et al. 2009). Since the 844ins68 allele is reported to result in skipping of the inserted sequence, the proposed mechanisms by which the 844ins68 allele could have an effect are: an increase in CBS activity, an up regulation of the amount of mRNA, or perhaps this polymorphism is in linkage disequilibrium with another functional polymorphism (Tsai et al. 1996; Tsai et al. 1999).

MTHFR 677C>T and *CBS* 844in68 genotypes were shown to interact and effect Hcy levels in two studies in European males. The first study included several countries in Europe and found that the *CBS* 844ins68 allele may counterbalance the Hcy raising effect of the *MTHFR* 677TT genotype (De Stefano et al. 1998). The second study was from the United Kingdom and confirmed this finding (Dekou et al. 2001) while a third study also in the UK did not replicate this finding in a population at high risk for coronary artery disease (Bowron et al. 2005). The Industrial Workers study was based on a population of Northern Irish males and the results supported the finding that *MTHFR* 677TT homozygotes who were also *CBS* 844ins68 allele carriers had significantly lower tHcy levels than 677TT/844ins68 noncarriers. In addition serum folate concentrations were higher in 677TT/844ins68 carriers and this was a novel finding. Concentrations of tHcy

and folate in 677TT/844ins68 carriers were similar to those found in 677CC and 677CT individuals. The interaction of these polymorphisms has an impact on Hcy and folate phenotypes, therefore it is possible that gene-gene interactions could contribute to risk for diseases such as cardiovascular disease. The excess risk conferred by *MTHFR* 677TT homozygosity may be negated by *CBS* 844ins68 carrier status. Large studies with enough power to examine gene-gene interactions will be required to test this observation and to examine the effects of gene-gene interactions on disease risk.

A high Hcy and low folate phenotype is associated with pregnancy complications such as neural tube defects (NTDs), which is relevant to the female population in the Premenopausal Women study. The first proof that multivitamins containing folic acid could prevent NTDs came from a nonrandomized controlled trial by Smithells et al. (1980), which showed 0.6% of mothers given multivitamins had a child with an NTD compared to 5% of unsupplemented mothers who had a child with an NTD. The Premenopausal Women study represents a relevant population in which to study the effects of genetic polymorphisms and lifestyle factors on Hcy and folate levels. In this study African American and Caucasian analyses were carried out separately for several reasons. Biochemical variables as well as genotype distributions were significantly different between the two groups. African American and Caucasian women have different prevalences of pregnancy related complications. Caucasians have a higher prevalence of carrying a child with spina bifida compared to African Americans (Feuchtbaum et al. 1999) and conversely, African Americans have a higher prevalence of preeclampsia (Eskenazi et al. 1991), low-birth-weight infants, and preterm birth (Heron et al. 2010). An individual woman's risk for poor pregnancy outcome is multifactorial and

complex, but may be due to lifestyle factors and genetic background. Therefore the Premenopausal Women Study was designed to use high precision methodology to compare the effect of folate related genotypes and lifestyle factors on biochemical phenotypes in a small number of African American and Caucasian women in order to elucidate the reasons for the differences in risk detailed above.

The MTHFR 677TT genotype is associated with high Hcy and low folate levels (Harmon et al. 1996; Jacques et al. 1996), and with increased risk for NTDs (van der Put et al. 1995; Whitehead et al. 1995). In the Premenopausal Women study there were no *MTHFR* 677TT homozygotes in African Americans and there were 5 (19.2%) in Caucasians. We observed that *MTHFR* 677TT homozygotes had higher tHcy levels and altered RBC folate distributions compared to 677C carriers. RBC folate distributions in 677TT homozygotes consisted of relatively high THF and 5,10-MTHF and relatively low 5-MTHF levels. These quantitative changes resulted in ratios of RBC 5-MTHF:THF that were lower in 677TT homozygotes. These altered RBC folate distributions are consistent with a study by Bagley and Selhub (1998) which found that *MTHFR* 677TT homozygotes had low RBC 5-MTHF and high RBC formylated THF levels. Since the 677TT genotype was responsible for a large amount of the variation present in tHcy and folate levels, biochemical and genetic analyses in the Premenopausal Women study were performed in the subset of Caucasians that excluded the 677TT homozygotes.

Logically polymorphisms in enzymes of the folate/Hcy pathway should be associated with risk for NTDs based on their association with changes in the levels of Hcy and folate. The following polymorphisms confer risk via the mother's genotype: *MTR* 2756G carriers, *MTRR* 66A carriers, *MTHFD1* 1958AA, *RFC1* 80GG, and *DHFR*

c.86+60_78 del/del (Brody et al. 2002; De Marco et al. 2003; Doolin et al. 2002; Johnson et al. 2004; Wilson et al. 1999). Polymorphisms which have an effect on Hcy and folate levels in the Premenopausal Women Study therefore are relevant to risk for carrying a child with NTDs. Genotype distributions for 5 of the 11 polymorphisms which we studied were significantly different between African American and Caucasian women. Of the above mentioned risk-associated genotypes only *MTHFR* 677TT and *MTHFD1* 1958AA had higher frequencies in Caucasians while the other above mentioned genotype distributions were not significantly different. In Caucasians, the *MTHFD1* 1958G>A polymorphism was the only one that was not in Hardy-Weinberg equilibrium. This observation has been documented in other control populations, such as the Irish (Parle-McDermott et al. 2006a).

In the Premenopausal Women study polymorphisms other than *MTHFR* 677C>T had a significant effect on folate/Hcy phenotype. The ratio of RBC 5-MTHF:THF may be a better indicator of alterations in RBC folate distribution than the absolute concentration of either of the individual folate derivatives. The following findings involving the ratio of 5-MTHF:THF have not been previously investigated and are therefore novel. The *MTHFR* 1298A>C polymorphism has conflicting associations in the literature. The 1298C allele has been associated with both increased RBC folate (Parle-McDermott et al. 2006b) and increased Hcy (Ulvik et al. 2007). In the Premenopausal Women study Caucasians 1298C carriers had increased tHcy and were consistent with the finding by Ulvik et al. (2007). Also we found that Caucasian 1298C carriers had increased RBC THF and a lower ratio of 5-MTHF:THF.

The *MTR* 2756A>G polymorphism is associated with decreased Hcy levels (Fredriksen et al. 2007; Harmon et al. 1999; Tsai et al. 2009) and in the Premenopausal Women study both African American and Caucasian 2756G carriers had lower tHcy levels. Only African American 2756G carriers had higher total RBC folate, in particular RBC 5-MTHF, and higher plasma 5-MTHF. This agrees with a study by Chen et al. (2001) which also found that *MTR* 2756GG homozygotes had higher plasma folate and lower Hcy levels.

The *TYMS* 1494del6 polymorphism has been associated with increased RBC folate and decreased Hcy levels (Kealey et al. 2005). Results from the Premenopausal Women study were consistent; Caucasian deletion carriers had higher total RBC folate, in particular RBC 5-MTHF, and also had higher plasma 5-MTHF concentrations. In contrast to Caucasians, African American insertion carriers had lower tHcy levels. African Americans had a different distribution of *TYMS* 1494del6 genotypes than Caucasians such that there were not enough ins/ins individuals (n=2) to be analyzed separately and were therefore combined with the heterozygous genotype for analysis. In Caucasians the same was true for del/del individuals (n=2) and hence the small number of study subjects may be limiting the analyses of 'rare' homozygotes. The other polymorphism in *TYMS* examined in the Premenopausal Women study was the 5' VNTR with 2 or 3 repeats (2R or 3R). African American 2R carriers had higher plasma 5-MTHF levels. This was in agreement with a study by Trinh et al. (2002), which showed that 3R/3R homozygotes had lower plasma folate levels. Also in the Premenopausal Women study African American 2R carriers had higher ratios of RBC 5-MTHF:THF.

The *RFC1* 80A>G polymorphism is associated with lower RBC folate concentrations (Stanislawska-Sachadyn et al. 2009). Our study found that African American 80G carriers had lower RBC THF levels and that in both African Americans and Caucasians 80G carriers had a higher ratio of RBC 5-MTHF:THF. It is unclear though how this polymorphism, which is in a transport protein, would have an effect on the distribution of RBC folates.

Multiple reports have established that Hcy and folate levels have an inverse relationship (Kang et al. 1987; Selhub et al. 1993). In the Premenopausal Women study total RBC folate, and in particular RBC 5-MTHF, had inverse relationships with tHcy in African Americans. In Caucasians RBC THF had a direct association with tHcy while the ratio of RBC 5-MTHF:THF had an inverse relationship with tHcy. This well established inverse relationship between Hcy and folate levels may not be so simple. Observations from our study indicate that individual folate derivatives or the ratio of RBC 5-MTHF:THF may be more useful in predicting Hcy concentrations. Short term pools of folate represented by plasma folate should be directly related to long term pools of folate represented by RBC folate. In the Premenopausal Women study plasma 5-MTHF levels had a direct association with RBC 5-MTHF levels in both African Americans and Caucasians.

Vitamin B_{12} levels were inversely associated with Hcy and directly associated with folate levels in several studies (Konstantinova et al. 2007; Thuesen et al. 2010). In African Americans in the Premenopausal Women study B_{12} levels had an inverse relationship with tHcy and with the ratio of RBC 5-MTHF:THF and a direct relationship with total RBC folate, RBC 5-MTHF, and RBC THF. In Caucasians B_{12} levels only had a

direct relationship with RBC THF. These relationships indicate that B_{12} levels are important determinants of the generation of RBC THF from 5-MTHF, because MTR requires B_{12} as a cofactor in this reaction.

Smoking has been associated with higher Hcy and lower folate levels (Brown et al. 2004b; Gabriel et al. 2006; Stanislawska-Sachadyn et al. 2008b) as well as poor pregnancy outcome, including orofacial clefts, preterm birth, and low-birth-weight infants (Meyer et al. 1976; Wyszynski et al. 1997). In the Premenopausal Women study African American smokers had lower total RBC folate, specifically 5-MTHF, as well as lower plasma 5-MTHF, which is consistent with other studies that found lower RBC folate in Caucasian smokers (Brown et al. 2004b; Gabriel et al. 2006).

In the literature alcohol use is associated with higher Hcy levels (Jacques et al. 2001; Laufer et al. 2004) and contradictorily with higher folate levels (Ubbink et al. 1998). African American alcohol users in the Premenopausal Women study had higher tHcy levels while Caucasian alcohol users had higher total RBC folate, in particular 5-MTHF. There are two plausible reasons that alcohol intake could be associated with higher folate levels. It is possible that the primary alcoholic beverage consumed was beer which contains folic acid (Ubbink et al. 1998) or that alcohol is capable of inhibiting MTR and causes a methylfolate trap, allowing for the accumulation of 5-MTHF (Mason and Choi 2005).

Caucasians have a higher risk of carrying a child with spina bifida compared to African Americans (CDC 2009). The explanation for this difference in prevalence may lie in the differences in genotype frequencies and in the effect of lifestyle factors on biochemical phenotypes. The quantitatively precise methods used in this study to

measure individual folate derivatives allowed a detailed description of the effect of the polymorphisms in enzymes of the folate/Hcy pathway. Genetic and lifestyle factors which contribute to a dysregulation of folate/Hcy metabolism may increase risk for various clinical conditions, such as carrying a child with spina bifida. This study was relatively small and these preliminary findings should be confirmed in larger studies using similar analytical methods.

Chapter 3: Polymorphisms Associated with the Risk of Neural Tube Defects

3.1 Abstract

Spina bifida is a neural tube defect which is associated with high Hcy and low folate. Spina bifida can be prevented with periconceptional supplementation with folic acid. The mechanism by which folate can prevent spina bifida is unknown. Common functional polymorphisms within genes of the enzymes of the folate/Hcy metabolic pathway are good candidates for examining the effect of genetic variants on the risk of spina bifida. These genetic variants are capable of conferring risk at both levels, the risk of the child and the risk of the mother for carrying a child with spina bifida.

In the Neural Tube Defects study, which included approximately 500 families, we examined 7 polymorphisms within 5 genes of the enzymes of the folate/Hcy pathway. Transmission disequilibrium tests (TDT) were applied to child trios and mother trios to check for preferential transmission of any allele at both the level of the child and the mother. Hardy-Weinberg equilibrium was also assessed within each of the five subject types, which included children, mothers, fathers, maternal grandmothers, and maternal grandfathers, as a measure of deviation from the expected, and hence a possible causative genotype.

We did not find any of the 7 polymorphisms to be significant within the TDT analyses. There were only two polymorphisms which deviated from Hardy-Weinberg equilibrium, which were *MTHFR* 677C>T in fathers and *TYMS* 1494del6 in children. In both instances the observed genotypes lacked a significant number of expected heterozygous genotypes while both wild-type and variant homozygous genotypes were equally overrepresented. This did not provide any clear evidence of at risk genotypes.

3.2 Introduction

3.2.1 Neural Tube Defects

Neural tube defect (NTD) is a general term which includes the birth defects spina bifida, anencephaly, and encephalocele. Spina bifida is itself a broad term for a group of medical conditions (such as myelomeningocele and meningocele). More specifically myelomeningocele is an open defect where a herniation of neural tissue is present as a saclike membrane on the spine due to failure of fusion of the neural tube. Meningocele is a closed defect, skin covered, where the meninges are herniated through the spine (Mitchell 2008). Anencephaly refers to failure of the neural tube to close resulting in missing parts of the brain and skull. Encephalocele refers to a saclike protrusion of the brain through the skull, again caused by failure of the neural tube to close completely.

Spina bifida is a common congenital defect with a prevalence ranging from 0.2-3 per 1,000 births according to regional and population-specific variation. The health costs associated with medical care for an individual with spina bifida are substantial because of increased risk for hydrocephalus, leg weakness and paralysis, bowel and bladder dysfunction, and orthopedic abnormalities such as hip dislocation (Mitchell et al. 2004).

3.2.2 Folic Acid Fortification

In 1991 a study was published by the Medical Research Council which showed that folic acid had a 72% protective effect (RR 0.28 [0.12-0.71]) in women who previously had a pregnancy affected by a NTD (Medical Research Council 1991). This established that periconceptional folic acid could prevent recurrence of NTDs. Subsequently a study by Cziezel and Dudas (1992) showed that first occurrence of NTDs could be prevented by periconceptional folic acid. Although the specific mechanism by

which folic acid provides a protective effect is unknown, the US Public Health service in 1992 recommended that women planning to get pregnant should have 400 μg of folic acid per day (CDC 1992). This recommendation was supported by the results of a large nonrandomized intervention study which took place in the north and south regions of China (Berry et al. 1999). The protective effect of 400 μg folic acid was greater in the northern region which had a higher prevalence of NTDs, but still showed a protective effect in the southern region.

United States

Mandatory folic acid fortification of grain products was in place by 1998. It was designed to improve the folate status of reproductive aged women by increasing folic acid intake by an estimated 100 μg a day. A study by Jacques et al. (Jacques et al. 1999) compared pre- and post-fortification levels of folate and Hcy and found significantly increased folate and decreased Hcy concentrations as well as a reduction in the prevalence of folate deficiency (<7 nmol/L in plasma) from 22.0% to 1.7% ($p<0.001$) and also a reduction in the prevalence of hyperhomocysteinemia (>13 μmol/L) from 18.7% to 9.8% ($p<0.001$). Rates of spina bifida births declined by approximately 20% after fortification (Honein et al. 2001; Williams et al. 2002).

Canada

Folic acid fortification of cereal grain products was mandatory by 1998 in Canada providing an estimated additional intake of 200 μg a day (Ray et al. 2002b). RBC folate levels in women of reproductive age increased by 41% after fortification (Ray et al. 2002a). The prevalence of NTDs had declined by 46% when pre- fortification was compared with post-fortification rates (De Wals et al. 2007).

South America

In Chile the Ministry of Health has required the addition of folic acid to wheat flour since 2000. The estimated additional intake of folic acid was designed to be 400 μg a day. Fortification caused both serum and RBC folate levels to increase in women of reproductive age when pre- and post-fortification levels were compared (Hertrampf et al. 2003). The prevalence of NTDs decreased by 31% after fortification (Castilla et al. 2003).

Concerns

Reservations about implementing folic acid fortification include the masking of B_{12} deficiency and increasing the risk for cancer. B_{12} deficiency is common in the elderly due to loss of ability to digest and absorb B_{12} from food. High intakes of folic acid may appear to cure macrocytic anemia, which is often the result of B_{12} deficiency (Johnson 2007). In a recent study in the elderly high serum folate levels accompanying B_{12} deficiency were associated with twice the rate of anemia and cognitive impairment when compared with normal serum folate levels and B_{12} deficiency (Morris et al. 2007). As for cancer risk, in a study in ischemic heart disease patients those taking folic acid had an increased rate of cancer, hazard ratio (HR) 1.21 [1.03-1.41] (Ebbing et al. 2009). Folic acid supplementation is also associated with increased risk of colorectal neoplasia in those with a recent history of colorectal adenomas (Cole et al. 2007), and hence the worry is that pre-existing cancers will grow.

3.2.3 Selected Polymorphisms Associated with NTDs

The process of neural tube closure involves tightly controlled expression of genes, which relies upon specific methylation patterns, proliferation/apoptosis, migration, and

differentiation of specific cells (Beaudin and Stover 2007). Many of these processes overlap with important functions of the folate/Hcy pathway such as methylation of DNA and proteins as well as nucleotide synthesis. Compared to mothers that had normal pregnancies, mothers that gave birth to children with spina bifida had lower folate levels and also higher Hcy concentrations (Lucock et al. 1998). This dysregulation of folate/Hcy phenotype has led to the examination of genetic variants in candidate genes, specifically of common functional polymorphisms within enzymes of the folate/Hcy metabolic pathway.

Family history of NTDs is a strong risk factor and in siblings of affected individuals risk of NTD ranges from 2.4-5.2%, which is much higher than prevalences in the general population (Carter and Evans 1973; Hunter 1984). There are two basic study types which are capable of analyzing the risk between genetic variants and NTDs. A case-control study compares genotype frequencies between these two groups and arrives at an estimate of the odds ratio (OR). A family-based study uses either the transmission disequilibrium test (TDT) or log-linear modeling to assess the transmission of alleles or the frequencies of mating types (parents' genotypes) and outcomes (child's genotype), respectively. Another less specific test for association is whether the genotypes are in Hardy-Weinberg equilibrium. Overrepresentation of a homozygous genotype above the expected value calculated from the Hardy-Weinberg equation could mean that the genotype is associated with the medical condition. This test is less specific because there is a range of reasons that genotype frequencies could deviate from Hardy-Weinberg equilibrium, including inbreeding, non-random mating, selection, or even a problem with the genotyping method itself.

MTHFR 677C>T and 1298A>C

Two studies initially found that the *MTHFR* 677TT genotype was associated with increased risk of spina bifida via case-control studies (van der Put et al. 1995; Whitehead et al. 1995). Some studies were not able to show that this polymorphism confers risk for NTDs (Mornet et al. 1997; Papapetrou et al. 1996). A meta-analysis by Botto and Yang (2000) confirmed that the *MTHFR* 677TT genotype carried increased risk for spina bifida in both infants as well as mothers with a pooled odds ratio of 1.8 and 2.0, respectively. Subsequently Kirke et al. (2004) showed that the 677CT genotype also carried increased risk in a case-control study. When the population attributable fraction was calculated the 677CT genotype was responsible for a similar number of NTDs within the Irish population as the 677TT genotype (14.9% vs 11.3%, respectively).

When MTHFR 1298A>C genotypes were studied in combination with the 677C>T genotypes in a NTD study from the Netherlands, none of the combination genotypes had a significantly increased OR (van der Put et al. 1998), but *in vitro* experiments on MTHFR enzyme activity showed the 677CT/1298AC double heterozygotes had decreased activity compared to heterozygosity for either polymorphism alone. An Italian NTD study found increased risk for 1298C carriers in children and mothers (OR 2.2 and 2.7, respectively) although there was no stratification by 677C>T genotypes (De Marco et al. 2002).

CBS 844ins68

CBS 844ins68 genotypes were not associated with the risk of NTDs in an Irish study (Ramsbottom et al. 1997). It was noted that in this study among those who were *MTHFR* 677TT homozygotes there was a higher percentage of *CBS* 844ins68 carriers in

children with NTDs compared to controls (27.0% vs 12.8%, respectively) but this was not statistically significant. Reanalysis of this study's data by Botto and Mostroiacovo (1998) found significantly increased risk of NTDs in *MTHFR* 677TT/*CBS* 844ins68 carriers, OR 5.2 [1.4-21.2]. Another study found similar results that the *CBS* 844ins68 polymorphism alone carries no significant risk but that the combination genotype *MTHFR* 677TT/*CBS* 844ins68 carrier had an OR of 3.69 [1.04-13.50] (de Franchis et al. 2002). A UK study also found that *MTHFR* 677C>T and *CBS* 844in68 interacted to significantly increase NTD risk in children (Relton et al. 2004 Apr). A study in Germany was unable to replicate these findings using case-control and TDT analyses (Richter et al. 2001). Overall the evidence is compelling that this combination of genotypes may increase risk for NTDs. Although this combination of genotypes has been associated with lower Hcy concentrations in men (De Stefano et al. 1998; Dekou et al. 2001; Summers et al. 2008b), it is possible that during development it could have a different effect.

TYMS 5' VNTR and 1494del6

A study by Volcik et al. (2003) found that in Caucasians both the *TYMS* 1494del6 ins/ins genotype, OR 3.6 [1.3-10.1], and the *TYMS* 5' VNTR 2R/2R genotype, OR 4.0 [1.8-8.8], were associated with increased NTD risk in children. Also the combined *TYMS* ins/ins and 2R/2R genotype was significantly associated with increased risk, OR 4.7 [1.1-19.8]. A subsequent study in the UK did not find any association between *TYMS* 5' VNTR genotypes and risk of NTDs using case-control and TDT analyses (Wilding et al. 2004).

DHFR c.86+60-78

In a US study by Johnson et al. (2004) found that the *DHFR* c.86+60_78 del/del genotype was more frequent in mothers than controls, OR 2.04 [0.94-4.29], and genotype frequencies were not in Hardy-Weinberg equilibrium in mothers. In contrast a study found that the *DHFR* c.86+60_78 deletion allele provided a protective effect in mothers in an Irish population (Parle-McDermott et al. 2007). A study in the Netherlands did to find any association between *DHFR* c.86+60_78 genotypes and NTD risk in a case-control study (van der Linden et al. 2007).

MTHFD1 1958G>A

A study by Brody et al. (2002) in the Irish showed that the *MTHFD1* 1958AA genotype was overrepresented in mothers of NTD children compared to controls, OR 1.52 [1.16-1.99]. A second Irish study confirmed that the *MTHFD1* 1958AA genotype was associated with increased NTD risk in mothers, OR 1.49 [1.07-2.09] (Parle-McDermott et al. 2006a), which was consistent with their earlier finding using a separate cohort. In an Italian population it was found that both 1958GA (OR 1.69) and 1958AA (OR 1.91) individuals had increased risk of NTD in children (De Marco et al. 2006). Also this study found that mothers who were carriers of the 1958A allele had increased risk (OR 1.67) and that TDT analyses confirmed there was a significant excess transmission of the 1958A allele.

The selected polymorphisms discussed above were chosen based on their association with NTD risk and were genotyped in a large family based study in the US population.

3.3 Methods

3.3.1 NTD Study

Subjects

The NTD study included 671 families with at least one member affected with spina bifida., as previously described (Mitchell 2008). The family structure that this study intended to capture was a "pent". A complete pent includes the affected child, mother, father, and maternal grandparents. This design allows for assessment of maternal and embryonic genetic effects. Families were recruited between November 1997 and April 2006 by the Spina Bifida Research Resource through the Children's Hospital of Philadelphia in Philadelphia, Pennsylvania; the Alfred I. Dupont Hospital for Children in Wilmington, Delaware; local chapters of the Spina Bifida Association of America; colleagues; and the study website. The study was approved by the Institutional Review Boards of the Texas A&M University, the University of Pennsylvania School of Medicine, and each of the participating hospitals. Each subject gave informed consent and when appropriate assent was given. Interview information included pregnancy history, supplement use, and details of the affected individual's lesion.

Affected individuals and their families were excluded if spina bifida was determined to be syndromic (due to pregestational diabetes, teratogenic exposure to anticonvulsant medication, or suspected co-occurring syndrome such as deletion of 22q11), failure to complete the interview, or type of neural tube defect. Subsequent analyses were based on affected individuals with open spina bifida. The characteristics of the affected individuals include a slight predominance of females and a majority of non-Hispanic Caucasians as previously described (Mitchell 2008). It should be noted that

interview data was not directly accessible during these statistical analyses and therefore subgroup analyses are not included.

Genotyping

Genomic DNA was extracted from whole blood using Generation Capture Columns (Gentra Systems) or using QIAamp DNA Mini Kits (Qiagen) according to the manufacturer's instructions. DNA was extracted from saliva samples using Oragene kits (DNA Genotek Inc, Ontario, Canada) according to the manufacturer's instructions. DNA was also extracted from buccal swabs using the following procedure. Samples were vortexed for 5min in 600ul 50mM NaOH, heated at 95℃ for 10min and neutralized with 120ul 1M Tris (pH 8.0).

TaqMan genotyping methods were performed as previously described for *MTHFR* 677C>T (Summers et al. 2008a), *MTHFR* 1298A>C (Summers et al. 2008a), and *MTHFD1* 1958G>A (Summers et al. 2010). Genotypes were only accepted as valid for TaqMan runs in which the Fam or Vic signals crossed the threshold, which was set based on background signals of Fam and Vic, with Ct fewer than 40 cycles. The *MTHFR* 677C>T assay had a 10% failure rate compared to 1-3% for the other TaqMan assays; most of the *MTHFR* 677C>T assay failures occurred in DNA derived from buccal samples.

Size difference PCR assays were performed essentially as previously described for *CBS* 844ins68 (Summers et al. 2010), *DHFR* c.86+60_78, *TYMS* 1494del6 (Summers et al. 2008a), and *TYMS* 5' VNTR (Summers et al. 2010) with the following changes. The CBS 844ins68 assay used modified primer sequences, forward (5'-GGTTTCTCATCCTGCCTCTG-3') and reverse (5'-GTTGTCTGCTCCGTCTGGTT-

3'). *TYMS* 5'UTR genotypes were deemed unreliable in buccal samples and therefore genotypes were accepted for only blood and saliva samples, which were considered to be high quality DNA samples.

Statistics

All statistical analyses were performed with SAS version 9.1 (SAS Institute, Cary, NC). Hardy-Weinberg equilibrium was tested for all 7 polymorphisms within each of the subject types. The transmission disequilibrium test (TDT) (Spielman et al. 1993) determines whether the frequency of allele transmission from heterozygous parents to affected offspring differs significantly from the Mendelian expectation of equal transmission of the alleles. TDT analyses were calculated for each polymorphism in child trios (child, mother, and father) and mother trios (mother, maternal grandmother, and maternal grandfather). Individuals with TYMS 5' VNTR genotypes containing the 4R allele were excluded from the TDT analyses. Families with more than one affected child were included with one trio for each child.

3.4 Results

3.4.1 NTD Study

After applying exclusion criteria there were 490 children with spina bifida, 501 mothers, 399 fathers, 240 maternal grandmothers, and 182 maternal grandfathers. Genotype frequencies within each of the subject types are listed in Table 3-1. All genotypes were in Hardy-Weinberg equilibrium except for *MTHFR* 677C>T in fathers and *TYMS* 1494del6 in children. Deviation from Hardy-Weinberg equilibrium would be expected if a polymorphism was associated with risk of spina bifida. In fathers the expected values for *MTHFR* 677CC, CT, and TT genotypes were 153, 164, and 44,

respectively. There was a lack of heterozygotes and an almost equal over abundance of CC and TT individuals, which does not indicate a clear at risk genotype. The same was true for *TYMS* 1494del6 genotypes in children for which the expected values for ins/ins, ins/del, and del/del were 231, 204, and 45, respectively.

Table 3-1. Genotype frequencies in the NTD study.

		Child	Mother	Father	Maternal Grandmother	Maternal Grandfather
MTHFR 677	CC	197 (44.7)	210 (44.5)	161 (44.6)*	87 (42.7)	77 (51.3)
	CT	188 (42.6)	204 (43.2)	147 (40.7)	91 (44.6)	57 (38.0)
	TT	56 (12.7)	58 (12.3)	53 (14.7)	26 (12.8)	16 (10.7)
MTHFR 1298	AA	234 (50.2)	243 (49.2)	190 (49.1)	108 (47.6)	82 (48.0)
	AC	190 (40.8)	201 (40.7)	154 (39.8)	101 (44.5)	68 (39.8)
	CC	42 (9.0)	50 (10.1)	43 (11.1)	18 (7.9)	21 (12.3)
CBS 844ins68	W/W	407 (86.2)	402 (81.7)	334 (86.5)	182 (79.8)	145 (84.3)
	W/I	62 (13.1)	84 (17.1)	50 (13.0)	44 (19.3)	24 (14.0)
	I/I	3 (0.6)	6 (1.2)	2 (0.5)	2 (0.9)	3 (1.7)
MTHFD1 1958	GG	147 (31.3)	161 (32.9)	112 (29.0)	75 (32.6)	63 (36.4)
	GA	239 (51.0)	234 (47.9)	197 (51.0)	118 (51.3)	78 (45.1)
	AA	83 (17.7)	94 (19.2)	77 (20.0)	37 (16.1)	32 (18.5)
DHFR c.86+60_78	Ins/ins	154 (32.2)	165 (33.7)	124 (32.5)	75 (33.0)	57 (33.7)
	Ins/del	222 (46.4)	229 (46.8)	183 (47.9)	109 (48.0)	80 (47.3)
	Del/del	102 (21.3)	95 (19.4)	75 (19.6)	44 (19.0)	32 (19.0)
TYMS 1494del6	Ins/ins	243 (50.6)*	221 (44.3)	196 (49.6)	107 (45.3)	85 (48.9)
	Ins/del	180 (37.5)	220 (44.1)	165 (41.8)	105 (44.5)	71 (40.8)
	Del/del	57 (11.9)	58 (11.6)	34 (8.6)	24 (10.2)	18 (10.3)
TYMS 5' VNTR	2R/2R	65 (23.6)	65 (21.2)	49 (21.3)	24 (22.0)	20 (27.0)
	2R/3R	120 (43.6)	153 (50.0)	116 (50.4)	58 (53.2)	31 (41.9)
	3R/3R	87 (31.6)	86 (28.1)	63 (27.4)	27 (24.8)	23 (31.1)
	2R/4R	1 (0.4)	0	0	0	0
	3R/4R	2 (0.7)	2 (0.7)	2 (0.9)	0	0

N (%). *Hardy-Weinberg equilibrium $p<0.05$.

MTHFR 677 and 1298 genotypes were combined and are displayed in Table 3-2 by subject type. The *MTHFR* 677T allele is in linkage disequilibrium with the 1298A allele, and therefore the 677T allele does not occur on the same chromosome as the 1298C allele unless a very rare crossover event has occurred. There was evidence of two such chromosomal crossover events in the genotypes of this study, evidenced by *MTHFR*

677TT/1298AC and *MTHFR* 677CT/1298CC genotypes, which were both found in mothers.

Table 3-2. *MTHFR* 677/1298 combined genotypes.

		MTHFR 1298		
Child		AA	AC	CC
	CC	63 (14.4)	93 (21.3)	39 (8.9)
MTHFR 677	CT	101 (23.1)	86 (19.7)	0
	TT	55 (12.6)	0	0
		MTHFR 1298		
Mother		AA	AC	CC
	CC	60 (12.8)	99 (21.1)	48 (10.2)
MTHFR 677	CT	111 (23.7)	92 (19.6)	1 (0.2)
	TT	57 (12.2)	1 (0.2)	0
		MTHFR 1298		
Father		AA	AC	CC
	CC	44 (12.4)	71 (19.9)	42 (11.8)
MTHFR 677	CT	75 (21.1)	72 (20.2)	0
	TT	52 (14.6)	0	0
Maternal		*MTHFR* 1298		
Grandmother		AA	AC	CC
	CC	24 (11.9)	47 (23.3)	16 (7.9)
MTHFR 677	CT	47 (23.3)	44 (21.8)	0
	TT	24 (11.9)	0	0
Maternal		*MTHFR* 1298		
Grandfather		AA	AC	CC
	CC	25 (16.8)	32 (21.5)	20 (13.4)
MTHFR 677	CT	34 (22.8)	23 (15.4)	0
	TT	15 (10.1)	0	0

N (overall %).

TYMS 5' VNTR and 1494del6 genotypes were combined and are displayed in Table 3-3 by subject type. Since *TYMS* 5' VNTR genotypes were completed only on high quality DNA samples, there were combined genotypes available for only 275 children, 305 mothers, 230 fathers, 109 maternal grandmothers, and 73 maternal grandfathers. The *TYMS* 5' VNTR 2R allele is in linkage disequilibrium with the *TYMS* 1494del6 insertion allele, and therefore the 2R allele does not occur on the same chromosome as the 1494del6 deletion allele unless a very rare crossover event has occurred. The *TYMS*

2R/2R and del/del combined genotype only appeared in mothers and children and not in the other subject types. Also the *TYMS* 2R/3R and del/del combined genotype frequency was highest in children (2.6%) compared to mothers (2.0%), fathers (1.7%), maternal grandmothers (1.8%), and maternal grandfathers (absent). The *TYMS* 5' VNTR 4R allele is rare and only appeared in children, mothers, and fathers, although this could be due to the smaller number of maternal grandparents in the study.

None of the 7 polymorphisms had significant results in the TDT analyses in child trios and in mother trios (data not shown). An example of the data used in the TDT analyses is presented in Table 3-4 for *MTHFR* 677C>T in child trios. There were 304 complete child trios that had no *MTHFR* 677C>T genotypes missing, and 147 trios were considered to be non-informative mostly because both parents were homozygous. There were 191 heterozygous parents which provided informative transmissions and 90 (47.1%) of those were for the 677T allele.

Table 3-3. *TYMS* 5'VNTR/1494del6 combined genotypes.

Children		*TYMS* 1494del6 Ins/ins	Ins/del	Del/del
TYMS 5' VNTR	2R/2R	52 (18.9)	11 (4.0)	2 (0.7)
	2R/3R	67 (24.4)	46 (16.7)	7 (2.6)
	3R/3R	24 (8.7)	35 (12.7)	28 (10.2)
	2R/4R	1 (0.4)	0	0
	3R/4R	0	2 (0.7)	0

Mothers		*TYMS* 1494del6 Ins/ins	Ins/del	Del/del
TYMS 5' VNTR	2R/2R	48 (15.7)	15 (4.9)	1 (0.3)
	2R/3R	66 (21.6)	81 (26.6)	6 (2.0)
	3R/3R	21 (6.9)	41 (13.4)	24 (7.9)
	2R/4R	0	0	0
	3R/4R	1 (0.3)	0	1 (0.3)

Fathers		*TYMS* 1494del6 Ins/ins	Ins/del	Del/del
TYMS 5' VNTR	2R/2R	41 (17.8)	8 (3.5)	0
	2R/3R	59 (25.7)	53 (23.0)	4 (1.7)
	3R/3R	17 (7.4)	32 (13.9)	14 (6.1)
	2R/4R	0	0	0
	3R/4R	1 (0.4)	0	1 (0.4)

Maternal Grandmothers		*TYMS* 1494del6 Ins/ins	Ins/del	Del/del
TYMS 5' VNTR	2R/2R	16 (14.7)	8 (7.3)	0
	2R/3R	33 (30.3)	23 (21.1)	2 (1.8)
	3R/3R	5 (4.6)	16 (14.7)	6 (5.5)
	2R/4R	0	0	0
	3R/4R	0	0	0

Maternal Grandfathers		*TYMS* 1494del6 Ins/ins	Ins/del	Del/del
TYMS 5' VNTR	2R/2R	17 (23.3)	3 (4.1)	0
	2R/3R	17 (23.3)	14 (19.2)	0
	3R/3R	5 (6.9)	10 (13.7)	7 (9.6)
	2R/4R	0	0	0
	3R/4R	0	0	0

N (overall %).

Table 3-4. *MTHFR* 677C>T mating types in child trios with number of allele transmissions.

Mating Type (Mother x Father = Child)	Transmission of Specific Allele	Number of Transmissions n(%)
Informative Trios		
CC x CT = CC	C	101 (52.9)
CT x CC = CC		
CT x CT = CC*		
TT x CT = CT		
CT x TT = CT		
CC x CT = CT	T	90 (47.1)
CT x CC = CT		
CT x CT = TT*		
TT x CT = TT		
CT x TT = TT		
Non-Informative Trios		
CC x CC = CC		
CT x CT = CT		
TT x TT = TT		
CC x TT = CT		
TT x CC = CT		

*Counts as transmission of 2 C or 2 T alleles, respectively.

3.5 Discussion

The NTD study investigated 7 polymorphisms in enzymes of the folate/Hcy metabolic pathway for risk of NTDs. TDT analyses did not find over transmission of any of the alleles. Hardy-Weinberg equilibrium was distorted for two of the polymorphisms but examination of expected values revealed no over representation of either homozygous genotype. In *MTHFR* 677/1298 combined genotypes two rare crossover events were observed only in mothers' genotypes. In *TYMS* 5'VNTR/1494del6 combined genotypes children and mothers were the only groups that had the rare 2R/2R and del/del combined genotypes. It is possible that these rare combination genotypes may carry increased risk.

A large study in NTDs based in California examined 118 SNPs and found that 15 polymorphisms in 7 genes were significantly associated in children with spina bifida (Shaw et al. 2009). This California based study found that the *MTHFR* 677C>T

polymorphism was significantly associated OR 2.0 [1.2-3.1]. Another US study in NTDs which examined 28 polymorphisms in 11 genes found that only a SNP in *betaine-homocysteine methyltransferase* (*BHMT*) was significantly associated with NTDs in the overall data set, although the *MTHFR* 677C>T polymorphism was not associated with increased risk. The association with the polymorphism in *BHMT* was stronger in mothers who took folate containing supplements before conception and when the *MTHFR* 677T allele was also transmitted from parent to offspring (Boyles et al. 2006).

There have been several publications which found that specific genotypes were associated with risk for NTDs using the early phases of the NTD study before sample collection had been completed. It was previously published that increased NTD risk was associated with transcription factor *T* IVS7C allele in children (Jensen et al. 2004), *NOS3* 894T in children (Brown et al. 2004a), *MCP1* -2518AA in mothers (Jensen et al. 2006a), and that loss of function polymorphisms in *NAT1* protect against spina bifida in mothers and children (Jensen et al. 2006b). Brown et al. (2004a) evaluated the *NOS3* 894G>T genotypes by TDT and log-linear analyses. The TDT revealed no association while the log-linear analyses revealed that the embryonic, but not maternal, genotype was associated with risk of spina bifida. While we have genotyped the full collection of samples, we have used the methodologically simpler TDT analyses and a lack of more complex analyses may have resulted in failure to see an association with any of these 7 polymorphisms. Another important classification which should be applied would be to subset the analyses by year of birth to account for folic acid fortification. Some genetic polymorphisms may have more of an influence on NTD risk in the pre-fortification era when folate levels were lower.

The primary strength of this study was the ability to assess the risk associated with 7 polymorphisms in 5 genes at the level of the child and the mother. The limitation of this study is that a control population was not collected during the study and hence the calculation of odds ratios and gene-gene interactions is absent. Although the results of the TDT analyses did not indicate that any allele was associated with the risk of spina bifida, further analyses of these genotypes using other statistical models or subsets of the study may reveal a significant impact.

Chapter 4: Polymorphisms in the Context of Inflammatory Diseases: Systemic Lupus Erythematosus and Rheumatoid Arthritis

4.1 Abstract

High Hcy and low folate levels are associated with inflammatory diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). In healthy individuals common functional polymorphisms of enzymes in the folate/Hcy pathway have a significant impact on these metabolite levels. The SLE study was designed to examine the effects of these polymorphisms on tHcy concentrations and coronary artery calcification (CAC) scores. Genotypes for 7 polymorphisms within 6 genes of the enzymes of the folate/Hcy pathway were completed in 163 female SLE cases and 160 matched controls. African American SLE cases had higher tHcy levels than Caucasian cases and African American controls. For 6 of the 7 polymorphisms genotype distributions were significantly different between African American and Caucasian controls. There were no significant differences in genotype distributions between SLE cases and controls even after stratification by race.

The effects of genetic polymorphisms on tHcy levels were examined within general linear models which adjusted for glomular filtration rate (GFR) as a covariate because it was strongly negatively correlated to tHcy levels. In SLE patients of both races none of the 7 polymorphisms had a significant impact on tHcy levels. In Caucasian controls *MTHFR* 677CT and 677TT individuals had higher tHcy levels than 677CC homozygotes. Also in Caucasian controls *MTHFR* 1298C carriers had lower tHcy levels than 1298AA homozygotes. None of the polymorphisms were associated with CAC scores in SLE patients or controls even after stratification by race. In SLE cases

polymorphisms in folate/Hcy metabolizing enzymes do not have an impact on tHcy concentrations or CAC scores.

Rheumatoid arthritis is a chronic inflammatory disease associated with elevated Hcy levels. Methotrexate (MTX), an anti-folate, is classified as a disease-modifying anti-rheumatic drug (DMARD), and used at low doses to treat inflammatory diseases. Polymorphisms in the enzymes of the folate/Hcy pathway are involved in the metabolism of MTX and may therefore influence efficacy and/or toxicity. The RA study was designed to examine the effects of these polymorphisms on tHcy and folate derivative levels in plasma and red blood cells. Also associations between these polymorphisms and clinical variables, such as physician global assessment (PGA) scores, were examined. In the RA study there were 252 RA subjects defined as current users of MTX and 37 RA subjects defined as discontinued users of MTX. Genotypes were completed for 11 polymorphisms in 9 genes in enzymes of folate/Hcy metabolism. THcy, plasma folic acid, and folate derivatives in plasma and red blood cells were measured using stable isotope dilution liquid chromatography multiple reaction monitoring mass spectrometry. C reactive protein and B_{12} levels were measured and PGA scores and American Rheumatism Association classes were assigned by physicians.

In African Americans *MTHFD1* 1958G>A genotypes had significantly different distributions when current users and discontinued users were compared. *MTHFD1* 1958G>A genotypes may be associated with discontinuation of MTX therapy. In Caucasians discontinuation of MTX therapy was associated with *MTHFR* 1298A>C genotypes. Several of the investigated polymorphisms were found to have a significant impact on tHcy and folate derivative levels in African American and Caucasian current

users. *MTR* 2756A>G genotypes were associated with attributable toxicities in African American current users. In Caucasian current users *MTHFD1* 1958G>A genotypes were associated with attributable toxicities. In both African American and Caucasian current users CRP levels were positively correlated with total RBC folate levels and with levels of specific RBC folate derivatives.

4.2 Introduction

4.2.1 Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a chronic inflammatory disease which predominates in women and African Americans (Simard and Costenbader 2007). Premature atherosclerotic cardiovascular disease is a common comorbidity in those with SLE (Manzi et al. 1997). Electron beam computed tomography (EBCT) is used to measure coronary artery calcification (CAC), which is a subclinical sign of premature atherosclerotic cardiovascular disease. CAC scores are positively correlated with risk of future cardiovascular events (Arad et al. 2005). Atherosclerosis progression depends on multiple factors and a low folate/high Hcy phenotype is associated with increased risk of cardiovascular disease. In the general population elevated Hcy levels are associated with a relative risk (RR) of 2.2 [1.6-2.9] for cardiovascular disease (Graham et al. 1997). SLE patients have higher Hcy concentrations compared to matched controls (Afeltra et al. 2005; Bruce et al. 2003; Von Feldt et al. 2006). Hcy levels were found to be positively correlated with CAC scores in SLE patients (Von Feldt et al. 2006). The SLE study was undertaken to examine if polymorphisms in enzymes of the folate/Hcy metabolic pathway have any effect on tHcy levels and/or CAC scores in SLE patients.

4.2.2 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a systemic inflammatory disease where inflammation leads to joint damage and eventually functional impairment. RA affects about 1% of the general population (Pugner et al. 2000). RA is associated with elevated Hcy levels compared to controls (Hernanz et al. 1999; Wallberg-Jonsson et al. 2002). High Hcy levels were associated with atherothrombotic events in a study in RA patients from Sweden (Berglund et al. 2009).

Methotrexate

Low dose methotrexate (MTX) therapy is one of the most commonly prescribed disease-modifying anti-rheumatic drugs (DMARDs) for the treatment of rheumatoid arthritis (Whittle and Hughes 2004). MTX therapy is associated with decreases in joint swelling and pain as well as stopping progression of joint damage (Brinker and Ranganathan 2010). MTX is an anti-folate drug, which at high doses is used to treat cancers due to its anti-proliferative effect. The anti-inflammatory mechanisms of MTX in the treatment of RA have not been clearly defined. MTX is transported into cells by reduced folate carrier 1 (RFC1) and once it enters the cell it becomes polyglutamated. MTX is a structural analog of folic acid that inhibits enzymes involved in folate metabolism and nucleotide synthesis. MTX inhibits the following enzymes: dihydrofolate reductase (DHFR), thymidylate synthase (TYMS), glycinamide ribonucleotide transformylase (GART), and aminoimidazolecarboxamide ribonucleotide transformylase (AICART) (Kremer 2004).

Folic acid

RA patients taking MTX also take folic acid supplements for the prevention of adverse events (Whittle and Hughes 2004). Evidence from a randomized placebo-controlled trial of folic acid supplementation showed that folic acid decreased toxicity without affecting efficacy in RA patients taking MTX (Morgan et al. 1990). The protective effect of folic acid was confirmed by a randomized, double-blind study in which the substitution of placebo for folic acid in RA patients that had been taking MTX and folic acid for a mean of 30 months showed that placebo was associated with discontinuation of MTX because of adverse events (Griffith et al. 2000). The use of MTX alone to treat RA is associated with increased Hcy levels and addition of folic acid is associated with decreased Hcy levels (van Ede et al. 2002). Concomitant folic acid is protective against adverse events and favorably affects Hcy levels.

Associations of specific polymorphisms with efficacy or toxicity

Genetic polymorphisms may be able to predict an individual's therapeutic outcome and this field of science is known as pharmacogenetics. Pharmacogenetics is the study of how interindividual differences in drug response are due to polymorphisms in genes for drug metabolizing enzymes, drug transporters, and drug targets (Evans and McLeod 2003). Several polymorphisms in enzymes of the folate/Hcy pathway are associated with either MTX efficacy or toxicity. An RA study in Slovenia found that the RFC1 80GG genotype was associated with increased risk of overall toxicity with OR 3.57 and that the *MTHFR* 1298CC genotype was associated with a protective effect OR 0.17 (Bohanec Grabar et al. 2008). A study in US RA patients found that a favorable response to MTX was associated with the *RFC1* 80AA genotype (Dervieux et al. 2004).

RFC1 80AA genotype was also found to be associated with decreased RA symptoms and the frequency of the 80A allele was higher in MTX responders (Drozdzik et al. 2007). The *RFC1* 80AA genotype seems to be consistently associated with MTX efficacy. In a study in Dutch RA patients *MTHFR* 1298AA genotype was associated with efficacy and less overall toxicities (Wessels et al. 2006), which is in contrast to the RA study from Slovenia. In a US-based RA study *TYMS* 5' VNTR 2R/2R genotype was associated with a specific toxicity, alopecia, OR 5.6 (Weisman et al. 2006). Polymorphisms in enzymes of folate/Hcy metabolism require further study to establish their predictive ability.

4.3 Methods

4.3.1 SLE Study

Subjects

Nonpregnant women attending University of Pennsylvania clinics with SLE and race and age matched controls were invited to participate in the study. There were 163 SLE cases and 160 controls enrolled in the study. SLE cases fulfilled at least 4 of the American College of Rheumatology revised criteria for the classification of SLE (Hochberg 1997). Controls had no underlying inflammatory disease or exposure to corticosteroids. The study was approved by the Institutional Review Board of the University of Pennsylvania. Each participant gave written informed consent. This study was previously described (Summers et al. 2008a).

Biochemical Assays

All subjects' medical histories and fasting blood samples were collected, as well as undergoing electron beam computed tomography (EBCT) (Von Feldt et al. 2006). Framingham point scores were calculated based on a published method of scoring various

cardiovascular risk factors (National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) 2002). Glomular filtration rate (GFR) was calculated using the Modification of Diet in Renal Disease equation (Levey et al. 2003). Plasma tHcy levels were measured by fluorescence polarization immunoassay (AxSYM Homocysteine; Abbott Laboratories, Abbott Park, IL), which was performed by the hospital's clinical laboratory.

Genotyping

Generation Capture Column kits (Gentra Systems, Minneapolis, MN) were used to isolate DNA from whole blood. A heteroduplex generator method was used for *MTHFR* 677C>T genotypes (Barbaux et al. 2000) and a portion of samples were repeated using the TaqMan assay. *MTHFR* 677C>T, *MTHFR* 1298A>C, *MTR* 2756A>G, and *MTRR* 66A>G TaqMan assays were previously described (Summers et al. 2008a). Briefly, real-time polymerase chain reaction (PCR) assays were performed on a DNA Engine Opticon 2 continuous fluorescence detection system (Bio-Rad, Hercules, CA). PCR amplifications took place in 20ul volumes containing 20ng genomic DNA, forward and reverse primers, Fam and Vic labeled allele-specific probes, and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Cycling conditions consisted of an initial incubation at 50℃ for 2min, 95℃ for 10min, and 50 cycles of assay specific temperatures for denaturation and extension. Dual fluorescence was measured at the end of each cycle. Genotype interpretations were performed with Opticon Monitor Analysis software, version 2.02 (Bio-Rad).

Size-difference PCR methods were used to genotype *CBS* 844ins68, *TYMS* 1494del6, and *DHFR* c.86+60_78 as previously described (Summers et al. 2008a). Briefly, PCR amplifications were performed using 50ng genomic DNA, forward and reverse primers, dNTPs, 10x PCR buffer (Applied Biosystems), $MgCl_2$, and AmpliTaq DNA polymerase (Applied Biosystems) in 25ul volumes. PCR products were separated on 3% agarose gels run for 45min at 140V then stained with ethidium bromide.

Statistics

All statistical analyses were performed with SAS version 9.1 and p values were considered significant if <0.05. Log-transformation of tHcy was necessary to better approximate normality. Hardy-Weinberg equilibrium for each polymorphism was assessed by chi-square test. Differences in genotype distributions between African American and Caucasian controls and between case and control groups were assessed by chi-square and Fisher's Exact test. Pearson's correlation coefficients for age, GFR, and Framingham point scores were assessed in relation to log tHcy. The effects of smoking status, use of B_6, B_{12}, and folic acid on log tHcy were assessed by Student's t-test. The above significantly correlated variables were used in general linear modeling as covariates in the assessment of the effect of genotype on log tHcy. Results of log tHcy analyses were back-transformed to report results in original measurement units (μmol/L).

4.3.2 RA Study

Subjects

Subjects were recruited who fulfilled the 1987 American College of Rheumatology revised criteria for rheumatoid arthritis (Arnett et al. 1988). This study was approved by the Institutional Review Board of the University of Pennsylvania

School of Medicine. All subjects provided written informed consent. Study subjects described as current users were taking MTX for a minimum of 17 weeks, and were excluded if they did not report that they were taking folic acid. Study subjects described as discontinued users had taken MTX previously but were not currently taking the drug. Subjects gave a blood sample for biochemical and genetic analysis and completed a short in-person interview. The interview questions covered alcohol use, smoking status, folic acid supplementation, and medical history. Doctors performed physical assessments and gave physician global assessment (PGA) scores and American Rheumatism Association (ARA) classes.

Biochemical Assays

THcy, FA, and folate derivatives were measured in plasma and RBCs using stable isotope dilution liquid chromatography multiple reaction monitoring mass spectrometry (LC/MRM/MS) as previously described (Huang et al. 2007; Huang et al. 2008).

C-reactive protein (CRP) levels were measured by the clinical laboratory of the Hospital of the University of Pennsylvania using VITROS MicroSlides (Ortho-Clinical Diagnostics, Rochester, NY). Vitamin B_{12} levels were measured with the Immulite 2000 Vitamin B_{12} assay (Diagnostic Products Corp., Los Angeles, CA).

Genotyping

QIAamp DNA Mini Kits (Qiagen, Santa Clarita, CA) were used to extract DNA from whole blood. TaqMan genotyping methods were previously described for *MTHFR* 677C>T, *MTHFR* 1298A>C, *MTR* 2756A>G, *MTRR* 66A>G (Summers et al. 2008a), *MTHFD1* 1958G>A, *RFC1* 80A>G, and *SHMT1* 1420C>T (Summers et al. 2010). Briefly, real-time polymerase chain reaction (PCR) assays were performed on a DNA

Engine Opticon 2 continuous fluorescence detection system (Bio-Rad, Hercules, CA). PCR amplifications took place in 20ul volumes containing 20ng genomic DNA, forward and reverse primers, Fam and Vic labeled allele-specific probes, and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Cycling conditions consisted of an initial incubation at 50℃ for 2min, 95℃ for 10min, and 50 cycles of assay specific temperatures for denaturation and extension. Dual fluorescence was measured at the end of each cycle. Genotype interpretations were performed with Opticon Monitor Analysis software, version 2.02 (Bio-Rad).

Size difference PCR methods were used to genotype *CBS* 844ins68, *TYMS* 1494del6, *TYMS* 5' VNTR, and *DHFR* c.86+60_78 as previously described (Summers et al. 2010). Briefly, PCR amplifications were performed using 50ng genomic DNA, forward and reverse primers, dNTPs, 10x PCR buffer (Applied Biosystems), and AmpliTaq DNA polymerase (Applied Biosystems) in 25µl volumes. PCR products were separated on 3% agarose gels run for 45min at 140V then stained with ethidium bromide.

Statistics

Statistical analyses were performed using SAS version 9.1 (SAS Institute, Cary, NC). Discrete variables were presented as counts and proportions and continuous variables were presented as medians and interquartile ranges. Total RBC folate was the sum of RBC 5-MTHF, THF, and 5,10-MTHF. THF values which were not detectable (n=4) were given a value corresponding to the lower limit of quantitation (4.5nmol/L), allowing both absolute THF levels and the ratio of RBC 5-MTHF:THF to be analyzed as continuous variables. RBC 5,10-MTHF levels were not detectable in a large portion of the samples and therefore was treated as a dichotomous (detectable/not detectable)

variable. CRP values reported by the clinical laboratory as <0.3 were given values of 0.3 and B_{12} values reported as >1000 were given values of 1000 in order to analyze the data with nonparametric tests, which is based on ranking values.

For the purposes of classification, severe toxicities included pneumonitis, hemorrhagic enteritis, and bone marrow depression; attributable toxicities included the severe toxicities and leucopenia, thrombocytopenia, abnormal mcv, abnormal ALP/ALT/AST, other abnormal LFTs, elevated homocysteine, photosensitivity/hyperpigmentation, urticaria/puritis, alopecia, and mucositis; and any toxicity was defined as having at least one of the twenty five defined events or otherwise listed as other event.

4.4 Results

4.4.1 SLE Study

Subject Characteristics

The study included 163 SLE cases and 160 controls, of which approximately 50% self-reported as African American, 39% as Caucasian, 5% as Asian, 4% as Hispanic, and 1% as other as presented in Table 4-1. The average age in SLE cases and controls was about 43 years. Median CAC scores were significantly higher in SLE cases than controls (p=0.0003). THcy levels were also higher in SLE cases than controls (10.4 vs 9.2 μmol/L, p<0.0001). When analyses were applied to specific races, African American SLE cases had higher tHcy levels than African American controls (12.1 vs 9.7 μmol/L, p<0.0001). Caucasian SLE cases had higher tHcy levels than Caucasian controls but this was not statistically significant (10.0 vs 9.0 μmol/L, p=0.12). African American SLE cases had higher tHcy levels than Caucasian SLE cases (p=0.0009), but African

American controls did not have significantly different tHcy levels than Caucasian controls (p=0.21). Framingham point scores and GFR were not significantly different between SLE cases and controls.

Table 4-1. SLE Study Subject Characteristics.

Characteristic	SLE cases	Controls	P value
No.	163	160	ND
Age, yrs, mean ± SD	43.3 ± 11.0	43.5 ± 10.5	0.90
Race, %(n)			
African American	50.3 (82)	50.6 (81)	1.00
Caucasian	39.3 (64)	39.4 (63)	
Asian	4.9 (8)	5.0 (8)	
Hispanic	4.3 (7)	3.8 (6)	
Other	1.2 (2)	1.2 (2)	
CAC, median [IQR]	0 [0-7.2]	0 [0-0]	0.0003
tHcy, µmol/L, mean ± SD (n)	10.4 ± 1.4	9.2 ± 1.4	<0.0001
African American*	12.1 ± 1.4 (82)	9.7 ± 1.3 (81)	<0.0001
Caucasian	10.0 ± 1.3 (64)	9.0 ± 1.5 (63)	0.12
Framingham point scores, median [IQR] (n)	8 [3-13] (161)	8 [2-12] (156)	0.39
GFR, ml/min/1.73m^2, mean ± SD	90.0 ± 32.5	94.5 ± 20.8	0.14

SD = standard deviation, ND = not determined, IQR = interquartile range.
*For African American SLE cases vs. Caucasian SLE cases p=0.0009; for African American controls vs. Caucasian controls p=0.21(Summers et al. 2008a)

Genotype frequencies in African American and Caucasian controls

SLE cases and controls were genotyped for 7 polymorphisms in 6 genes in the folate/Hcy metabolic pathway and included: *MTHFR* 677C>T and 1298A>C, *MTR* 2756A>G, *MTRR* 66A>G, *CBS* 844ins68, *TYMS* 1494del6, and *DHFR* c.86+60_78. All genotypes were in Hardy-Weinberg equilibrium for SLE cases and controls even after stratification by race. The genotype frequency distributions were significantly different between African American and Caucasian controls for all of the polymorphisms except for *MTR* 2756A>G (Table 4-2). For example, *MTHFR* 677TT homozygotes made up 2.5% of African American controls compared to 19.0% of Caucasian controls

(p=0.0003). The wildtype genotype tends to be the higher frequency homozygote and the lower frequency homozygote is usually the variant genotype. For 3 of the polymorphisms (*MTRR* 66A>G, *TYMS* 1494del6, and *DHFR* c.86+60_78) the homozygous genotype that had the higher frequency in African Americans had the lower frequency in Caucasians. In analyses involving genotypes African Americans and Caucasians were therefore analyzed separately.

Risk of SLE as determined by genotype frequency

Specific genotypes may be associated with development of SLE if SLE cases have an overrepresentation in genotype frequency when compared to the control frequency. None of the carrier frequencies of the 7 polymorphisms were significantly different when stratified by race (Table 4-3). The only polymorphism that approached significance was in Caucasians, *MTR* 2756G carriers were overrepresented in controls (p=0.07) meaning that 2756AA homozygotes are at increased risk of developing SLE.

Table 4-2. Distributions of genotype frequencies in African American and Caucasian controls.

Polymorphism	Genotype	African Americans	Caucasians	P
MTHFR 677C>T	CC	67.9 (55)	39.7 (25)	0.0003
	CT	29.6 (24)	41.3 (26)	
	TT	2.5 (2)	19.0 (12)	
MTHFR 1298A>C	AA	69.1 (56)	55.6 (35)	0.0347
	AC	30.9 (25)	38.1 (24)	
	CC	0	6.3 (4)	
MTHFR 677/1298	CC/AA	46.9 (38)	11.1 (7)	<0.0001
	CC/AC	21.0 (17)	22.2 (14)	
	CC/CC	0	6.3 (4)	
	CT/AA	19.7 (16)	25.4 (16)	
	CT/AC	9.9 (8)	15.9 (10)	
	TT/AA	2.5 (2)	19.0 (12)	
CBS 844ins68	WW	58.0 (47)	87.3 (55)	0.0005
	WI	39.5 (32)	12.7 (8)	
	II	2.5 (2)	0	
MTR 2756A>G	AA	58.0 (47)	47.6 (30)	0.19
	AG	33.3 (27)	47.6 (30)	
	GG	8.7 (7)	4.8 (3)	
MTRR 66A>G	AA	48.2 (39)	20.6 (13)	0.0009
	AG	40.7 (33)	50.8 (32)	
	GG	11.1 (9)	28.6 (18)	
TYMS 1494del6	Ins/ins	19.7 (16)	39.7 (25)	0.0012
	Ins/del	53.1 (43)	54.0 (34)	
	Del/del	27.2 (22)	6.3 (4)	
DHFR c.86+60_78	Ins/ins	19.7 (16)	38.1 (24)	0.0068
	Ins/del	45.7 (37)	47.6 (30)	
	Del/del	34.6 (28)	14.3 (9)	

%(n). P values by χ^2 test. (Summers et al. 2008a)

Table 4-3. Distributions of carrier frequencies between SLE cases and controls by race.

Genotype	SLE cases	Controls	p
MTHFR 677T carriers			
African American	31.7 (26)	32.1 (26)	1.00
Caucasian	65.6 (42)	60.3 (38)	0.58
MTHFR 1298C carriers			
African American	32.9 (27)	30.9 (25)	0.87
Caucasian	54.7 (35)	44.4 (28)	0.29
CBS 844ins68 carriers			
African American	48.8 (40)	42.0 (34)	0.43
Caucasian	15.6 (10)	12.7 (8)	0.80
MTR 2756G carriers			
African American	46.3 (38)	42.0 (34)	0.64
Caucasian	35.9 (23)	52.4 (33)	0.07
MTRR 66G carriers			
African American	43.9 (36)	51.9 (42)	0.35
Caucasian	78.1 (50)	79.4 (50)	1.00
TYMS 1494del6 ins carriers			
African American	63.4 (52)	72.8 (59)	0.24
Caucasian	89.1 (57)	93.7 (59)	0.53
DHFR c.86+60_78 ins carriers			
African American	59.8 (49)	65.4 (53)	0.52
Caucasian	78.1 (50)	85.7 (54)	0.36

Carriers of alleles subset by race %(n). P values by Fisher's exact test. (Summers et al. 2008a)

Predictors of tHcy concentrations

The continuous variables which were selected for correlation analysis with tHcy concentrations were age, GFR, and Framingham point scores. Displayed in Table 4-4 are the Pearson's correlation coefficients and corresponding p values for all study subjects. All 3 continuous variables were significantly correlated with tHcy concentrations. Age and Framingham point scores were positively correlated while GFR was negatively correlated with tHcy concentrations. Uncontrolled sources of variation were categorical variables and the following were selected for analysis: folic acid use, B_6 use, B_{12} use, and smoking status. None of these variables were significantly associated with tHcy concentrations (data not shown). The results of the above analyses were similar when

stratified by group (SLE cases and controls) except that smoking status was significantly associated with tHcy concentrations in controls (smokers 11.8 (n=25) vs nonsmokers 9.5 (n=133), p=0.0046).

Table 4-4. Correlations with homocysteine levels.

Variable	Pearson's Correlation Coefficient	n	p
Age	0.22917	323	<0.0001
GFR	-0.42930	323	<0.0001
Framingham Point Scores (Summers et al. 2008a)	0.22249	317	<0.0001

From the above analysis the explanatory variables age, GFR, and Framingham point scores along with race and group were put into a general linear model with tHcy as the dependent variable, with no interactions between any of the terms. In this analysis of covariance GFR along with race and group were the only variables significantly associated with tHcy (Table 4-5). These variables were used in modeling the effect of genotype on tHcy concentrations.

Table 4-5. Analysis of covariance for homocysteine.

Source Variation	Sum of Squares	Df	F	p
GFR	1.11	1	60.18	<0.0001
Group*	5.53	1	12.04	0.0006
Race†	2.14	1	23.28	<0.0001

*Refers to case and control groups
†Refers to African Americans and Caucasians(Summers et al. 2008a)

Modeling the effects of polymorphisms of folate/Hcy metabolizing enzymes on tHcy concentrations

More complex models were explored and a clinically sound parsimonious model was arrived at with variables that significantly contributed to the variation in tHcy. For each polymorphism a general linear model was used with tHcy as the dependent variable and classified by race, group, and genotype with the addition of the covariate GFR and its

interaction with that genotype. The classification resulted in 4 categories, which were African American SLE cases, African American controls, Caucasian SLE cases, Caucasian controls. The least-square mean estimates of tHcy were adjusted for the uncontrolled variable GFR. Two polymorphisms (*MTHFR* 1298A>C and *CBS* 844ins68) were modeled based on carriers of the variant allele, which is the combination of heterozygotes and variant homozygotes. This was because there were no variant homozygotes present in at least one of the four categories. Out of the 7 polymorphisms only 2 were significantly associated with tHcy concentrations. In Caucasian controls *MTHFR* 677CC homozygotes had lower tHcy levels than 677CT heterozygotes (7.7 vs 9.4 μmol/L, p=0.0196) and 677CC homozygotes also had lower tHcy levels than 677TT homozygotes (7.7 vs 9.8 μmol/L, p=0.0275). Also *MTHFR* 1298AA homozygotes had higher tHcy levels than 1298C carriers (9.6 vs 7.8 μmol/L, p=0.0083) (Table 4-6).

Table 4-6. Analysis of covariance with two factors (group, race) and one covariate (GFR).

Category	Genotype	Adjusted means of tHcy	P*
Caucasian Controls	MTHFR 677 CC	7.7	-
	CT	9.4	0.0196
	TT	9.8	0.0275
Caucasian Controls	MTHFR 1298 AA	9.6	-
	AC/CC†	7.8	0.0083

*p value for comparison to wildtype genotype
†These genotypes were combined because of low numbers of homozygotes(Summers et al. 2008a)

CAC scores and polymorphisms of folate/Hcy metabolizing enzymes

None of the genotypes were associated with median CAC scores even after stratification by race (data not shown).

4.4.2 RA Study

Subject Characteristics

The RA study included 252 subjects classified as current (MTX) users, of which 47 (18.7%) were African American and 37 subjects classified as discontinued (MTX) users, of which 11 (29.7%) were African American (Table 4-7). Toxicities were reported and categorized into several categories. Severe toxicities would require discontinuation of MTX, and therefore were present only in the discontinued users group. Attributable toxicities are those known to be associated with MTX therapy, such as elevated liver function tests (LFTs), and could require a decrease in MTX dose. Non-attributable toxicities were side effects which could have come from a different source, such as headache and diarrhea. ARA class 1 was defined as RA having almost no impact on the subject's daily activities while class 4 was defined as RA having a severe impact on the subject's daily activities resulting in limitations in ability to perform daily activities. RBC 5,10-MTHF levels were measured and due to a large number of undetectable values were determined to be either detectable or undetectable for subsequent analyses.

Table 4-7. RA study subject characteristics for categorical variables.

	African American		Caucasian	
	Current Users	**Discontin-ued Users**	**Current Users**	**Discontin-ued Users**
Gender – Male	6 (12.8)	1 (9.1)	62 (30.2)	6 (23.1)
Female	41 (87.2)	10 (90.9)	143 (69.8)	20 (76.9)
Smoker – Yes	8 (17.8)	1 (11.1)	29 (14.6)	0
No	37 (82.2)	8 (88.9)	170 (85.4)	23 (100.0)
Drinker – Yes	7 (16.3)	4 (44.4)	88 (44.0)	11 (50.0)
No	36 (83.7)	5 (55.6)	112 (56.0)	11 (50.0)
Severe Toxicity	0	3 (27.3)	0	2 (7.7)
Attributable Toxicity	5 (10.6)	4 (36.4)	28 (13.7)	6 (23.1)
Non-Attributable Toxicity	12 (25.5)	4(36.4)	49 (23.9)	13 (50.0)
No Toxicity	30 (63.8)	0	128 (62.4)	5 (19.2)
Morning Stiffness – Yes	36 (80.0)	10 (90.9)	132 (68.0)	17 (68.0)
No	9 (20.0)	1 (9.1)	62 (32.0)	8 (32.0)
ARA Class I	15 (35.7)	1 (10.0)	75 (40.5)	12 (52.2)
II	18 (42.9)	3 (30.0)	89 (48.1)	8 (34.8)
III	7 (16.7)	5 (50.0)	13 (7.0)	2 (8.7)
IV	2 (4.8)	1 (10.0)	8 (4.3)	1 (4.4)
RBC 5,10-MTHF – D*	16 (39.0)	3 (50.0)	96 (54.9)	3 (27.3)
ND	25 (61.0)	3 (50.0)	79 (45.1)	8 (72.7)

N (%)
*D=Detectable, ND=Not Detectable

Continuous variables were assessed for differences between African American current users and Caucasian current users (Table 4-8). The following observations were associated with p values <0.05. African Americans were taking slightly higher doses of MTX than Caucasians (17.5 vs 15.0 mg, respectively). PGA scores were slightly higher in African Americans (32 vs 24). RBC THF levels were slightly lower in African Americans (24.7 vs 29.4 nmol/L) and therefore the ratio of 5-MTHF:THF was slightly higher (39.1 vs 29.4). When current users were compared to discontinued users in African Americans PGA scores were much higher in discontinued users (61 vs 32). Since current users were taking folic acid, levels of plasma folic acid were higher in current

users (12.4 vs 0 nmol/L). Total RBC folate levels were decreased in current users (993.1 vs 1283.4 nmol/L) and this possibly due to the anti-folate effects of MTX. Also 5-MTHF levels were lower in current users (957.8 vs 1004.3 nmol/L). When Caucasian current users were compared to discontinued users plasma 5-MTHF levels were lower in current users (35.9 vs 66.1 nmol/L). In Caucasians similar observations were seen as those observed in African Americans with respect to plasma folic acid (FA), total RBC folate, and RBC 5-MTHF.

Clinical variables of RA severity are associated

The clinical continuous variables PGA score and CRP levels were stratified by the clinical categorical variables morning stiffness and ARA class to assess differences in median levels of the continuous variables within current users (Table 4-9). In Caucasians PGA scores were higher in those who experienced morning stiffness compared to those that did not experience morning stiffness (27 vs 17, p=0.0003). In African Americans PGA scores were higher in those who experienced morning stiffness but this was not statistically significant. PGA scores in African Americans were higher in individuals categorized as ARA classes II-IV compared to those categorized as ARA class I (41 vs 18, p=0.0005).

Table 4-8. RA study subject characteristics for continuous variables.

	Current Users		Discontinued Users	
	African American	**Caucasian**	**African American**	**Caucasian**
Age, years	57 [46-67] (47)	56 [48-66] (202)	59 [54-64] (10)	56 [38-71] (26)
Current MTX Dose, mg	17.5 [12.5-20.0] (46)	15.0 [10.0-17.5] (202)*	-	-
Cumulative MTX Dose, mg	539.8 [346.5-3038.6] (40)	556.1 [325.0-3513.6] (194)	851.6 [480.0-2780.0] (10)	556.1 [267.5-2380.0] (22)
PGA score	32 [18-50] (41)	24 [12-35] (187)*	61 [41-72] (10) †	29 [11-56] (23)
Plasma 5-MTHF, nmol/L	33.4 [21.3-46.0] (41)	35.9 [24.9-47.7] (178)	58.6 [26.2-66.2] (6)	66.1 [33.6-96.4] (11) ‡
Plasma Folic Acid, nmol/L	12.4 [1.0-35.1] (41)	12.6 [1.1-52.7] (178)	0 [0-0.8] (6) †	1.1 [0-5.4] (11) ‡
Total RBC folate°, nmol/L	993.1 [855.2-1160.9] (41)	1095.4 [863.2-1382.0] (175)	1283.4 [1108.8-1752.9] (6) †	1489.8 [1132.5-2251.5] (11) ‡
RBC 5-MTHF, nmol/L	957.8 [746.9-1055.0] (41)	1004.3 [768.0-1273.8] (175)	1249.3 [1049.0-1700.5] (6) †	1464.1 [1033.4-2220.4] (11) ‡
RBC THF, nmol/L	24.7 [16.9-37.4] (41)	29.4 [15.8-44.7] (175)*	34.9 [24.2-43.2] (6)	35.5 [25.7-71.7] (11)
Ratio 5-MTHF:THF	39.1 [26.6-48.8] (41)	29.4 [15.8-44.4] (176)*	41.9 [38.1-44.0] (6)	39.2 [20.8-60.8] (11)
CRP°, mg/L	0.7 [0.4-1.2] (44)	0.5 [0.3-1.0] (174)	1.3 [0.7-2.4] (8)	0.4 [0.3-0.9] (11)
B_{12}°, pmol/L	600 [354-855] (37)	615 [388-839] (169)	907 [708-1000] (6)	736 [356-1000] (12)
tHcy, µmol/L	10.5 [9.3-13.6] (41)	10.4 [9.0-12.3] (178)	10.2 [7.0-12.1] (6)	12.9 [9.3-14.3] (11)

Median [IQR] (n)

* p values <0.05 for comparison of African American current users to Caucasian current users by Wilcoxon

† p values <0.05 for comparison of African American current users to African American discontinued users by Wilcoxon

‡ p values <0.05 for comparison of Caucasian current users to Caucasian discontinued users by Wilcoxon
° Total RBC folate = RBC 5-MTHF + RBC THF + RBC 5,10-MTHF; CRP values of "<0.3" were given the value of 0.3; B_{12} values of ">1000" were given values of 1000

In Caucasians the same observation was significant, individuals in ARA classes II-IV had higher PGA scores (31 vs 12, p<0.0001). CRP levels in Caucasians were higher in individuals in ARA classes II-IV (0.7 vs 0.4, p=0.001). Although CRP levels in African Americans were higher in individuals in ARA classes II-IV, this was not statistically significant. It was observed that worse RA severity in clinical continuous variables was associated with worse RA severity in clinical categorical variables.

Table 4-9. Analyses of selected clinical variables in current users: continuous variables stratified by categorical variables.

Race	Continuous Variables	Categorical Variables		P Values
		Morning Stiffness – Yes	**No**	
African American	PGA	34 [19-58] (32)	26 [10-41] (9)	0.17
Caucasian	PGA	27 [14-37] (123)	17 [8-28] (58)	**0.0003**
		ARA Class I	**ARA Classes II-IV**	
African American	PGA	18 [12-22] (14)	41 [30-61] (27)	**0.0005**
Caucasian	PGA	12 [5-19] (75)	31 [22-38] (109)	**<0.0001**
African American	CRP*	0.5 [0.4-0.7] (13)	1.0 [0.3-1.8] (26)	0.18
Caucasian	CRP*	0.4 [0.3-0.7] (64)	0.7 [0.3-1.2] (92)	**0.001**

*CRP values of "<0.3" were given the value of 0.3

Differences in genotype distribution between races

Genotyping was completed for 11 polymorphisms in 9 genes of the enzymes of the folate/Hcy pathway. Genotype distributions are presented in Table 5-10. In current users the genotype frequencies were compared by Fisher's Exact test. There were significant differences in genotype distributions between African American and Caucasian current users. Only 3 of the 11 polymorphisms were not significantly different.

Genotype distribution differences between current and discontinued users

Overall there were 3 polymorphisms which were not in Hardy-Weinberg equilibrium. In African American current users there were two polymorphisms, which deviated from Hardy-Weinberg equilibrium: *MTHFR* 677C>T (p=0.047) and *DHFR* c.86+60_78 (p=0.045). In Caucasian current users there was only one polymorphism not in Hardy-Weinberg equilibrium and that was *MTHFR* 1298A>C (p=0.021). Deviation from Hardy-Weinberg equilibrium suggests that there may be increased risk of RA associated with these polymorphisms. Without a healthy control group with which to compare genotype frequencies the conclusion cannot be made that these genotypes increase risk for RA. The discontinued users served as a reference group for assessing the tolerability of MTX therapy. African American discontinued users had significantly different genotype frequencies for the polymorphism *MTHFD1* 1958A>G when compared to African American current users. Examination of these genotype frequencies suggests that there were more *MTHFD1* 1958GA and 1958AA individuals. This suggests that the 1958A allele may be associated with discontinuation of MTX therapy in African Americans. In Caucasian discontinued users the distribution of *MTHFR* 1298A>C genotypes were significantly different from current users. There seemed to be more 1298CC homozygotes when compared to current users, which suggests that this genotype may be associated with discontinuation of MTX therapy.

Table 4-10. RA study genotype distributions.

		Current Users		Discontinued Users	
		African American	Caucasian	African American	Caucasian
MTHFR 677C>T	CC	37 (82.2)	83 (40.7)*	8 (72.7)	10 (43.5)
	CT	6 (13.3)	102 (50.0)	2 (18.2)	10 (43.5)
	TT	2 (4.4)	19 (9.3)	1 (9.1)	3 (13.0)
MTHFR 1298A>C	AA	33 (73.3)	93 (45.6)*	6 (54.6)	9 (39.1) ‡
	AC	12 (26.7)	98 (48.0)	5 (45.4)	7 (30.4)
	CC	0	13 (6.4)	0	7 (30.4)
CBS 844ins68	WW	30 (66.7)	177 (86.8)*	7 (63.6)	18 (78.3)
	WI	12 (26.7)	26 (12.8)	3 (27.3)	5 (21.7)
	II	3 (6.7)	1 (0.5)	1 (9.1)	0
MTR 2756A>G	AA	29 (64.4)	137 (67.2)	7 (63.6)	16 (69.6)
	AG	14 (31.1)	61 (29.9)	4 (36.4)	6 (26.1)
	GG	2 (4.4)	6 (2.9)	0	1 (4.4)
MTRR 66A>G	AA	18 (40.0)	45 (22.1)*	7 (63.6)	4 (17.4)
	AG	24 (53.3)	95 (46.6)	4 (36.4)	15 (65.2)
	GG	3 (6.7)	64 (31.4)	0	4 (17.4)
MTHFD1 1958G>A	GG	33 (73.3)	68 (33.3)*	4 (36.4) †	7 (30.4)
	GA	10 (22.2)	91 (44.6)	4 (36.4)	10 (43.5)
	AA	2 (4.4)	45 (22.1)	3 (27.3)	6 (26.1)
SHMT1 1420C>T	CC	22 (48.9)	98 (48.0)	6 (54.6)	9 (39.1)
	CT	21 (46.7)	87 (42.7)	5 (45.4)	11 (47.8)
	TT	2 (4.4)	19 (9.3)	0	3 (13.0)
DHFR c.86+60_78	del/del	12 (26.7)	44 (21.6)*	3 (27.3)	5 (21.7)
	ins/del	30 (66.7)	104 (51.0)	8 (72.7)	13 (56.5)
	ins/ins	3 (6.7)	56 (27.5)	0	5 (21.7)
TYMS 5' VNTR	2R/2R	7 (15.9)	41 (20.1)	4 (36.4)	8 (34.8)
	2R/3R	25 (56.8)	103 (50.5)	5 (45.5)	8 (34.8)
	3R/3R	12 (27.3)	59 (28.9)	1 (9.1)	6 (26.1)
	2R/4R	0	1 (0.5)	0	0
	3R/4R	0	0	1 (9.1)	1 (4.4)
TYMS 1494del6	del/del	14 (31.1)	23 (11.3)*	3 (27.3)	3 (13.0)
	ins/del	23 (51.1)	86 (42.2)	5 (45.4)	12 (52.2)
	ins/ins	8 (17.8)	95 (46.6)	3 (27.3)	8 (34.8)
RFC1 80A>G	AA	27 (60.0)	41 (20.1)*	6 (54.5)	5 (21.7)
	AG	16 (35.6)	101 (49.5)	4 (36.4)	15 (65.2)
	GG	2 (4.4)	62 (30.4)	1 (9.1)	3 (13.0)

n(%)

* p values <0.05 for comparison of African American current users to Caucasian current users by Fisher's Exact Test

† p values <0.05 for comparison of African American current users to African American discontinued users by Fisher's Exact Test

‡ p values <0.05 for comparison of Caucasian current users to Caucasian discontinued users by Fisher's Exact Test

MTHFR 677C>T substantially impacts biochemical variables

In African American current users there were only 2 *MTHFR* 677TT homozygotes and this was not enough to support further analyses. In Caucasian current users there were enough *MTHFR* 677TT homozygotes to permit the analysis of the effect of this genotype on biochemical variables. *MTHFR* 677TT individuals had marginally higher tHcy levels than 677C carriers but this was not statistically significant (Table 5-11). Although *MTHFR* 677TT homozygotes had slightly more total RBC folate, which was not significant, they had drastically lower levels of RBC 5-MTHF (679.8 vs 1022.3 nmol/L, p=0.002). *MTHFR* 677TT homozygotes had substantially more RBC THF (447.8 vs 34.0 nmol/L, p<0.0001). This alteration of the distribution of RBC folate derivatives led to a dramatically lower ratio of 5-MTHF:THF in 677TT individuals (1.8 vs 30.9, p<0.0001). RBC 5,10-MTHF levels were detectable in 16 out of 18 (88.9%) *MTHFR* 677TT individuals compared to 80 out of 157 (51.0%) 677C carriers (p=0.002). Vitamin B_{12} levels were also impacted by this polymorphism. *MTHFR* 677TT homozygotes had lower levels of B_{12} (541 vs 634 pmol/L, p=0.002). Since the 677TT genotype had such a widespread and substantial impact on folate/Hcy metabolism, all *MTHFR* 677TT individuals were excluded for analyses which involved metabolites of the folate/Hcy pathway.

Table 4-11. Effects of *MTHFR* 677C>T genotypes in Caucasian current users.

MTHFR 677	CC/CT	TT	P Value
tHcy, µmol/L	10.4 [9.0-12.3] (160)	11.2 [9.2-14.1] (18)	0.50
Total RBC Folate*, nmol/L	1088.2 [869.2-1361.2] (157)	1155.5 [863.2-1745.4] (18)	0.29
RBC 5-MTHF, nmol/L	1022.3 [832.5-1279.5] (157)	679.8 [537.0-1068.5] (18)	**0.002**
RBC THF, nmol/L	34.0 [22.6-60.5] (157)	447.8 [118.6-492.6] (18)	**<0.0001**
Ratio 5-MTHF:THF	30.9 [18.7-47.6] (157)	1.8 [0.9-7.5] (18)	**<0.0001**
RBC 5,10-MTHF - D†	80 (51.0)	16 (88.9)	**0.002**
ND	77 (49.0)	2 (11.1)	
B_{12}*, pmol/L	634 [401-858] (152)	541 [289-600] (17)	**0.018**

Median [IQR] (n)
*Total RBC folate = RBC 5-MTHF + RBC THF + RBC 5,10-MTHF; B_{12} values of ">1000" were given values of 1000
†D=Detectable, ND=Not Detectable

Effects of other polymorphisms on biochemical variables

In African American current users the *DHFR* c.86+60_78 polymorphism had an effect on RBC THF levels and the ratio of 5-MTHF:THF. *DHFR* del/del homozygotes had lower THF levels than ins carriers (15.3 vs 27.7 nmol/L, p=0.008, Table 5-12). This difference influenced the ratio of 5-MTHF:THF such that del/del individuals had higher ratios (53.3 vs 37.4, p=0.010). *DHFR* del/del individuals were also being prescribed lower doses of MTX (15.0 vs 20.0 mg, p=0.025). Another polymorphism was also associated with currently prescribed dose. *TYMS* 1494del6 ins/ins individuals were prescribed 12.5 mg, which was lower than 20.0 mg for del/ins and 16.3 mg for del/del individuals (p=0.028).

Table 4-12. Effects of selected genotypes on biochemical variables and current MTX dose in African American current users.

Genotype		Biochemical Variable	P Value
		RBC THF, nmol/L	
DHFR c.86+60_78	Ins carriers	27.7 [19.1-37.9] (27)	0.008
	Del/del	15.3 [13.5-32.3] (11)	
		Ratio of 5-MTHF:THF	
DHFR c.86+60_78	Ins carriers	37.4 [25.9-45.7] (27)	0.010
	Del/del	53.3 [39.1-72.1] (11)	
		Current MTX Dose, mg	
DHFR c.86+60_78	Ins carriers	20.0 [15.0-20.0] (31)	0.025
	Del/del	15.0 [10.0-17.5] (11)	
TYMS 1494del6	Del/del	16.3 [12.5-17.5] (14)	0.028
	Del/ins	20.0 [15.0-20.0] (20)	
	Ins/ins	12.5 [11.3-17.5] (8)	

Median [IQR] (n)

In Caucasian current users total RBC folate concentrations were lowest in *RFC1* 80AA homozygotes (934.5 vs 1071.5 for AG vs 1293.0 for GG, p=0.001, Table 5-13). In particular RBC 5-MTHF levels were lowest in 80AA homozygotes (859.4 vs 1002.1 for AG vs 1252.1 for GG, p=0.0005). The *MTRR* 66A>G polymorphism altered RBC folate derivative distributions. *MTRR* 66GG individuals had the lowest level of RBC THF (22.7 vs 42.8 for AG vs 43.1 for AA, p<0.0001). Consequently the ratio of 5-MTHF:THF was highest in GG individuals (44.8 vs 24.7 for AG vs 23.7 for AA, p<0.0001). B_{12} levels were lower in *MTR* 2756AA homozygotes compared to 2756G carriers (590.0 vs 784.0, p=0.006).

The *MTRR* 66A>G polymorphism not only affected RBC THF levels and the ratio of 5-MTHF:THF but also determined whether RBC 5,10-MTHF levels were detectable or not. In Caucasian current users there were 16 out of 52 (32.7%) *MTRR* 66GG individuals who had detectable RBC 5,10-MTHF levels compared to 60% of 66AA and 66AG individuals (p=0.006, Table 5-14).

Table 4-13. Effects of selected genotypes on biochemical variables in Caucasian current users.

Genotype		Biochemical Variable	P Value
		Total RBC Folate*, nmol/L	
RFC1 80A>G	AA	934.5 [763.4-1156.7] (33)	0.001
	AG	1071.5 [875.4-1306.5] (80)	
	GG	1293.0 [997.4-1514.3] (44)	
		RBC 5-MTHF, nmol/L	
RFC1 80A>G	AA	859.4 [680.2-1085.6] (33)	0.0005
	AG	1002.1 [836.1-1242.4] (80)	
	GG	1252.1 [946.9-1464.3] (44)	
		RBC THF, nmol/L	
MTRR 66A>G	AA	43.1 [28.0-79.4] (35)	<0.0001
	AG	42.8 [25.5-72.3] (70)	
	GG	22.7 [17.5-35.9] (52)	
		Ratio 5-MTHF:THF	
MTRR 66A>G	AA	23.7 [14.1-34.4] (35)	<0.0001
	AG	24.7 [16.5-37.5] (70)	
	GG	44.8 [32.3-56.3] (52)	
		B_{12}*, pmol/L	
MTR 2756A>G	AA	590.0 [359.0-793.0] (103)	0.006
	G carriers	784.0 [514.0-908.0] (49)	

Median [IQR] (n)
*Total RBC folate = RBC 5-MTHF + RBC THF + RBC 5,10-MTHF; B_{12} values of ">1000" were given values of 1000

Table 4-14. Distribution of *MTRR* 66A>G genotypes in Caucasian current users by detection of RBC 5,10-MTHF.

	MTRR 66A>G		
RBC 5,10-MTHF	AA	AG	GG
Detectable	21 (60.0)	42 (60.0)	17 (32.7)
Not Detectable	14 (40.0)	28 (40.0)	35 (67.3)

n(%), Fishers Exact P=0.006

Genotypes associated with toxicities

Genotype distributions were compared between various categories of toxicities. In African American current users *MTR* 2756G carriers had 5 out of 16 (31.2%) individuals experience an attributable toxicity compared to 0 out of 29 individuals who were 2756AA homozygotes (p=0.004, Table 5-15). The same association was significant for attributable toxicities compared to no toxicities (data not shown). In Caucasian current

users *MTHFD1* 1958GG homozygotes had 14 out of 68 (20.6%) individuals experience an attributable toxicity compared to 12.1% of 1958GA and 4.4% of 1958AA individuals (p=0.043, Table 5-16). The same association was significant for attributable toxicities compared to no toxicities (data not shown).

Table 4-15. Distribution of *MTR* 2756A>G genotypes in African American current users by type of toxicity.

	MTR 2756A>G	
	AA	AG/GG
Attributable Toxicity	0	5 (31.2)
Non-Attributable and No Toxicities	29 (100.0)	11 (68.8)

n (%), Fishers exact p=0.004

Table 4-16. Distribution of *MTHFD1* 1958G>A genotypes in Caucasian current users by type of toxicity.

	MTHFD1 1958G>A		
	GG	GA	AA
Attributable Toxicity	14 (20.6)	11 (12.1)	2 (4.4)
Non Attributable and No Toxicities	54 (79.4)	80 (87.9)	43 (95.6)

n (%), Fishers exact p=0.043

Associations among biochemical and other continuous variables

In African American current users tHcy levels were negatively correlated with plasma 5-MTHF levels (p=0.011) and positively correlated with age (p=0.003, Table 5-17). Plasma 5-MTHF levels were positively correlated with total RBC folate levels (p=0.003) and specifically there was a positive correlation with RBC 5-MTHF (p=0.005). Total RBC folate was positively correlated with CRP levels (p=0.004) and B_{12} levels (0.027). In particular RBC 5-MTHF was positively correlated with CRP levels (p=0.034) and B_{12} levels (p=0.042). Plasma folic acid was positively associated with age (p=0.021).

There was a profile which develops and consists of the following relationships being associated with high tHcy: low plasma 5-MTHF, older age, low total RBC folate, low RBC 5-MTHF, low CRP, low B_{12}, and high plasma folic acid.

Table 4-17. Correlation of selected continuous variables in African American current users.

	Spearman Correlation Coefficient (n)	Significant P values
	tHcy	
Plasma 5-MTHF	-0.39 (41)	0.011
Age	0.46 (41)	0.003
	Plasma 5-MTHF	
Total RBC folate	0.46 (41)	0.003
RBC 5-MTHF	0.43 (41)	0.005
	Total RBC folate*	
CRP*	0.45 (41)	0.004
B_{12}*	0.36 (37)	0.027
	RBC 5-MTHF	
CRP*	0.33 (41)	0.034
B_{12}*	0.34 (37)	0.042
	Plasma FA	
Age	0.36 (41)	0.021

*Total RBC folate = RBC 5-MTHF + RBC THF + RBC 5,10-MTHF; CRP values of "<0.3" were given the value of 0.3; B_{12} values of ">1000" were given values of 1000

In Caucasian current users tHcy levels were positively correlated with age (p=<0.0001) and plasma folic acid (p=0.013, Table 5-18). Plasma 5-MTHF was positively correlated with total RBC folate (p<0.0001) and in particular RBC 5-MTHF (p<0.0001). Plasma 5-MTHF was negatively correlated with plasma folic acid (p=0.047). Total RBC folate was positively correlated with the following: plasma folic acid (p=0.025), CRP (p<0.0001), and age (p=0.002). In particular it was RBC 5-MTHF which was positively correlated with the following: plasma folic acid (p=0.018), CRP (p=0.0003), and also B_{12} (p=0.029). RBC THF was another folate derivative also positively correlated with plasma folic acid (p=0.020) and CRP (p=0.046). In addition

RBC THF was positively correlated with age (p=0.011). Plasma folic acid was positively correlated with age (p<0.0001) and B_{12} (p=0.0002). PGA scores were positively correlated with CRP levels (p=0.003). CRP was negatively correlated with B_{12} (p=0.014). B_{12} was positively correlated with age (p=0.006). There were no clear profiles which developed because plasma folic acid was negatively correlated with plasma 5-MTHF and positively correlated with total RBC folate but plasma 5-MTHF was positively correlated with total RBC folate.

Table 4-18. Correlation of selected continuous variables in Caucasian current users.

	Spearman Correlation Coefficient (n)	Significant P values
	tHcy	
Age	0.35 (175)	<0.0001
Plasma FA	0.19 (178)	0.013
	Plasma 5-MTHF	
Total RBC folate	0.32 (175)	<0.0001
RBC 5-MTHF	0.34 (175)	<0.0001
Plasma FA	-0.15 (178)	0.047
	Total RBC folate*	
Plasma FA	0.17 (175)	0.025
CRP*	0.34 (168)	<0.0001
Age	0.24 (172)	0.002
	RBC 5-MTHF	
Plasma FA	0.18 (175)	0.018
CRP*	0.29 (168)	0.0003
B_{12}*	0.17 (167)	0.029
	RBC THF	
Plasma FA	0.18 (175)	0.020
CRP*	0.15 (168)	0.046
Age	0.19 (172)	0.011
	Plasma FA	
Age	0.37 (175)	<0.0001
B_{12}	0.28 (168)	0.0002
	PGA	
CRP*	0.24 (157)	0.003
	CRP*	
B_{12}*	-0.19 (161)	0.014
	B_{12}*	
Age	0.21 (166)	0.006

* Total RBC folate = RBC 5-MTHF + RBC THF + RBC 5,10-MTHF; CRP values of "<0.3" were given the value of 0.3; B_{12} values of ">1000" were given values of 1000

4.5 Discussion

The SLE study was designed to study associations between polymorphisms of enzymes in the folate/Hcy pathway and elevated Hcy levels. Since elevated Hcy is predictive of CAC scores (Von Feldt et al. 2006), associations between genetic variants and CAC scores was also analyzed. African Americans had significantly different genotype distributions than Caucasians and therefore the analyses were performed within each race. Previous reports have shown that African American and Caucasian genotype frequencies are significantly different. For example *MTHFR* 677C>T and 1298A>C polymorphisms have higher frequencies of the variant genotype in Caucasians as compared to African Americans (Tsai et al. 2009). Also *MTR* 2756A>G (Tsai et al. 2009), *MTRR* 66A>G (Shi et al. 2003; Tsai et al. 2009), *CBS* 844ins68 (Pepe et al. 1999; Tsai et al. 2009), and *TYMS* 1494del6 (Ranganathan et al. 2004) have all been reported to have different genotype frequencies when African Americans and Caucasians were compared. In the SLE study frequencies of *DHFR* c.86+60_78 genotypes also differed between races. None of the polymorphisms which were examined had significantly different distributions when comparing SLE cases and controls in each race. This indicated that none of the polymorphisms in the SLE study were related to increased risk of SLE. The literature suggests that in some populations these polymorphisms may carry increased risk. An Italian study reported that the *MTHFR* 677TT genotype was overrepresented in SLE patients (Afeltra et al. 2005). While a Polish study could not replicate the finding regarding *MTHFR* 677C>T genotypes but instead observed an overrepresentation of the *MTR* 2756G allele in SLE patients (Burzynski et al. 2007).

As far as the effects of polymorphisms on Hcy levels of SLE patients there was a report in the literature which was consistent with our findings. A study by Fijnheer et al. (1998) found that *MTHFR* 677C>T genotypes were not associated with Hcy levels in SLE patients. In our control group the *MTHFR* 677TT genotype was associated with higher tHcy levels. Also in the control group *MTHFR* 1298C carriers had lower tHcy levels. These findings are consistent with reports from the literature. In healthy Irish men from the Industrial Workers study the *MTHFR* 677TT genotype was associated with elevated tHcy concentrations (Harmon et al. 1996). A study by Parle-McDermott et al. showed that the *MTHFR* 1298C allele was associated with increased RBC folate levels (Parle-McDermott et al. 2006b). There was a report from a large scale study that was not consistent with the *MTHFR* 1298 allele having a decreased Hcy/increased folate phenotype. The *MTHFR* 1298C allele was associated with higher Hcy levels and lower serum folate concentrations in a large scale study in healthy individuals (Ulvik et al. 2007). In the SLE study folate, B_{12}, and B_6 levels were not available but instead use of vitamin supplements was studied as a surrogate. Vitamin use did not have any effect on Hcy levels. Kidney function as measured by GFR was negatively correlated with Hcy levels, which was consistent with reports from the literature. A study in the US showed that low GFR was significantly associated with elevated Hcy concentrations (Francis et al. 2004).

Although the SLE study has some limitations, the magnitude of the difference in tHcy levels between SLE cases and controls suggested that genetic factors might be responsible for the elevation of tHcy in SLE cases. THcy concentrations of Caucasian controls were associated with *MTHFR* 677C>T and 1298A>C genotypes. In SLE cases

tHcy concentrations were not associated with any of the 7 polymorphisms even with adjustment for covariates, such as GFR. Also none of the 7 polymorphisms were associated with CAC scores. We acknowledge that the size of our study population precludes a conclusion that there are no contributing genetic factors for elevated tHcy levels. Our SLE study suggests that if genetic factors are involved it is likely that they would have a relatively small effect. The higher tHcy levels observed in SLE cases is likely due to inflammatory aspects of the SLE disease process itself, which might dominate over any genetic effects.

In the RA study, which measured tHcy and folate derivatives using a high precision method, several polymorphisms in enzymes of the folate/Hcy metabolism were associated with levels of these metabolites in current MTX users. In Caucasians the *MTHFR* 677C>T polymorphism had a large impact on folate derivative levels. The distribution of RBC folate derivatives was significantly altered. RBC 5-MTHF levels in *MTHFR* 677TT homozygotes were much lower and RBC THF levels were much higher than 677C carriers. The ratio of 5-MTHF:THF was therefore impacted and was dramatically lower in 677TT individuals. Detection of RBC 5,10-MTHF was associated with the 677TT genotype. These results are similar to those observed in Caucasians in the Premenopausal Women study {{597 Summers,C.M. 2010}}, except that 677TT individuals in the Premenopausal Women study had increased tHcy levels while there was no such association in RA patients currently taking MTX. The impact of this polymorphism was large enough to warrant the removal of individuals with the 677TT genotype from further analyses involving tHcy and folate derivatives in the RA study.

In Caucasian current users *RFC1* 80A>G was another polymorphism which was associated with altered levels of folate derivatives. *RFC1* 80AA homozygotes had lower total RBC folate and lower RBC 5-MTHF levels. This seems to be consistent with findings in the Premenopausal Women study where African American *RFC1* 80G carriers had lower RBC THF levels and higher ratios of 5-MTHF:THF (Summers et al. 2010). In Caucasian current users the *MTRR* 66A>G polymorphism was found to be associated with lower RBC THF, higher ratios of 5-MTHF:THF, and also was associated with non-detectable RBC 5,10-MTHF. These were novel associations.

In African Americans the *DHFR* del/del genotype was associated with lower RBC THF levels and higher ratios of 5-MTHF:THF. *DHFR* del/del genotype is associated in the literature with higher serum and RBC folate in healthy women (Stanislawska-Sachadyn et al. 2008a). Our finding seems to be inconsistent, but since total RBC folate levels were not significantly different in African Americans with RA currently taking MTX it is hard to draw any final conclusion. African American *DHFR* del/del individuals were being prescribed lower doses of MTX. The *TYMS* 1494del6 polymorphism in African Americans was also associated with current MTX dose. TYMS ins/ins individuals were being prescribed lower doses of MTX. These polymorphisms may be associated with efficacy at a lower dose of MTX but further studies will be needed to validate this observation.

A Middle Eastern study in RA patients found that the *MTR* 2756GG genotype was overrepresented in RA patients when compared to controls (Berkun et al. 2007). The RA study did not have controls available to replicate this finding but instead found MTR 2756G carriers were associated with attributable toxicities while taking MTX. It is

possible that this polymorphism may increase risk for RA as well as increase toxicities while on MTX therapy.

In an RA study in Slovenia the *MTHFR* 1298CC genotype was associated with a protective effect, a decreased risk of overall toxicity (Bohanec Grabar et al. 2008). While the RA study did not replicate this finding we instead found that the *MTHFR* 1298CC genotype in Caucasians was associated with discontinuation of MTX therapy. This finding in Caucasians is consistent with a study in Dutch RA patients that found the *MTHFR* 1298AA genotype was associated with efficacy and less overall toxicities (Wessels et al. 2006). The RA study also found that the *MTHFD1* 1958GG genotype in Caucasians was associated with increased attributable toxicities. This finding was contradictory to the finding that the *MTHFD1* 1958AA genotype in African Americans was associated with discontinuation of MTX therapy. There is not any biological explanation as to why the same polymorphism in different races would carry very different associations.

The *RFC1* 80GG genotype in an RA study in Slovenia was associated with increased risk of overall toxicity (Bohanec Grabar et al. 2008). A study in US RA patients found that a favorable response to MTX was associated with the *RFC1* 80AA genotype (Dervieux et al. 2004). Although the RFC1 80G>A polymorphism has consistent results in the literature, this finding was not replicated in the RA study. Overall the RA study has found that different polymorphisms in African Americans and Caucasians are associated with differences in tHcy and folate metabolites, being prescribed lower doses of MTX, and increased risk for attributable toxicities and discontinuation of MTX.

Elevated CRP levels are indicative of systemic inflammation, are associated with presence of an inflammatory disease, and are also associated with increased risk for cardiovascular disease (Windgassen et al. 2011). In the RA study CRP levels were positively associated with total RBC folate and specific RBC folate derivatives in both African American and Caucasian subjects. This association is not easily explained and it is possible that folic acid taken with MTX may be blocking some of its anti-inflammatory effect, although further studies are required to support this finding. The RA study has provided evidence that polymorphisms in enzymes of the folate/Hcy metabolic pathway may be able to predict therapeutic outcome. Larger studies with special attention to race and which make use of high precision methods for measurement of folate/Hcy metabolites will be required to validate these findings.

Chapter 5: The Effect of Methotrexate Alone and on the Activation of Endothelial Cells

5.1 Abstract

Mild folate deficiency is known to be associated with a proatherosclerotic phenotype in endothelial cells. EA.hy 926 endothelial cells grown in low (Lo) folate conditions had higher levels of monocyte chemoattractant protein 1 (MCP-1) then cells grown in normal (Hi) folate conditions. Methotrexate (MTX) is an anti-folate drug used to treat a broad range of diseases including inflammatory diseases. MTX seems to have conflicting properties of anti-folate and anti-inflammatory, therefore Hi and Lo cells were treated with MTX at concentrations relative to peak plasma concentrations after low dose administration in rheumatoid arthritis patients. Tumor necrosis factor alpha (TNF-α) is a cytokine which is involved in systemic inflammation. The effect of MTX in the presence of TNF- α, an inflammatory stimulus, was examined by pre-treating the cells with MTX for 24hrs before exposing the cells to TNF-α. Activation of endothelial cells by TNF-α stimulates inflammatory gene expression. It was investigated whether MTX treatment was associated with an anti-folate effect or an anti-inflammatory effect at concentrations relative to those seen in the treatment of rheumatoid arthritis.

When Hi and Lo cells were treated with MTX cellular proliferation was inhibited, total intracellular folate was depleted, the composition of intracellular folate derivatives was altered, and levels of unmetabolized folic acid were higher. Microarray analysis was used to examine MTX induced gene expression in Hi and Lo cells. There were over 100 two-fold or higher differentially expressed genes. Specifically there was differential gene expression of several inflammatory genes which included up regulation of C3 and IL-8, and down regulation of a large set of genes involved in mitosis. Dihydrofolate reductase

(DHFR), an enzyme in folate metabolism and target of MTX, was also down regulated. There was no change in MCP-1 levels. Selected targets were confirmed by quantitative RT-PCR and ELISA. MTX treatment may involve a more complex modulation of inflammation than previously assumed.

5.2 Introduction

Elevated circulating levels of the intermediate amino acid Hcy, referred to as hyperhomocysteinemia, has been associated with a wide range of human pathologies including cardiovascular disease, stroke (Refsum et al. 1998), Alzheimer's disease (Mattson and Shea 2003), some cancers (Weinstein et al. 2001), and birth defects (van der Put et al. 2001). Hyperhomocysteinemia is generally underpinned by low folate status, in which both the absolute (Kang et al. 1987; Selhub et al. 1996) and relative (Mitchell et al. 2009) concentrations of intracellular folate derivatives may be altered. Historically elevated Hcy was considered to be the pathogenic component in the conditions with which hyperhomocysteinemia has been associated because of its direct toxic effects on redox thiol status and ER stress response (Koch et al. 1998). However, alternative causative mechanisms implicating low folate concentrations and their negative impact on processes such as nucleic acid synthesis and methylation have been suggested (Lucock 2000).

Many of the medical conditions which are associated with a high Hcy and low folate phenotype have inflammatory aspects and involve damage to, or dysfunction of, the vasculature and its constituent cell types, in particular endothelial cells. Inappropriate or sustained activation of immunologically active endothelial cell products might contribute to ongoing pathology at the local and possibly also systemic level. In recent

studies, EA.hy 926 endothelial cells grown under low folate conditions adopted a 'proatherosclerotic' phenotype compared to cells grown under high folate conditions without displaying any significant differences in intracellular or extracellular Hcy levels (Brown et al. 2006). This low folate phenotype was characterized by elongated cell morphology with prominent networks of stress fibers and impaired barrier function. A significantly increased synthesis and export of monocyte chemoattractant protein 1 (MCP-1) was observed in the cells grown under low folate conditions (Brown et al. 2006). MCP-1, a potent chemokine encoded by the *CCL2* gene, is synthesized by various cell types including vascular smooth muscle cells in response to oxidized lipid. MCP-1 facilitates the transmigration of monocytes from the circulation across the endothelium and is a key contributor to the early stages of atheroma formation (Schwartz et al. 1991). In cultured human monocytes Hcy induced the secretion of MCP-1 and IL-8, a neutrophil chemoattractant (Zeng et al. 2003). The above *in vitro* observation has been corroborated *in vivo* in a study of young healthy adults in whom serum MCP-1 levels were inversely associated with serum and red blood cell folate concentrations, and positively associated with circulating tHcy concentrations (Hammons et al. 2009). These finding have reinforced speculation that folate stress, which is indicative of poor nutritional status, might augment aspects of baseline inflammatory preparedness to facilitate a more vigorous initial responses to infectious challenges in individuals weakened by malnutrition (Lu et al. 2009).

The central role of folate in nucleotide synthesis has been exploited pharmacologically via the development of potent anti-folate drugs for the treatment of neoplastic and auto-immune conditions. One of the most widely used anti-folate drugs is

methotrexate (MTX), which inhibits the key enzymes dihydrofolate reductase (DHFR), thymidylate synthase (TYMS), glycinamide ribonucleotide transformylase (GART), and aminoimidazolecarboxamide ribonucleotide transformylase (AICART) (Kremer 2004)(Figure 5-1). High dose MTX is a component of diverse therapeutic regimens for several cancers including acute lymphoblasic leukemia, while lower doses are used to treat inflammatory diseases such as rheumatoid arthritis (RA). In the latter condition, MTX tends to be well tolerated with relatively minor side effects and there is clear therapeutic benefit in reducing the inflammatory aspects of the disease that contribute to joint damage (Coury and Weinblatt 2010). However, RA patients have significant cardiovascular comorbidity (Nurmohamed 2009) and there is controversy as to whether MTX exacerbates or ameliorates this serious source of co-mortality. An early study in the use of MTX in the treatment of rheumatoid arthritis patients with existing CVD indicated that mortality was increased (Landewe et al. 2000). Conversely, several more recent studies have suggested that MTX use is associated with a decrease in the incidence of CVD events and mortality (Choi et al. 2002; Naranjo et al. 2008; van Halm et al. 2006), although it remains unclear whether such a decrease would reflect a full or only partial amelioration of inflammation-attributable CVD.

Over expression of TNF-α is involved in many inflammatory diseases including rheumatoid arthritis and atherosclerosis (Parameswaran and Patial 2010). The possibility that low folate status, due to nutritional variables or the use of anti-folate drugs, contributes to human disease by inducing a subset of potentially pathogenic inflammation-associated molecules, including MCP-1, is of considerable public health interest. The characterization of changes to the inflammatory profile that might be

induced by drugs such as MTX, alone and prior to exposure to TNF-α, would serve as the foundation for future studies to define the precise relationship between dysregulation of folate metabolism and inflammation. This study was designed to investigate the effect of pharmacologically relevant doses of MTX on the absolute and relative concentrations of key folate derivatives and gene expression in the EA.hy 926 endothelial cell line. The potential implications of observations concerning the up-regulation of key inflammatory proteins are discussed.

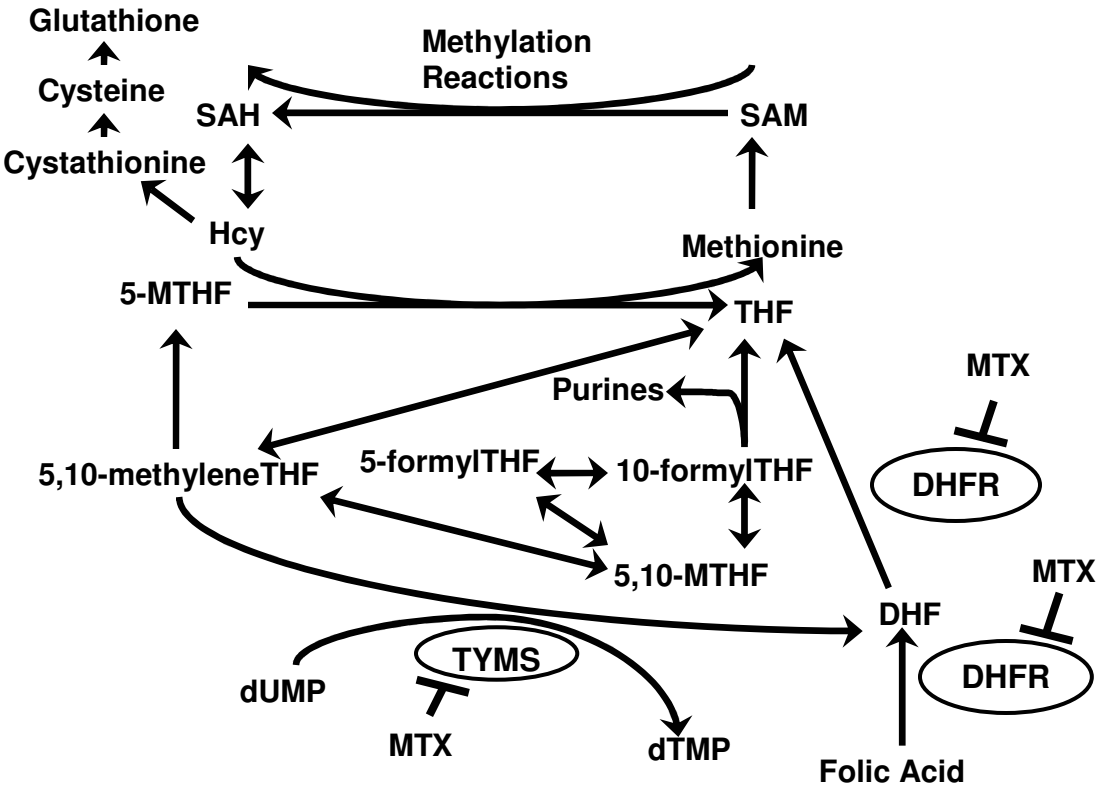


Figure 5-1. Folate/Homocysteine pathway and MTX inhibition. 5-MTHF, 5-methyltetrahydrofolate; 5,10-MTHF, 5,10-methenyltetrahydrofolate; DHF, dihydrofolate; DHFR, dihydrofolate reductase; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; Hcy, homocysteine; MTX, methotrexate; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; THF, tetrahydrofolate; TYMS, thymidylate synthase.

5.3 Methods

5.3.1 EA.926 cell line experiments

Cell Culture

The EAhy.926 cell line is a fusion product between human umbilical vascular endothelial cells (HUVECs) and the epithelial cell line A549 derived from a human lung carcinoma (Edgell et al. 1983). EA.hy 926 cells were maintained in DMEM with 10% fetal calf serum (FCS), gentamycin, penicillin, streptomycin, and fungizone. Lo cells

were adapted to growth under low folate conditions for three weeks in Medium 199 (Gibco, Invitrogen, Carlsbad, CA), which contains 23nM folic acid, and supplemented with 10% FCS, non-essential amino acids, gentamycin, penicillin, streptomycin, and fungizone. Hi cells were cultured in parallel in Medium 199 with the addition of 4mg/L (9uM) folic acid and supplemented as above as previously described (Brown et al. 2006).

BrdU Cell Proliferation Assays

Hi and Lo cells were seeded in their respective media into 96-well plates at a density of 1x10^4 cells per well. After overnight incubation triplicate cultures were given fresh media containing 0, 0.1, 0.25, or 0.5uM MTX (Sigma-Aldrich, St. Louis, MO). After 24 and 48 hours media was removed and adherent cells were fixed and stained using the Cell Proliferation ELISA, BrdU Colorimetric kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. Colorimetric analyses were performed with an ELISA plate reader (Dynex Technologies, Chantilly, VA).

Cell Viability Assays

Hi and Lo cells, grown to confluence in 6-well plates, were maintained for 24 hours in fresh medium prior to the addition of 0, 0.1, 0.25, or 0.5μM MTX. After a further 48 hours the numbers of live cells remaining were determined in duplicate with an electronic cell counter (Scepter, Millipore, Bedford, MA). For experiments involving TNF-α (Invitrogen), cells were treated for 24hrs with 0.5μM MTX followed by the addition of 10ng/mL TNF- α for an additional 24hrs. The numbers of live and dead cells in each treatment group were also determined by counting using a hematocytometer and a dye exclusion assay using 0.4% w/v trypan blue solution (Mediatech, Herndon, VA).

Alamar Blue Assays

Fresh medium was added to confluent Hi and Lo cell cultures grown in 96-well plates, and treated 24 hours later with 0, 0.1, 0.25, 0.5μM MTX. After 2, 8, 16, 24, and 48 hours metabolic activity was measured in biological triplicates by Alamar Blue Assay (Trek Diagnostic Systems, West Lake, OH) according to the manufacturer's directions. For experiments involving TNF-α, cells were treated for 24hrs with 0.5μM MTX followed by the addition of TNF- α for an additional 24hrs.

Biochemical Phenotyping

Confluent Hi and Lo cells were maintained for 24 hours in fresh medium prior to treatment with 0.5μM MTX for 48hrs. For experiments involving TNF-α, cells were treated for 24hrs with 0.5μM MTX followed by the addition of TNF- α for an additional 24hrs. Intracellular folate derivatives, i.e. 5-MTHF, THF, 5,10-methenyltetrahydrofolate (5,10-MTHF), and unmetabolized folic acid (FA), were measured in biological triplicates by stable isotope dilution liquid chromatography, multiple reaction monitoring, mass spectrometry (LC/MRM/MS) as described previously (Huang et al. 2008).

Affymetrix Microarrays

RNA was isolated from biological triplicates with the RNeasy kit (Qiagen Inc., Valencia, CA) and reverse transcribed to cDNA using the Affymetrix WT Expression kit (Ambion, Austin, TX). Subsequent experimental procedures and statistical analyses of microarray signals were performed by the University of Pennsylvania Microarray and Bioinformatics core facilities respectively as follows. The purity and size distribution of cDNA was assessed using the Agilent Bioanalyzer and RNA6000 Nano LabChips (Agilent, Palo Alto, CA), and quantitation was performed using a Nanodrop

spectrophotometer (Thermo Scientific, Wilmington, DE), prior to their hybridization to Affymetrix Human Gene 1.0 ST microarrays. Washing and staining of the microarrays was done on an Affymetrix 450 series fluidics machine. Scanning of each microarray was performed with an Affymetrix Gene Chip Scanner 3000 to produce Cel files that were imported into Partek Genomics Suite v6.5 (Partek Inc., St Louis, MO) where robust multi-array analysis (RMA) was applied. A fold change of >2 and false discovery rate of 5%, corresponding to a corrected p-value of <0.05 were chosen as the cutoffs. The microarray data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO, http://www.nlm.nih.gov/geo/) and are accessible through GEO Series accession number [will be available soon].

Quantitative Real Time PCR (qRT-PCR)

RNA was prepared from biological triplicates of control and MTX treated Hi and Lo cells using the RNeasy kit (Qiagen Inc.). For experiments involving TNF-α, RNA was prepared from biological triplicates of control, MTX treated, TNF-α treated, and MTX/TNF-α treated Hi and Lo cells. Reverse transcription was carried out with MMLV reverse transcriptase (Promega, Madison, WI) as described previously (Brown et al. 2006). To determine the amount of target mRNA relative to that transcribed from the housekeeping gene *GAPDH*, qRT-PCR was carried out in 20ul reactions containing 1ul cDNA, 1ul Taqman Gene Expression Assay (Applied Biosystems, Foster City, CA) in Taqman Universal master mix (Applied Biosystems). The Applied Biosystems assay ID numbers were Hs01100879_m1 for C3, Hs00174103_m1 for IL-8 (encoded by *CXCL8*), Hs00758822_s1 for DHFR, Hs00365486_m1 for VCAM-1, Hs00164932_m1 for ICAM-1, and Hs00174057_m1 for E-selectin. GAPDH and MCP-1 (encoded by *CCL2*) qRT-

PCR assays were performed as previously described (Brown et al. 2006). All samples were assayed in duplicate.

ELISA Assays

Confluent Hi and Lo cells were maintained for 24 hours in fresh medium prior to treatment with 0.5μM MTX. After a further 48hrs MCP-1, IL-8, and C3 concentrations in medium from MTX treated and untreated control cells were measured in biological triplicates. For experiments involving TNF-α, cells were treated for 24hrs with 0.5μM MTX followed by the addition of TNF- α for an additional 24hrs. Secreted proteins were measured using an MCP-1 ELISA kit (PeproTech, Inc, Rocky Hill, NJ), an IL-8 ELISA kit (BD Biosciences, San Diego, CA), and a C3 ELISA kit (Innovative Research, Novi, MI) with adjustment for protein content in the corresponding cell fraction. All samples were assayed in duplicate.

Statistics

Differences between means were compared by two-tailed Student's t-test. Statistical analyses were performed using SAS version 9.1 (SAS Institute, Cary, NC).

5.4 Results

5.3.1 EA.926 cell line experiments

MTX Dose finding

Peak concentrations of plasma MTX in patients undergoing treatment for RA have been reported to fall within a range of 0.37-1.36μM (Lebbe et al. 1994). This guided our selection of test MTX doses (i.e. 0.1, 0.25, and 0.5μM) that were applied to Ea.hy 926 cells to identify a concentration that was pharmacologically relevant but did

not cause excessive cell death or compromise overall metabolic activity. Several methods were used as detailed below.

The Effect of MTX on EA.hy 926 Cell Proliferation

BrdU cell proliferation assays were performed on Hi and Lo cells following 24 and 48 hours of treatment with all test doses of MTX. A dose dependent inhibition of proliferation in MTX treated Hi cells was observed relative to control untreated cells (Figure 5-2A), whereas MTX significantly inhibited proliferation at all concentrations tested in Lo cells ($p<0.05$) (Figure 5-2B). Comparable proliferation readouts were observed for untreated Hi and Lo cells and similar levels of inhibition (approximately 84%) were observed for both using the 0.5μM dose of MTX.

The Effect of MTX and TNF-α on EA.hy 926 Cell Viability

The proportions of Hi and Lo cells that remained viable after treatment with 0.5μM MTX, TNF-α, and MTX/TNF-α were determined using two independent methods. By Trypan Blue exclusion assay, the percentages of live cells treated with MTX, TNF-α, MTX/TNF-α, and untreated Hi cells were 96%, 95%, 92%, and 99% respectively. The percentages of live cells treated with MTX, TNF-α, MTX/TNF-α, and untreated Lo cells were 67%, 94%, 53%, and 96% respectively (data not shown). Direct counting using a handheld electronic cell counter, the Scepter, indicated that MTX treated Hi cells had 77% as many live cells as controls ($p=0.001$) and TNF-α treated had 81% ($p=0.001$) and MTX/TNF-α treated had 66% ($p<0.0001$). The percent of live cells for MTX treated Lo cells was 69% compared to controls ($p=0.002$) and TNF-α treated had 84% ($p=0.020$) and MTX/TNF-α had 53% ($p<0.0001$) (data not shown). Thus, by both methods 0.5μM MTX had only a minimal impact on Hi cell viability and a moderate impact on Lo cell

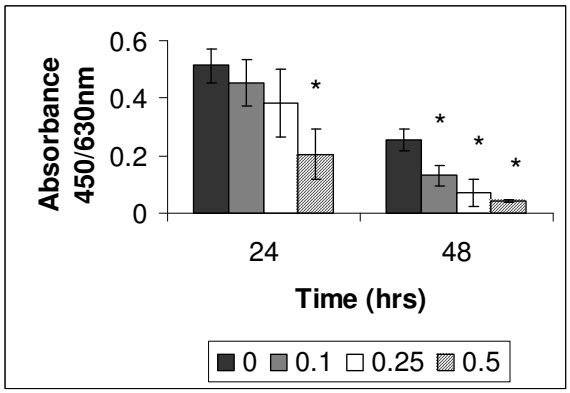
viability. TNF-α had a minimal impact on viability alone and therefore MTX/TNF- α treated cells showed similar viability to MTX treatment alone.

The Effect of MTX on EA.hy 926 Cell Metabolic Activity

Alamar Blue assays were performed on confluent Hi and Lo cell cultures following treatment with all test concentrations of MTX (i.e. 0.1, 0.25, 0.5μM) for 2, 8, 16, 24, and 48 hours in order to estimate reductions in basic metabolic activity over time. None of the MTX concentrations had a substantial impact on metabolic activity in either series (Figure 5-2C and 5-2D). Hi and Lo cells treated with the highest dose of MTX (0.5μM) for 48 hours retained more than 79% (p= 0.005) and 82% (p=0.029) of the untreated control Alamar Blue readouts respectively, indicating that metabolism remained largely intact in MTX treated cells.

Alamar blue assays were performed on Hi and Lo cells treated with 0.5μM MTX alone, TNF-α alone, and the combination of MTX/TNF-α and were compared to control readouts. MTX treated Hi cells maintained 78% metabolic activity (p=0.003) (Figure 5-2E). TNF-α treated Hi cells had 99% and MTX/TNF-α treated Hi cells had 70% (p=0.005) metabolic activity compared to controls. In Lo cells treated with MTX metabolic activity was 72% (p=0.006) compared to controls. TNF-α treated Lo cells had 83% (p=0.007) and MTX/TNF-α treated Lo cells had 67% (p=0.004) metabolic activity compared to controls.

2A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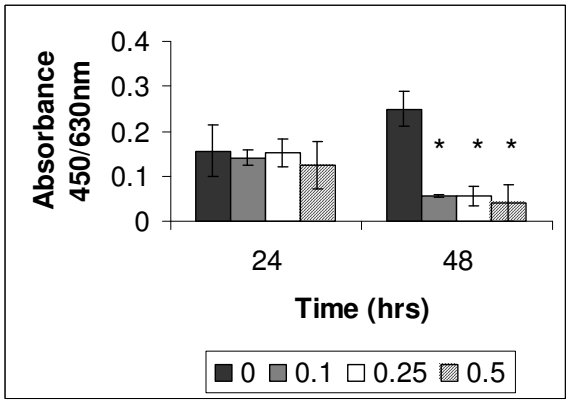
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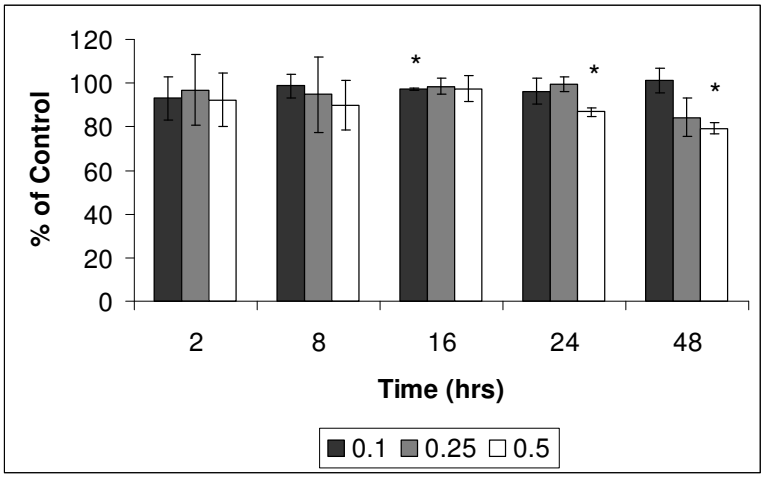
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100
80
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8
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Time (hrs)
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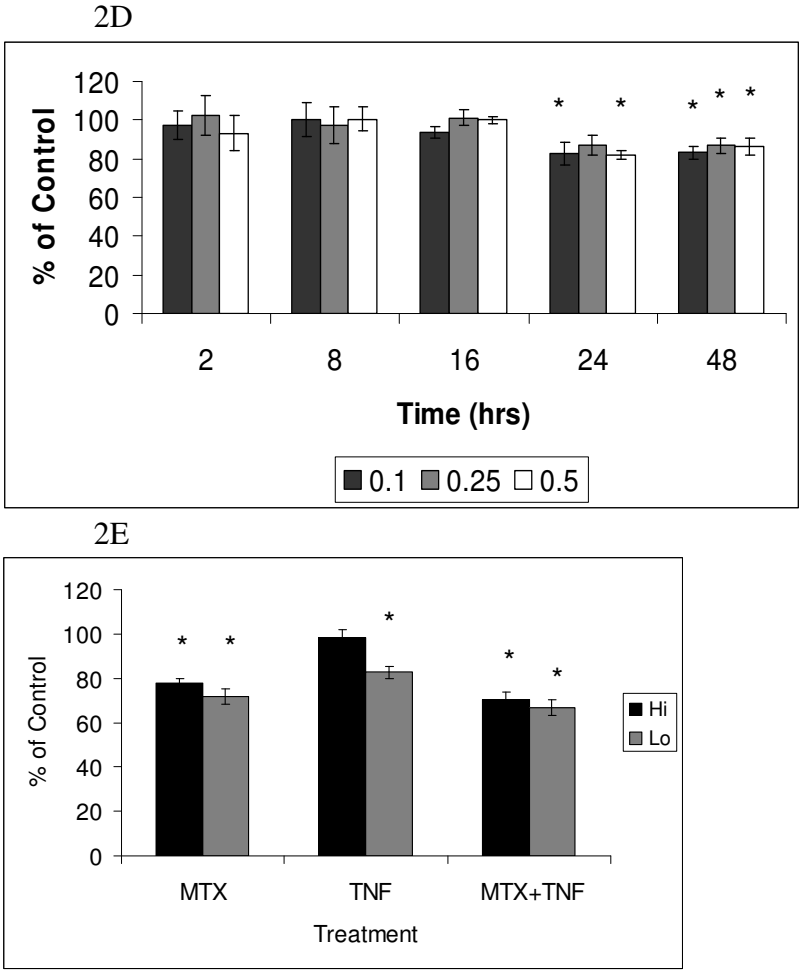


Figure 5-2. MTX dose finding and viability experiments. *A*, Recently plated EA.hy 926 Hi cells were incubated overnight and plating medium was replaced with Hi medium containing various concentrations of MTX (0.1, 0.25, 0.5uM). After 24 and 48 hours the medium was removed and adherent cells were fixed and stained according to the manufacturer's instructions for BrdU quantitation. Each bar represents the mean±SD of three samples. This experiment is representative of a total of three experiments with similar results. *B*, Lo cells were submitted to the above described experiments for BrdU quantitation. *C*, Hi confluent cells received a media change containing various concentrations of MTX (0.1, 0.25, 0.5uM). Cells were incubated with alamar blue for 2 hours. After 2, 8, 16, 24, and 48 hours of exposure to MTX absorbance was measured and calculated as the percentage of control. Each bar represents the mean±SD of three samples. This experiment is representative of a total of three experiments with similar results. *D*, Lo cells were submitted to the above described experiments for the alamar blue assay. *E*, Confluent Hi and Lo cells received a media change, 24hrs later 0.5uM MTX was added. Then 24hrs later 10ng/mL TNF-α was added and after 22hrs alamar blue was incubated with the cells for 2hrs. Absorbance was measured and calculated as the percentage of control. Each bar represents the mean±SD of three samples. This experiment is representative of a total of three experiments with similar results. *P values <0.05 compared with respective control.

Taken together the above results suggested that MTX at a concentration of 0.5μM would be an appropriate, pharmacologically relevant dose at which to explore the impact of anti-folate drug treatment on folate phenotype and gene expression profile. Also the above results suggested that TNF-α did not substantially impact cell viability and metabolic activity beyond that of MTX.

Modulation of Folate Phenotype in EA.hy 926 Cells by MTX

Exposure of confluent Hi and Lo EA.hy 926 cell cultures to 0.5μM MTX for 48 hours resulted in qualitative and quantitative changes to folate phenotype in both. There was a dramatic almost 20-fold increase in unmetabolized folic acid from a mean of 32.9 ng per mg protein in untreated Hi cells to 630.4 ng per mg protein in MTX treated Hi cells ($p=0.003$, Figure 5-3A). This indicated that Hi cells were able to take up folic acid from the medium in the presence of MTX and that the drug efficiently inhibited DHFR activity to prevent the step-wise conversion of folic acid to DHF and THF and its subsequent entry into the cellular pool of natural folates. The accumulation of folic acid in MTX treated Hi cells was accompanied by a quantitative reduction in total intracellular folate (i.e. the sum of the three derivatives 5-MTHF, THF, and 5,10-MTHF) from 2099.1 ng per mg protein in untreated cells to 144.2 ng per mg protein ($p=0.003$) in MTX treated cells, which is a reduction to only 7% of the levels observed in untreated control cells. Although the concentrations of each of the individual folate analytes also fell (i.e. 5-MTHF levels from 901.8 to 8.9 ng per mg protein $p<0.001$, THF levels from 506.3 to 45.5 ng per mg protein $p=0.001$, and 5,10-MTHF levels from 690.0 to 89.8 ng per mg protein $p=0.008$) they did so with different degrees. MTX treated Hi cells had 5-MTHF, THF, and 5,10-MTHF concentrations that were respectively 1%, 9%, and 13% those

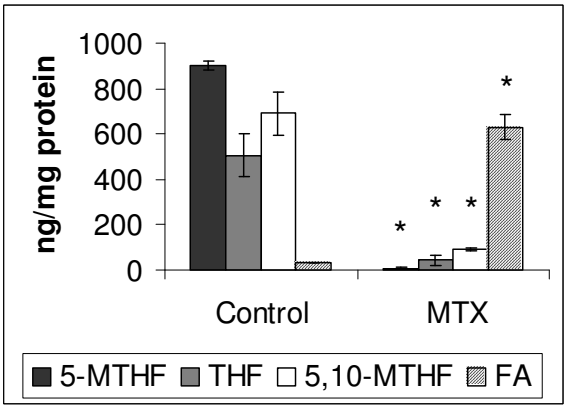
observed in untreated cells. Thus the individual folates represented very different proportions of total folate in untreated Hi cells compared to MTX treated Hi cells (43% to 6% for 5-MTHF, 24% to 32% for THF, and 33% to 62% for 5,10-MTHF).

Folic acid levels were estimated to be 12.4 ng per mg protein in untreated Lo cells compared to 22.6 ng per mg protein in MTX treated Lo cells (Figure 5-3B). Although this increase was not statistically significant Lo cells were able to take up folic acid in the presence of MTX but were unable to process it due to inhibition of DHFR. Total folate levels did not change significantly and went from 102.1 ng per mg protein in untreated Lo cells to 107.5 ng per mg protein in MTX treated Lo cells. Estimates of individual folate derivatives also did not change significantly. 5-MTHF levels were estimated to be 16.8 ng per mg protein in untreated Lo cells compared to an estimate of 15.8 ng per mg protein in MTX treated Lo cells. THF levels were estimated to be 12.4 ng per mg protein in untreated Lo cells compared to an estimate of 15.8 ng per mg protein in MTX treated cells ($p=0.005$). 5,10-MTHF levels were 72.9 ng per mg protein in untreated Lo cells compared to 75.8 ng per mg protein in MTX treated cells.

TNF-α treated Hi and Lo cells had folate derivative distributions that were similar to control cells (Figure 3C and 3D). MTX/TNF-α treated Hi and Lo cells had folate derivative distributions that were similar to MTX treated cells. The results described above regarding the effect of MTX alone were similar in this experiment. In Hi cells total folate decreased from 1241.7 ng per mg protein in control cells to 111.3 ng per mg protein in MTX treated cells, which represented a drop to 9% of control levels. Folic acid levels increased from 5.0 ng per mg protein in control Hi cells to 338.2 ng per mg protein in MTX treated Hi cells. This was a 67.6 fold increase in folic acid levels. In Lo control

cells total folate levels decreased from 19.1 ng per mg protein to 1.9 ng per mg protein in MTX treated Lo cells. Total folate levels dropped to 9.9% of control levels. Folic acid levels were not significantly different; they were 2.3 ng per mg protein in control cells and 3.8 ng per mg protein in MTX treated cells. This was a 1.7 fold increase in folic acid levels.

3A



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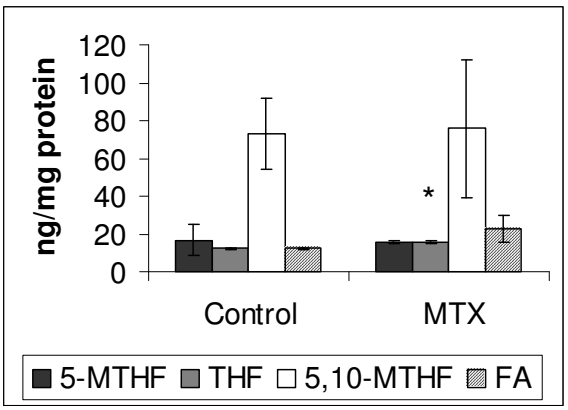


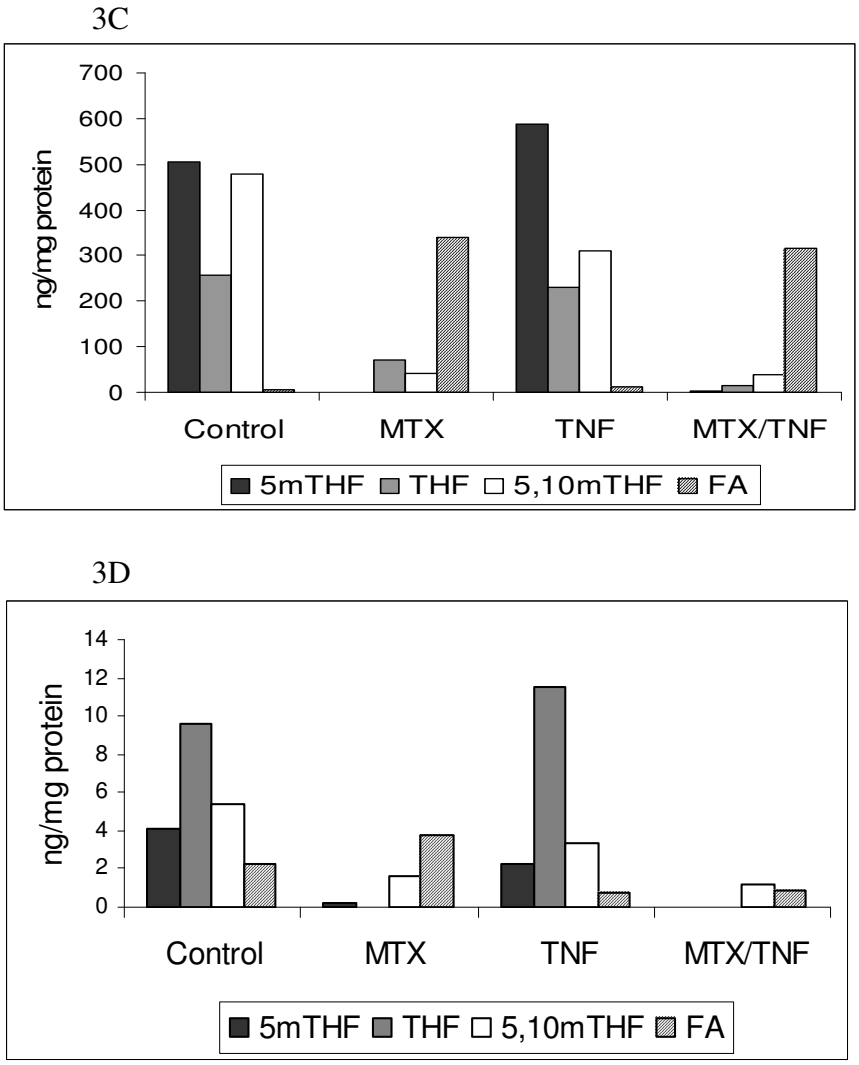


Figure 5-3. Folate derivatives measured by LC/MRM/MS. *A*, Hi confluent cells were treated with 0.5uM MTX for 48 hours. Each bar represents the mean±SD of three samples. This experiment is representative of a total of three experiments with similar results. *B*, Lo confluent cells were treated with 0.5uM MTX for 48 hours. Each bar represents the mean±SD of three samples. This experiment is representative of a total of three experiments with similar results except that in some experiments MTX treated Lo cells did have decreased levels of folate derivatives. *C*, Confluent Hi cells received a media change, 24hrs later 0.5uM MTX was added. Then 24hrs later 10ng/mL TNF-α was added and after 24hrs cells were harvested. Each bar represents a single sample and hence p values were not calculated. This experiment is representative of a total of three experiments with similar results. *D*, Lo cells were treated as in the above described experiment. *P values <0.05 compared with respective control.

In Hi cells it was observed that MTX not only dramatically increased folic acid and decreased total folate, but also modified the relative proportions of folate derivatives,

while in Lo cells MTX was estimated to have little effect on folate derivative levels and only a small increase in folic acid.

MTX Attributable Changes in Gene Expression

The impact of the anti-folate drug, MTX, on gene expression was profiled in both Hi and Lo cells using Affymetrix Human Gene 1.0 ST microarrays. Using an arbitrary threshold of at least 2.0 fold change in microarray signal in reference to untreated controls and adjusted p value <0.05, expression doubled for 47 genes in MTX treated Hi cells and 48 genes in MTX treated Lo cells (Table 5-1); of these 37 were in common. When the threshold was broadened to include the top 200 positive fold changes which were significant, there were 22 inflammatory related genes in MTX treated Hi cells and 18 in MTX treated Lo cells.

Table 5-1. Genes Up Regulated by MTX in Hi and Lo Cells.

			Hi MTX vs. Hi Control		Lo MTX vs. Lo Control	
Gene name	Gene Symbol	RefSeq	Corrected p-value	Fold Change	Corrected p-value	Fold Change
complement component 3	C3	NM_000064	0.004518	6.09	0.012688	4.26
interleukin 1 receptor-like 1	IL1RL1	NM_016232	0.03152	5.44	0.064945	4.16
DNA-damage-inducible transcript 3	DDIT3	NM_004083	0.000816	5.10	0.000896	4.59
amphiregulin	AREG	NM_001657	0.036354	4.74	0.037236	4.74
spermidine/spermine N1-acetyltransferase 1	SAT1	NR_027783	0.004794	4.47	0.007019	3.98
interleukin 8	IL8	NM_000584	0.035674	4.06	0.095036	3.00
interleukin 1, alpha	IL1A	NM_000575	0.018629	3.32	0.021271	3.21
myosin, heavy chain 16 pseudogene	MYH16	NR_002147	0.006217	3.20	0.012298	2.75
ankyrin repeat, family A, 2	ANKRA2	NM_023039	0.003436	3.14	0.003602	3.10
Rho family GTPase 3	RND3	NM_005168	0.014241	2.82	0.053128	2.15
arginase, type II	ARG2	NM_001172	0.000208	2.76	0.000332	2.45
small nucleolar RNA host gene 12 (non-protein coding)	SNHG12	AY277594	0.019755	2.68	0.018825	2.72
enkurin, TRPC channel interacting protein	ENKUR	NM_145010	0.029449	2.63	0.073982	2.17
basic helix-loop-helix family, member e40	BHLHE40	NM_003670	0.017717	2.61	0.045509	2.17
TNF receptor-associated factor 1	TRAF1	NM_005658	0.004209	2.59	0.001149	3.43
CCAAT/enhancer binding protein (C/EBP),	CEBPB	NM_005194	0.004179	2.57	0.009084	2.22

beta						
tumor necrosis factor receptor superfamily, member 9	TNFRSF9	NM_001561	0.000725	2.50	0.000255	3.15
interleukin 11	IL11	NM_000641	0.005749	2.45	0.006503	2.38
NIPA-like domain containing 4	NIPAL4	NM_001099287	0.007065	2.44	0.012427	2.20
jumonji C domain containing histone demethylase 1 homolog D	JHDM1D	NM_030647	0.032722	2.42	0.042911	2.30
leukemia inhibitory factor (cholinergic differentiation factor)	LIF	NM_002309	0.026925	2.37	0.021945	2.48
baculoviral IAP repeat-containing 3	BIRC3	NM_001165	0.017913	2.36	0.007347	2.82
chromosome 9 open reading frame 72	C9orf72	NM_018325	0.000208	2.33	0.000185	2.40
ATP-binding cassette, sub-family A (ABC1), member 1	ABCA1	NM_005502	0.002318	2.32	0.00375	2.12
nuclear factor, interleukin 3 regulated	NFIL3	NM_005384	0.018743	2.30	0.067535	1.85
kelch repeat and BTB (POZ) domain containing 8	KBTBD8	NM_032505	0.031108	2.30	0.025622	2.40
GTP binding protein overexpressed in skeletal muscle	GEM	NM_005261	0.003204	2.24	0.005476	2.04
ectonucleoside triphosphate diphosphohydrolase 7	ENTPD7	NM_020354	0.004725	2.23	0.0067	2.10
sestrin 2	SESN2	NM_031459	0.000397	2.21	0.000584	2.04
arylacetamide deacetylase (esterase)	AADAC	NM_001086	0.02442	2.20	0.027336	2.17
carcinoembryonic antigen-related cell adhesion molecule 1	CEACAM1	NM_001712	0.070458	2.20	0.039634	2.49
ribosomal RNA processing 12 homolog	RRP12	NM_015179	0.004518	2.18	0.00673	2.05
tumor protein p53 inducible protein 3	TP53I3	NM_004881	0.032257	2.16	0.037508	2.11
protein phosphatase 1, regulatory (inhibitor) subunit 15A	PPP1R15A	NM_014330	0.000131	2.14	5.13E-05	2.28
growth arrest and DNA-damage-inducible, alpha	GADD45A	NM_001924	0.000544	2.13	0.000302	2.32
GTP cyclohydrolase 1	GCH1	NM_000161	0.011519	2.12	0.004697	2.45
dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 3	DYRK3	NM_001004023	0.001218	2.12	0.001422	2.05
major facilitator superfamily domain containing 2	MFSD2	NM_001136493	0.0273	2.10	0.034258	2.02
nuclear factor of kappa light polypeptide gene enhancer in B-cells 2	NFKB2	NM_002502	0.002867	2.09	0.00178	2.25
zinc finger protein 79	ZNF79	NM_007135	0.003083	2.09	0.003641	2.04
MAX dimerization protein 1	MXD1	NM_002357	0.024596	2.08	0.022273	2.12
TAF4b RNA polymerase	TAF4B	NM_005640	0.005503	2.06	0.007019	1.99

II, TATA box binding protein (TBP)-associated factor						
ankyrin repeat domain 20B	ANKRD20B	NR_003366	0.016499	2.06	0.032682	1.86
ring finger protein 114	RNF114	NM_018683	0.000884	2.03	0.001126	1.92
interleukin 32	IL32	NM_001012631	0.001376	2.02	0.000794	2.15
kelch-like 21	KLHL21	NM_014851	0.00111	2.01	0.001354	1.92
alkaline ceramidase 2	ACER2	NM_001010887	0.018718	2.01	0.063154	1.69
				Greater than 2 fold change in Lo only		
SH2 domain protein 2A	SH2D2A	NM_001161441	0.382362	1.69	0.049093	2.90
CD274 molecule	CD274	NM_014143	0.121863	1.81	0.034584	2.27
activating transcription factor 3	ATF3	NM_001040619	0.004794	1.91	0.001585	2.26
FBJ murine osteosarcoma viral oncogene homolog B	FOSB	NM_006732	0.070182	1.75	0.016166	2.21
nuclear receptor subfamily 1, group D, member 1	NR1D1	NM_021724	0.000599	1.89	0.000276	2.11
NUAK family, SNF1-like kinase, 2	NUAK2	NM_030952	0.012792	1.76	0.003594	2.09
SERTA domain containing 1	SERTAD1	NM_013376	0.007894	1.81	0.002907	2.07
interleukin 13 receptor, alpha 2	IL13RA2	NM_000640	0.001225	1.80	0.000469	2.05
tribbles homolog 3	TRIB3	NM_021158	0.032254	1.91	0.02104	2.05
testis derived transcript	TES	NM_015641	0.001225	1.95	0.000796	2.03
poliovirus receptor-related 4	PVRL4	NM_030916	0.009434	1.80	0.003985	2.00

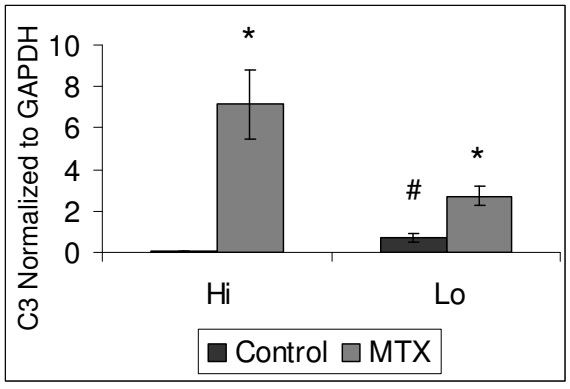
Two inflammatory genes, C3 and IL-8 topped the lists in both MTX treated Hi and Lo cells. C3 was number 1 on the list and was up regulated by 6.09 fold (p=0.005) in MTX treated Hi cells. C3 was number 3 on the list in MTX treated Lo cells and up regulated by 4.26 fold (p=0.013) (Table 5-1). C3 mRNA levels were verified by qRT-PCR methods. C3 mRNA levels were confirmed to be significantly up regulated in MTX treated Hi and Lo cells (Figure 5-4A). Specifically C3 mRNA levels were up regulated by 158.4-fold in MTX treated Hi cells (p=0.018) and 3.9-fold in MTX treated Lo cells (p=0.003). Also Lo control cells had 15.4 fold more C3 mRNA than Hi control cells (p=0.034). C3 mRNA levels were also measured in TNF-α and MTX/TNF-α treated Hi

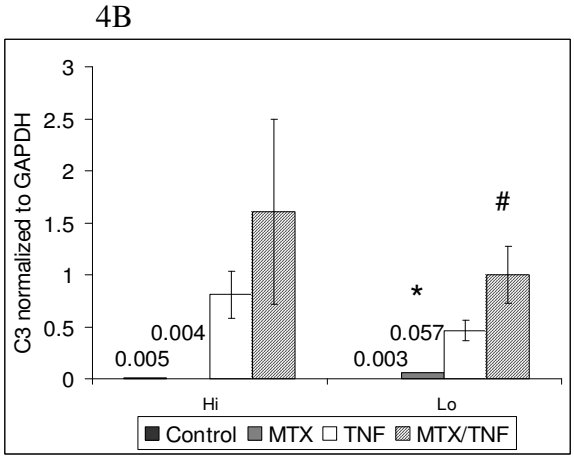
and Lo cells. MTX/TNF-α treated Lo cells had 2.2 fold more C3 mRNA than TNF-α treated Lo cells (p=0.0329) (Figure 5-4B).

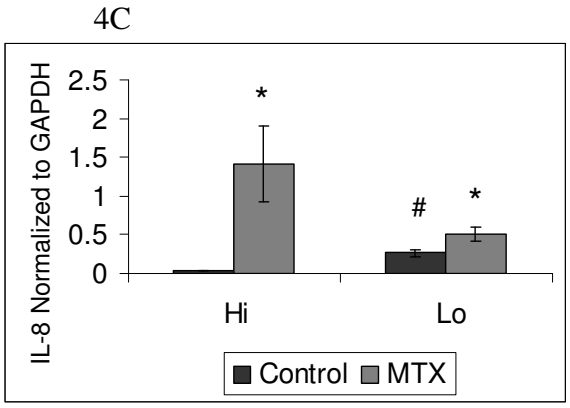
IL-8 was number 6 on the list in MTX treated Hi cells and was up regulated by 4.06 fold (p=0.036). IL-8 was number 11 on the list and up regulated by 3.00 fold (p=0.095) in MTX treated Lo cells (Table 5-1). IL-8 mRNA levels were also confirmed by qRT-PCR to be significantly up regulated (Figure 5-4C), specifically IL-8 mRNA levels were up regulated by 36.2-fold in MTX treated Hi cells (p=0.041) and 1.9-fold in MTX treated Lo cells (p=0.015). Also Lo control cells had 6.7 fold more IL-8 mRNA than Hi control cells (p=0.010).

Another inflammatory gene, MCP-1, that was previously shown to be regulated by folate status (Brown et al. 2006), was not significantly different in the microarray analysis of MTX treated Hi or Lo cells compared to their respective controls (data not shown).

4A







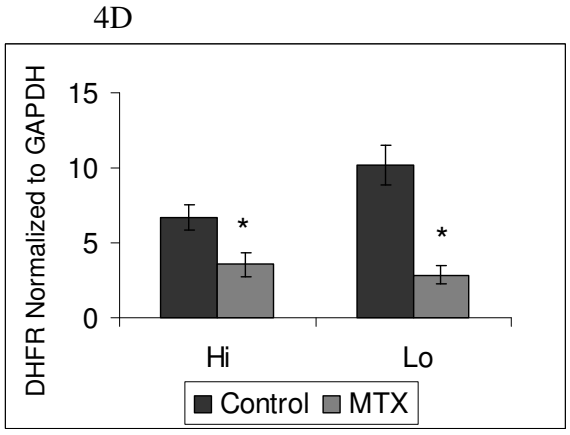


Figure 5-4. QRT-PCR. The mRNA expression of selected targets was studied by qRT-PCR. Gene expression of respective genes was calculated using the standard curve method using the endogenous housekeeping control gene GAPDH for normalization. Each bar represents mean±SD target expression levels normalized to GAPDH levels of three samples. These experiments are representative of a total of three experiments with similar results. *A*, C3. Hi and Lo cells were exposed to 0.5μM MTX for 48 hours. *B*, C3. Hi and Lo cells were treated with MTX prior to treatment with TNF-α. *C*, IL-8. Hi and

Lo cells were exposed to 0.5µM MTX for 48 hours. *D*, DHFR. Hi and Lo cells were exposed to 0.5µM MTX for 48 hours. *P values <0.05 compared with respective control. #P values <0.05 for Hi control compared to Lo control.

Conversely, expression was reduced by at least 50% for 83 genes in MTX treated Hi cells and 85 genes in MTX treated Lo cells (Table 5-2); of these 69 were in common. The most prominent in the list are a range of cell cycle related products as might be expected following treatment with an anti-folate drug. Also of interest DHFR, which is inhibited by MTX, was down regulated by 2.22 fold (p=0.003) in MTX treated Hi cells and 2.43 fold (p=0.002) in MTX treated Lo cells (Table 5-2). DHFR mRNA levels were confirmed by qRT-PCR to be down regulated, specifically DHFR mRNA levels were down regulated by 1.9-fold in MTX treated Hi cells (p=0.010) and 3.6-fold in MTX treated Lo cells (p=0.009) (Figure 5-4D).

A complete list of MTX regulated genes has been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO, http://www.nlm.nih.gov/geo/) and is accessible through GEO Series accession number [will be available soon].

Candidate inflammatory gene mRNA levels were examined in TNF-α and MTX/TNF-α treated cells. The chosen candidate genes were ICAM1, VCAM1, and E-selectin. None of the mRNA levels of these genes were significantly different between TNF-α and MTX/TNF-α treated cells (data not shown).

Table 5-2. Genes Down Regulated by MTX in Hi and Lo Cells.

			Hi MTX vs. Hi Control		Lo MTX vs. Lo Control	
Gene name	Gene Symbol	RefSeq	Corrected p-value	Fold-Change	Corrected p-value	Fold-Change
polo-like kinase 1	PLK1	NM_005030	1.88E-05	-5.25	3.80E-05	-4.68
histone cluster 1, H2bm	HIST1H2BM	NM_003521	0.000397	-4.98	0.000584	-4.26
kinesin family member 20A	KIF20A	NM_005733	0.009434	-4.11	0.028454	-3.03
budding uninhibited by benzimidazoles 1	BUB1	NM_004336	0.00019	-3.80	0.000206	-3.55

homolog						
protein regulator of cytokinesis 1	PRC1	NM_003981	7.72E-05	-3.80	5.13E-05	-3.74
DEP domain containing 1	DEPDC1	NM_001114120	0.00138	-3.79	0.002273	-3.30
topoisomerase II alpha	TOP2A	NM_001067	0.000673	-3.63	0.000794	-3.30
kinesin family member 15	KIF15	NM_020242	0.000884	-3.56	0.000502	-4.06
cyclin A2	CCNA2	NM_001237	0.000891	-3.47	0.001298	-3.08
proline rich 11	PRR11	NM_018304	0.000251	-3.40	0.000271	-3.43
transmembrane protein 71	TMEM71	NM_144649	0.001489	-3.40	0.00321	-2.82
NUF2, NDC80 kinetochore complex component, homolog	NUF2	NM_145697	0.000247	-3.33	0.000469	-2.84
family with sequence similarity 111, member B	FAM111B	NM_198947	0.000616	-3.22	0.000449	-3.43
centromere protein I	CENPI	NM_006733	0.000208	-3.15	0.000143	-3.40
histone cluster 1, H2bb	HIST1H2BB	NM_021062	0.002254	-3.04	0.003288	-2.77
NDC80 kinetochore complex component, homolog	SPC25	NM_020675	0.000431	-3.00	0.000372	-3.06
cyclin B2	CCNB2	NM_004701	0.001084	-2.94	0.001676	-2.61
discs, large (Drosophila) homolog-associated protein 5	DLGAP5	NM_014750	0.000691	-2.86	0.001149	-2.50
cell division cycle 2, G1 to S and G2 to M	CDC2	NM_001786	0.002365	-2.84	0.003357	-2.63
WD repeat domain 76	WDR76	NM_024908	0.001858	-2.84	0.00217	-2.72
nei endonuclease VIII-like 3	NEIL3	NM_018248	0.001214	-2.79	0.002907	-2.34
cyclin-dependent kinase inhibitor 3	CDKN3	NM_005192	0.010796	-2.79	0.01432	-2.61
non-SMC condensin I complex, subunit H	NCAPH	NM_015341	0.000343	-2.78	0.000297	-2.87
SHC SH2-domain binding protein 1	SHCBP1	NM_024745	0.004586	-2.75	0.007303	-2.49
centromere protein F	CENPF	NM_016343	0.000241	-2.69	0.000297	-2.58
budding uninhibited by benzimidazoles 1 homolog beta	BUB1B	NM_001211	0.000867	-2.62	0.000614	-2.70
collagen and calcium binding EGF domains 1	CCBE1	NM_133459	0.004156	-2.59	0.004322	-2.54
hospholipid scramblase 4	PLSCR4	NM_001128304	0.02513	-2.54	0.075709	-2.05
KIAA0101	KIAA0101	NM_014736	0.000947	-2.54	0.000693	-2.62
anillin, actin binding protein	ANLN	NM_018685	0.026609	-2.53	0.028161	-2.51
NDC80 homolog, kinetochore complex component	NDC80	NM_006101	0.00131	-2.53	0.002907	-2.19
shugoshin-like 2	SGOL2	NM_152524	0.001592	-2.51	0.000776	-2.84
Rho GTPase activating protein 11A	ARHGAP11A	NM_014783	0.001195	-2.51	0.000796	-2.63
PDZ binding kinase	PBK	NM_018492	0.001148	-2.48	0.000584	-2.78
citron (rho-interacting, serine/threonine kinase 21)	CIT	NM_007174	0.00357	-2.48	0.003553	-2.48

centromere protein K	CENPK	NM_022145	0.005066	-2.43	0.003837	-2.56
NDC80 kinetochore complex component, homolog	SPC24	NM_182513	0.001261	-2.42	0.000622	-2.72
kinesin family member 11	KIF11	NM_004523	0.000301	-2.41	0.000321	-2.36
forkhead box M1	FOXM1	NM_202002	0.00153	-2.40	0.001491	-2.39
hyaluronan-mediated motility receptor	HMMR	NM_001142556	0.044808	-2.38	0.089897	-2.08
membrane protein, palmitoylated 1	MPP1	NM_002436	0.03532	-2.38	0.083699	-2.02
cancer susceptibility candidate 5	CASC5	NM_170589	0.000884	-2.36	0.000802	-2.32
cell division cycle 20 homolog	CDC20	NM_001255	0.031901	-2.35	0.033656	-2.34
kinesin family member C1	KIFC1	NM_002263	0.000947	-2.33	0.000584	-2.49
primase, DNA, polypeptide 1	PRIM1	NM_000946	0.00197	-2.31	0.000799	-2.67
meiosis-specific nuclear structural 1	MNS1	NM_018365	0.005897	-2.31	0.002181	-2.80
kinesin family member 4A	KIF4A	NM_012310	0.001548	-2.31	0.000802	-2.54
kinesin family member 18B	KIF18B	NM_001080443	0.000257	-2.30	0.000332	-2.19
sperm associated antigen 5	SPAG5	NM_006461	0.009803	-2.29	0.017348	-2.08
minichromosome maintenance complex component 6	MCM6	NM_005915	0.001497	-2.27	0.00036	-2.99
histone cluster 1, H1b	HIST1H1B	NM_005322	0.00767	-2.26	0.004734	-2.44
nucleolar and spindle associated protein 1	NUSAP1	NM_016359	0.002992	-2.23	0.003799	-2.15
excision repair cross-complementing rodent repair deficiency, complementation group 6-like	ERCC6L	NM_017669	0.004792	-2.23	0.004222	-2.27
dihydrofolate reductase	DHFR	AK293146	0.002732	-2.22	0.001585	-2.43
G-protein signaling modulator 2	GPSM2	NM_013296	0.039037	-2.22	0.056481	-2.08
non-SMC condensin II complex, subunit G2	NCAPG2	NM_017760	0.008014	-2.21	0.006481	-2.28
transmembrane protein 106C	TMEM106C	NM_001143842	0.005389	-2.21	0.003569	-2.38
spindle and kinetochore associated complex subunit 3	SKA3	NM_145061	0.020449	-2.20	0.02316	-2.16
cyclin B1	CCNB1	NM_031966	0.004518	-2.20	0.001875	-2.56
aldehyde dehydrogenase 1 family, member A1	ALDH1A1	NM_000689	0.002022	-2.18	0.005222	-1.88
asp (abnormal spindle) homolog, microcephaly associated	ASPM	NM_018136	0.001036	-2.18	0.000728	-2.25
polymerase, epsilon 2	POLE2	NM_002692	0.001716	-2.16	0.000932	-2.35
BRCA1 interacting protein C-terminal helicase 1	BRIP1	NM_032043	0.016101	-2.15	0.033478	-1.92
integrin, beta-like 1	ITGBL1	NM_004791	0.017809	-2.15	0.035844	-1.92
fidgetin	FIGN	NM_018086	0.000544	-2.14	0.001157	-1.88

lamin B1	LMNB1	NM_005573	0.00394	-2.13	0.00321	-2.20
histone cluster 1, H3i	HIST1H3I	NM_003533	0.001916	-2.13	0.001864	-2.12
Fanconi anemia, complementation group D2	FANCD2	NM_033084	0.003083	-2.12	0.001318	-2.45
ribonucleotide reductase M2	RRM2	NM_001034	0.037498	-2.10	0.029957	-2.20
kinesin family member 23	KIF23	NM_138555	0.001204	-2.10	0.001972	-1.93
maternal embryonic leucine zipper kinase	MELK	NM_014791	0.000952	-2.09	0.000622	-2.18
antigen identified by monoclonal antibody Ki-67	MKI67	NM_002417	0.00284	-2.08	0.012216	-1.71
ASF1 anti-silencing function 1 homolog B	ASF1B	NM_018154	0.007787	-2.08	0.009392	-2.01
Opa interacting protein 5	OIP5	NM_007280	0.003731	-2.07	0.00454	-2.00
transmembrane protein 195	TMEM195	NM_001004320	0.028216	-2.06	0.157843	-1.59
cell division cycle associated 8	CDCA8	NM_018101	0.001225	-2.06	0.000505	-2.36
GINS complex subunit 2	GINS2	NM_016095	0.002318	-2.05	0.00058	-2.56
S-phase kinase-associated protein 2	SKP2	NM_005983	0.000131	-2.05	5.13E-05	-2.23
histone cluster 1, H2bh	HIST1H2BH	NM_003524	0.009348	-2.05	0.007019	-2.12
aurora kinase A	AURKA	NM_198433	0.002761	-2.04	0.002068	-2.13
spindle and kinetochore associated complex subunit 2	SKA2	NM_182620	0.001868	-2.04	0.003569	-1.86
GTPase, IMAP family member 2	GIMAP2	NM_015660	0.029118	-2.02	0.058697	-1.81
E2F transcription factor 8	E2F8	NM_024680	0.038974	-2.01	0.052401	-1.93
				Greater than 2 fold change in Lo only		
ubiquitin-conjugating enzyme E2C	UBE2C	NM_181802	0.000673	-1.95	0.000255	-2.27
ubiquitin-like with PHD and ring finger domains 1	UHRF1	NM_001048201	0.016634	-1.81	0.003569	-2.27
replication factor C 4	RFC4	NM_002916	0.001858	-1.73	0.0003	-2.23
high-mobility group box 2	HMGB2	NM_001130688	0.00745	-1.94	0.003641	-2.15
transmembrane protein 97	TMEM97	NM_014573	0.004009	-1.72	0.000796	-2.09
transforming, acidic coiled-coil containing protein 3	TACC3	NM_006342	0.003552	-1.84	0.001491	-2.07
chromosome 12 open reading frame 48	C12orf48	AK302724	0.00728	-1.72	0.001774	-2.06
histone cluster 1, H3a	HIST1H3A	NM_003529	0.0481	-1.65	0.009468	-2.06
kinesin family member 14	KIF14	NM_014875	0.008309	-1.95	0.005476	-2.05
thyroid hormone receptor interactor 13	TRIP13	NM_004237	0.010858	-1.87	0.005458	-2.05
breast cancer 1, early onset	BRCA1	NR_027676	0.012441	-1.72	0.003357	-2.05
chromosome 1 open reading frame 110	C1orf110	NM_178550	0.032012	-1.97	0.028158	-2.02
single-minded homolog 1	SIM1	NM_005068	0.008161	-1.84	0.003979	-2.02
butyrylcholinesterase	BCHE	NM_000055	0.061941	-1.71	0.021168	-2.00

KDEL (Lys-Asp-Glu-Leu) containing 2	KDELC2	NM_153705	0.000208	-1.86	9.71E-05	-2.00
minichromosome maintenance complex component 10	MCM10	NM_182751	0.010962	-1.83	0.005348	-2.00

Secreted Protein Quantification

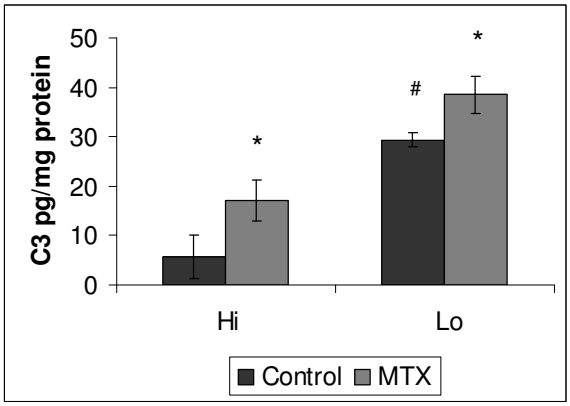
Secreted C3 levels were measured in the media of Hi and Lo cells treated with 0.5 μM MTX for 48 hours. MTX treated Hi cells had 17.1 ng of C3 per mg protein, which was significantly higher than 5.7 ng per mg protein in untreated Hi control cells, this represented an up regulation of 3.0 fold (p=0.032) (Figure 5-5A). MTX treated Lo cells had a secreted C3 level of 38.5 ng per mg protein, which was significantly higher than 29.3 ng per mg protein in untreated Lo control cells, this represented an up regulation of 1.3 fold (p=0.018). Also Lo control cells had significantly more secreted C3 than Hi control cells, which represented an up regulation of 5.1 fold (p=0.001).

Secreted IL-8 levels were measured in the media of Hi and Lo cells treated with 0.5 μM MTX for 48 hours. MTX treated Hi cells had a secreted IL-8 level of 46.2 ng per mg protein, which was significantly higher than 3.7 ng per mg protein in untreated cells, which represented an up regulation of 12.5 fold (p=0.016) (Figure 5-5B). MTX treated Lo cells had a secreted IL-8 level of 42.5 ng per mg protein, which was significantly higher than 25.0 ng per mg protein in untreated cells, which represented an up regulation of 1.7 fold (p=0.008). Also Lo control cells had significantly more secreted IL-8 than Hi control cells, which represented an up regulation of 6.8 fold (p=0.007). Secreted IL-8 levels were also measured in TNF-α and MTX/TNF-α treated Hi and Lo cells, but there was no significant difference (data not shown).

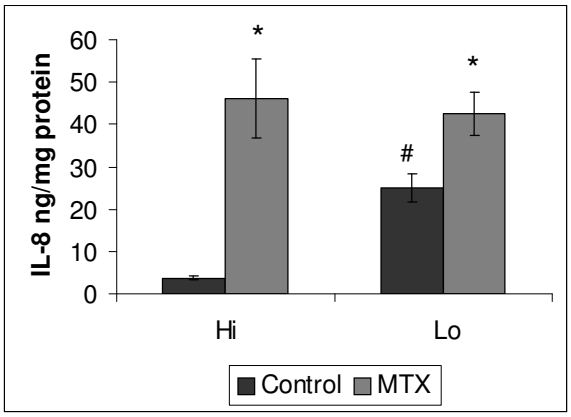
Secreted MCP-1 levels were measured in the media of Hi and Lo cells treated with 0.5 μM MTX for 48 hours. MTX treated Hi cells had a secreted MCP-1 level of 14.4

ng per mg protein, which was higher but not significantly different from 11.4 ng per mg protein in untreated Hi cells (p=0.26) (Figure 5-5C). MTX treated Lo cells had a secreted MCP-1 level of 21.1 ng per mg protein, which was higher but not significantly different from 19.7 ng per mg protein in untreated Lo cells (p=0.53). Also Lo control cells had significantly more secreted MCP-1 than Hi control cells, which represented an up regulation of 1.7 fold (p=0.033). Secreted MCP-1 levels were also measured in TNF-α and MTX/TNF-α treated Hi and Lo cells, but there was no significant difference (data not shown).

5A



5B



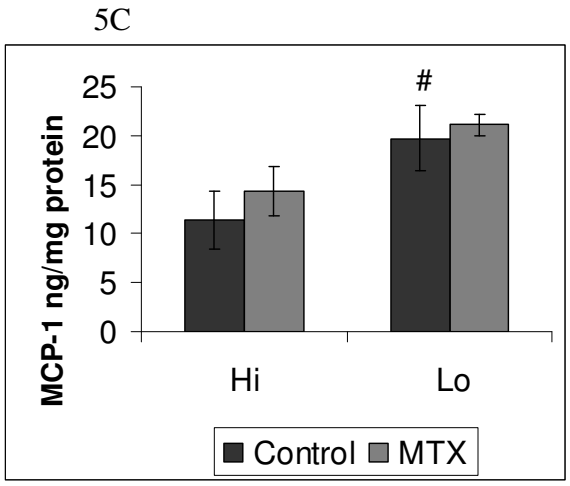


Figure 5-5. ELISAs. Hi and Lo cells were exposed to 0.5uM MTX for 48 hours. Secreted target protein was measured in the media. Each bar represents the mean±SD of three samples. *A*, C3. *B*, IL-8. *C,* MCP-1. These experiments are representative of a total of three experiments with similar results. *P values <0.05 for MTX compared with control. #P values <0.05 for Hi control compared to Lo control.

5.5 Discussion

Elevated homocysteine is often accompanied by low folate status but is also influenced by dietary intake of vitamins, lifestyle, and genetic factors (Refsum et al. 2006). A high homocysteine and low folate phenotype is associated with many conditions which also involve inflammation such as rheumatoid arthritis (RA) (Wallberg-Jonsson et al. 2002). Chronic inflammation of synovium in rheumatoid arthritis involves migration of leukocytes which interact with the endothelium via cell adhesion molecules and cytokine/chemokine signaling. The increase in inflammation in RA leads to increased comorbidity of cardiovascular disease (CVD) (Sattar et al. 2003). Methotrexate (MTX) is an anti-folate drug used to treat RA, which may have an effect on CVD comorbidity and mortality. The emerging consensus as reflected in a review by Westlake et al. (Westlake et al. 2010) is that the use of MTX in the treatment of RA is associated with a reduction in CVD comorbidity and mortality, due to its anti-inflammatory effects. We wanted to

examine how MTX treatment may influence the expression of inflammatory genes because of its conflicting properties of anti-folate and anti-inflammatory. We chose to investigate the effects of MTX within the context of a well defined model system, Hi and Lo EA.hy 926 endothelial cells adapted to normal and low folate, respectively.

Doses of MTX were chosen in the range of concentrations relevant to the treatment of rheumatoid arthritis (Lebbe et al. 1994). In this study MTX was shown to inhibit proliferation of endothelial cells in the range of concentrations from 0.1 to 0.5 μM, and the 0.5 μM dose was chosen for further study. A study in human umbilical vein endothelial cells (HUVECs) showed an inhibition of proliferation when treated with doses of MTX comparable to *in vivo* levels after low dose administration (0.1-1.0 μM) (Yamasaki et al. 2003).

Total intracellular folate was decreased in Hi cells when treated with 0.5 μM MTX. In MTX treated Hi cells the composition of intracellular folate derivatives was modified by MTX such that 5-MTHF levels were lower and folic acid levels were higher. The retention of unmetabolized folic acid, which cannot be utilized, indicates that dihydrofolate reductase (DHFR) was inhibited resulting in a change in the distribution of folate derivatives. These results are consistent with studies by Baram et al. (Baram et al. 1987) on cultured human myeloid precursor cells (MPCs) that found MTX treated cells had decreased levels of 5-MTHF, 10-formylTHF, and 5-formylTHF and increased levels of DHF and 10-formylDHF, evident of inhibition of DHFR. We observed that Hi and Lo cells treated with MTX down regulated DHFR, which could also account for the increase in folic acid.

The effect of the anti-folate, MTX, was investigated using microarrays which covered most of the genes within the human genome. In the present study it was demonstrated that MTX increased the production of C3 and IL-8 in Hi as well as Lo cells with no change in MCP-1 levels. C3 activation is responsible for local inflammation and levels of which are known to be increased in synovial fluids of patients with rheumatoid arthritis (Okroj et al. 2007). Although not direct evidence, a small study showed that combination treatment with MTX and cyclophosphamide of pediatric SLE resulted in increased C3 levels in serum (Lehman et al. 2004). Hi cells treated with MTX had a much greater fold increase in C3 than MTX treated Lo cells, which could be related to the dramatic drop in folate levels in Hi cells. In Lo cells in the presence of an inflammatory stimulator, TNF-α, pre-treatment with MTX increased C3 levels. The anti-folate properties of MTX seem to be capable of up regulating specific inflammatory proteins.

It was previously published that Lo cells secreted more MCP-1 than Hi cells (Brown et al. 2006), therefore chronic folate insufficiency was able to up regulate an inflammatory protein. We have found that in Lo control cells C3 and IL-8 were more highly expressed and had increased synthesis and export of protein compared to Hi control cells. This finding may indicate that folate dysfunction is associated with a heightened state of immune readiness.

In a study by Cascao et al. (2010) synovial fluid of rheumatoid arthritis patients had increased IL-8, a neutrophil chemoattractant. Rheumatoid arthritis patients also had increased circulating IL-8 levels, but after 4 months of MTX treatment circulating IL-8 levels were not significantly changed. MTX treatment is associated with a serious side

effect, inflammation of the lung called pneumonitis, which has been shown to be related to an elevation of IL-8 (Yoshida et al. 1999). Subsequently Yamauchi et al. (2004) has shown that bronchial and alveolar epithelial cells treated with MTX up regulated IL-8. We have shown that IL-8 was up regulated with a greater fold increase in MTX treated Hi cells than MTX treated Lo cells, which again could be related to the dramatic drop in folate levels in Hi cells.

MCP-1 is involved in both the recruitment of monocytes to atherosclerotic lesions and to synovium in RA patients. Although MTX did not influence MCP-1 levels in endothelial cells in this study, it remains to be seen whether MTX can influence MCP-1 levels in RA patients. An etiological component of RA is up regulation of systemic inflammation, consequently these patients have an increased risk of developing CVD (Sattar et al. 2003). Treatment of RA with MTX has been associated with an overall decrease in CVD comorbidity and mortality in a recent meta-analysis (Westlake et al. 2010), although *in vitro* evidence presented in this set of experiments points to increases in specific inflammatory products such as C3 and IL-8. This paradoxical decrease in CVD comorbidity observed in RA patients and up regulation of specific inflammatory genes in endothelial cells may mean that MTX treatment induces folate dysregulation which may counteract some of its anti-inflammatory actions. We have found C3 and IL-8 to be up regulated in endothelial cells, but well designed studies in RA patients beginning MTX will be needed to validate this observation, and to assess the benefit to CVD risk to see if levels of these inflammatory products are related to modulation of CVD risk.

Chapter 6: General Discussion and Future Directions

6.1 General Discussion

The overall conclusions that can be drawn from the research work in this thesis are:

A) There was gene-gene interaction between CBS 844ins68 genotypes and MTHFR 677C>T genotypes on tHcy and folate levels in healthy men. In healthy women 11 polymorphisms were genotyped and several were associated with altered levels of tHcy as well as plasma and RBC folate derivatives.

In healthy males from the Industrial Workers study *CBS* 844ins68 genotypes had no effect on tHcy and folate levels when considered alone but when considered in the context of *MTHFR* 677C>T genotypes there was an impact on tHcy and folate levels only within *MTHFR* 677TT individuals. The tHcy raising and folate lowering effect of the *MTHFR* 677TT genotype was counteracted in those who were *CBS* 844ins68 carriers.

In the Premenopausal Women study tHcy levels in African Americans were associated with the polymorphisms *MTR* 2756A>G and *TYMS* 1494del6. THcy levels in Caucasians were associated with the polymorphisms *MTHFR* 677C>T, *MTHFR* 1298A>C, and *MTR* 2756A>G. Plasma and RBC folate derivatives in African Americans were associated with the polymorphisms *MTR* 2756A>G, *MTRR* 66A>G, *RFC1* 80A>G, and *TYMS* 5'VNTR. In Caucasians plasma and RBC folate derivatives were associated with the polymorphisms *MTHFR* 677C>T, *MTHFR* 1298A>C, *MTR* 2756A>G, *TYMS* 1494del6, and *RFC1* 80A>G.

B) None of the 7 polymorphisms tested were associated with increased NTD risk in children and mothers by TDT analyses.

Although TDT analyses did not find any significant associations there was evidence that rare crossover events between *MTHFR* 677 and 1298 polymorphisms may increase risk of NTDs because two such genotypes were present in mothers only. Also rare crossover events between *TYMS* 5'VNTR and 1494del6 were more frequent in children and mothers.

C) None of the polymorphisms tested were associated with elevated tHcy levels or CAC scores in SLE cases. In RA patients taking MTX several polymorphisms were associated with altered levels of RBC folate derivatives. Several polymorphisms were associated with discontinuation of MTX and attributable toxicities.

In the SLE study GFR was negatively correlated to tHcy levels and was therefore used as a covariate in general linear models which examined the effects of genetic polymorphisms on tHcy levels. Although none of the polymorphisms were found to impact tHcy levels in SLE cases, the polymorphisms *MTHFR* 677C>T and 1298A>C were associated with tHcy levels in controls.

In the RA study the polymorphisms *MTHFD1* 1958G>A and *MTHFR* 1298A>C were associated with discontinuation of MTX therapy in African Americans and Caucasians, respectively. The polymorphisms *MTR* 2756A>G and *MTHFD1* 1958G>A were associated with attributable toxicities in African American and Caucasian current users, respectively. The polymorphisms *DHFR* c.86+60_78 and *TYMS* 1494del6 were associated with currently prescribed MTX dose in African Americans. RBC folate derivatives were associated with the polymorphism *DHFR* c.86+60_78 in African Americans. In Caucasians RBC folate derivatives were associated with the polymorphisms *MTHFR* 677C>T, *RFC1* 80A>G, and *MTRR* 66A>G.

D) In EA.hy 926 endothelial cells MTX treatment resulted in depletion of total folate, altered composition of folate derivatives, and increased expression of the inflammatory genes, C3 and IL-8.

Hi and Lo cells were treated with MTX and cellular proliferation was inhibited. MTX treated Hi cells had depleted levels of total folate, an altered distribution of folate derivatives, and higher levels of unmetabolized folic acid. Microarray analyses identified a number of inflammatory genes which were up regulated in MTX treated Hi and Lo cells. C3 and IL-8 were verified by qRT-PCR to be up regulated in MTX treated cells. ELISAs also confirmed that secreted protein levels of C3 and IL-8 were higher in MTX treated cells.

6.2 Future Directions

6.2.1 Healthy subjects

In the Industrial Workers study there are several polymorphisms which have been genotyped and remain to be analyzed and published even if there are no associations with tHcy and folate levels. These polymorphisms are: *MTHFR* 1298A>C, *SHMT1* 1420C>T, *RFC1* 80G>A, *TYMS* 1494del6, and *MTHFD1* 1958G>A. *TYMS* 5'VNTR and *DHFR* c.86+60_78 polymorphisms also need to be genotyped and analyzed. Although this study consists of only men and some of these polymorphisms are associated with differences in Hcy and folate levels only in women, the Industrial Workers study is large enough to permit the analyses of gene-gene interactions.

The Premenopausal Women study showed that polymorphisms in the enzymes of the folate/Hcy pathway impact tHcy and folate derivative levels. The use of high precision methods to measure individual folate derivatives should be expanded to studies

on healthy men. Also any subsequent studies should consider the number of subjects of each race that would need to be recruited because a large scale study would enable analyses of rare homozygotes. C3 and IL-8 levels could be measured in healthy individuals and examined for associations with tHcy and folate levels.

6.2.2 Families with a child affected by NTDs

As previously mentioned TDT analyses on specific subsets of the NTD study need to be performed. Subsets which may reveal increased genetic risk include maternal smoking status, maternal supplementation status, race, and year of birth, which would examine effects of genotypes prior to and after folic acid fortification in the US. TDT analyses are relatively simple and a more complex analysis such as log-linear modeling may uncover increased risk for NTDs associated with a particular genotype. Polymorphisms in C3 and IL-8 could be searched for and genotyped in the NTD study.

6.2.3 Inflammatory diseases

In the SLE study only tHcy concentrations were measured. If another study was planned then whole blood should be collected in a method compatible with measurement of folate derivatives using LC/MRM/MS, which would require that whole blood be immediately diluted in ascorbic acid to prevent the oxidation of folate derivatives. The association between CAC scores and folate derivative levels could then be analyzed. The current SLE study has serum and/or plasma samples available and candidate inflammatory genes such as C3 and IL-8 could be measured and compared to controls. C3 and IL-8 would be considered because MCP-1 was another inflammatory gene which was first found to be up regulated in Lo compared to Hi cells. In the SLE study MCP-1

levels were shown to be higher in SLE cases compared to controls (Brown et al. 2007).C3 and IL-8 levels should also be analyzed for association with CAC scores.

In the RA study analyses are underway which include baseline measurements of tHcy and folate derivative levels prior to MTX therapy. Other future directions include restricting the analyses of current users to just females because a few of the polymorphisms seem to be associated with differences in Hcy and folate levels only in women. Since C3 and IL-8 were up regulated in MTX treated Hi and Lo cells, levels of these proteins should be measured in plasma samples and analyzed for associations with tHcy and folate levels. The changes in C3 and IL-8 levels as RA patients begin MTX therapy will also be of interest.

6.2.4 Cell culture

In cell culture experiments involving Hi and Lo cells treated with MTX and TNF-α a future direction would be to measure secreted C3 levels in TNF-α and MTX/TNF-α treated cells. Since MTX treatment was associated with an inhibition of proliferation, an assay specific to cell death would be a better measure of MTX induced apoptosis than counting the number of live cells. The trypan blue assay was able to dye the dead cells but the length of time need to complete each assay limited the utility of this method. Even though MTX treatment was applied when cells were confluent it was apparent that cell division continued to take place after confluence and therefore the difference between the number of live control cells and live MTX treated cells after 48 hours may have been overestimated. Finally there are other inflammatory genes which were found to be significantly up regulated by MTX treatment in the microarray analysis, such as IL-1A, which need to be validated by qRT-PCR and ELISA.

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