NOTE TO USERS

Pharmacogenetic studies of methotrexate and metafolin in a mouse model of severe and mild 5, 10-methylenetetrahydrofolate reductase deficiency

By

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In memory of geneticists of the former Soviet Union, whose work and lives were destroyed in the period of 1927-1965.

Abstract

Methylenetetrahydrofolate reductase (MTHFR) is a critical enzyme in folate metabolism. Severe enzymatic deficiency results in metabolic disease with occasional early demise. Mild deficiency is due to a common polymorphism (A222V) which increases risk for several common disorders. Pharmacogenetic studies of the antifolate methotrexate on *Mthfr* heterozygous knockout mice (*Mthfr* +/-), a model of the mild human deficiency, revealed significantly higher apoptosis rates in several tissues compared to wildtype mice (*Mthfr* +/+). These results suggest that mild MTHFR deficiency is a risk factor for methotrexate toxicity in mice and possibly in humans. A new medication, metafolin, was administered to *Mthfr* +/- mothers to assess survival of their *Mthfr*-/- pups. Survival rate of pups at 5 weeks of age from treated mothers was significantly higher than that from untreated mothers (54% versus 20%). The results of this study suggest that metafolin could be useful for treatment of severe MTHFR deficiency in humans

Résumé

La méthylènetétrahydrofolate réductase (MTHFR) est une enzyme jouant un rôle-clé lors du métabolisme du folate. Des défauts enzymatiques sévères résultent en une maladie métabolique accompagnée parfois de mortalité en jeune âge. Une déficience plus légère est fréquemment provoquée par un polymorphisme (A222V) qui constitue un facteur de risque pour plusieurs maladies courantes. Des etudes pharmacogénétiques impliquant le methotrexate (un antifolate) et des souris dont on a aboli une des deux copie du gène *Mthfr* (*Mthfr* +/-; un modèle animal du défaut mineur observé pour les humains) a révélé une augmentation significative de l'apoptose dans plusieurs tissues en comparaison avec des souris normales (*Mthfr* +/+). Ces observations suggèrent qu'un léger deficit en MTHFR est un facteur de risque pour la toxicité du methotrexate dans ce modèle animal et possiblement pour les sujets humains. Une nouvelle médication, la métafoline, a été administrée à des mères *Mthfr* +/-, afin d'en tester l'effet sur la survie de leurs rejetons *Mthfr* -/-. Le taux de survie (jugé à un âge de 5 semaines) était significativement plus élevé pour les souriceaux de mères traitées que lorsque les mères n'avaient pas reçu le traitement (54% comparé à 20%). Les resultats de cette etude suggèrent que la métafoline pourrait être utile pour le traitement du défaut sévère de *MTHFR* observé pour des sujets humains.

ABBREVIATIONS

AI	Apoptotic Index
ALT	Alanine Aminotransferase
AST	Aspartate aminotransferase
BHMT	Betaine-Homocysteine Methyltransferase
CBS	Cystathionine-beta-synthase
DHF(R)	Dihydrofolate (Reductase)
(t)Hcy	Total plasma homocysteine
HPLC	High Performance Liquid Chromatography
dTMP	Deoxythymidinemonophosphate
dUMP	Deoxyuridinemonophosphate
LD	Lethal Dose
MTHFR	Methylenetetrahydrofolate Reductase
MS	Methionine Synthase
MTX	Methotrexate
NTD	Neural Tube Defect
PCR	Polymerase Chain Reaction
ROS	Reactive Oxygen Species
SAM	S-adenosylmethionine
SAH	S-adenosylhomocysteine
THF	Tetrahydrofolate
TS	Thymidylate Synthase
TUNEL	Terminal Deoxynucleotidyl Transferase-Mediated X-dUTP Nick End Labeling

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CHAPTER I

INTRODUCTION

I.1 Homocysteine and folate metabolism

Homocysteine is a sulfur amino acid derived from methionine. There are two major pathways of homocysteine metabolism (Fig.1). The first one is remethylation to methionine. It requires folate and cobalamin (vitamin B_{12}) or betaine. The second one is transsulfuration to cystathionine. It requires pyridoxal-5-phosphate (vitamin B_6).

I.2 Metabolism of Folate

Dietary folate is reduced, first to dihydrofolate (DHF) and then to tetrahydrofolate (THF). The enzyme catalyzing these reactions is dihydrofolate reductase (DHFR), which uses NADPH as a cofactor. When tetrahydrofolate enters cells, 4 or 5 additional glutamate residues are added. Reduced polyglutamate derivatives of THF are the forms that participate as cofactors in biochemical reactions. Reduction of DHF by NADPH, catalyzed by dihydrofolate reductase, regenerates THF (Fig.1).

The main function of THF is the transfer of one-carbon groups obtained from serine, glycine, histidine, formaldehyde, and formate. While attached to THF, these one-carbon units are oxidized and reduced. The most oxidized form is 10–formyl-THF (Marks et al. 1996).

10 –formyl-THF releases a water molecule to produce the 5, 10-methenyl derivative, which is further reduced to produce 5, 10-methylene-THF and serves as a source of carbon for methylation of deoxyuridinemonophosphate (dUMP) to form deoxythymidine monophosphate (dTMP).

Another important reaction is the irreversible reduction of 5, 10-methylene-THF to 5-methyl-THF. This reaction is catalyzed by the enzyme 5, 10-methylenetetrahydrofolate reductase (MTHFR Enzyme Commission 1.5.1.20). The methyl group is then transferred to vitamin B_{12} and is used in homocysteine methylation.

I.3 Remethylation pathway

In the remethylation pathway, 5-methyl-THF is used to methylate homocysteine to methionine via a B_{12} -dependent reaction, catalyzed by the enzyme methionine synthase (MS). Betaine serves as an alternative methyl donor for the second methylation reaction converting homocysteine to methionine. This reaction is catalyzed by the enzyme betaine-homocysteine methyltransferase (BHMT) and is limited to the liver and kidney. (Rozen 2000) Methionine is then activated by ATP to form S-adenosylmethionine (SAM). THF is regenerated in this reaction. SAM serves as a universal methyl donor to a variety of acceptors.

S-adenosylhomocysteine (SAH), the by-product of these methylation reactions, is subsequently hydrolyzed, thus regenerating homocysteine, which then becomes available for another cycle of methyl-group transfer (Selhub 1999).

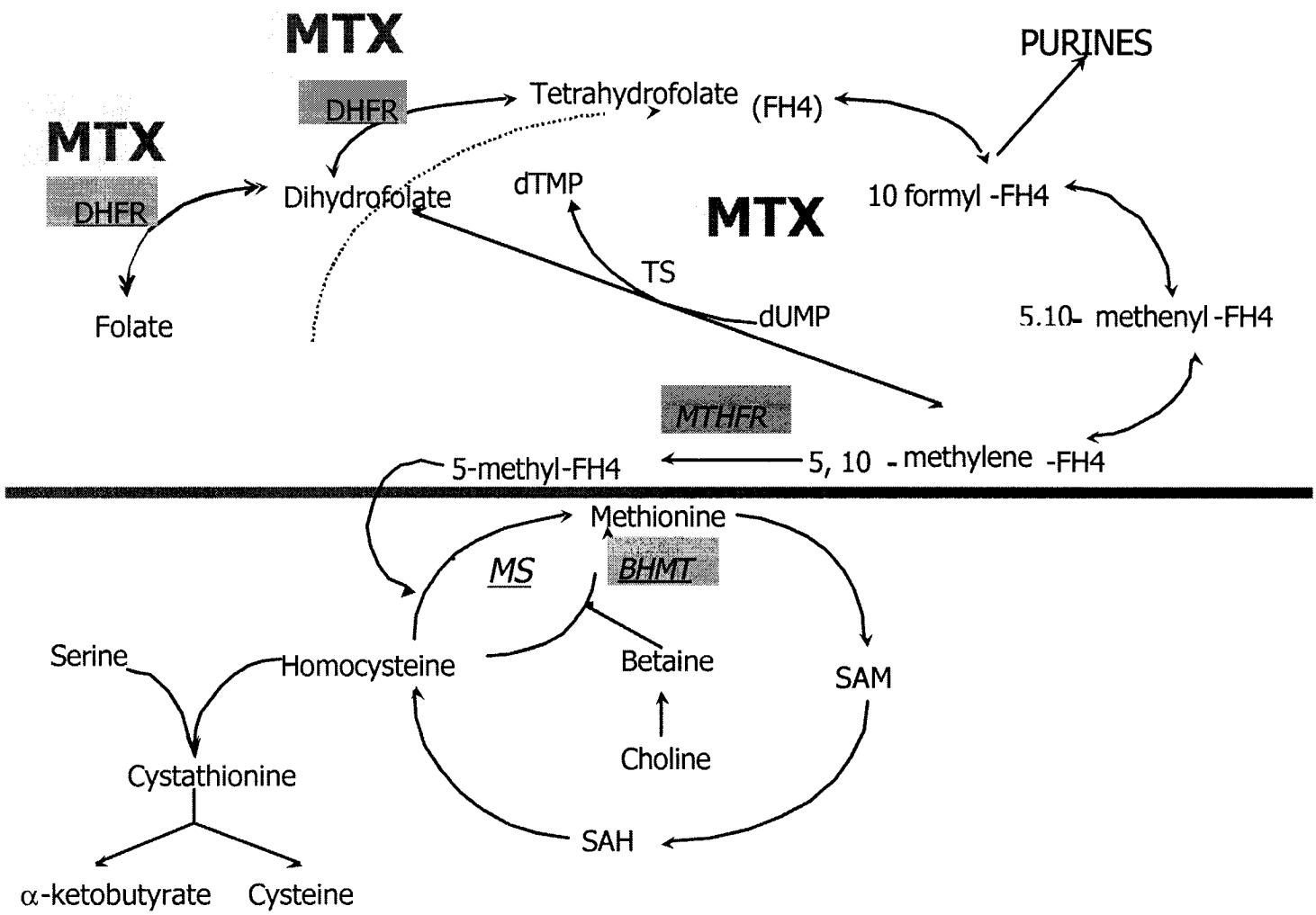
I.4 Transsulfuration pathway

In the transsulfuraton pathway, homocysteine condenses with serine to form cystathionine in an irreversible reaction catalyzed by the vitamin B_6-containing enzyme cystathionine-beta-synthase (CBS). Cystathionine is hydrolyzed by a second vitamin B_6-containing enzyme γ-cystathionase, to form cysteine and α-ketobutyrate. Excess cysteine is oxidized to taurine or inorganic sulfates or excreted in the urine. Thus, in addition to the synthesis of cysteine, this transsulfuration pathway effectively catabolizes excess homocysteine (Selhub 1999).

I.5 Nutritional regulation of homocysteine metabolism

When dietary methionine is high, the level of SAM will increase. This leads to inhibition of MTHFR, which results in suppressed 5-methyltetrahydrofolate synthesis, and activation of cystathionine β-synthase (CBS), thus increasing the rate of homocysteine catabolism. Conversely, when dietary methionine is low, remethylation will be favored over transsulfuration

Figure 1. Folate metabolism. Abbreviations: BHMT—betaine-homocysteine methyltransferase; TS-thymidylate synthase; MS-methionine synthase; DHFR- dihydrofolate reductase; MTHFR- methylenetetrahydrofolate reductase; SAM-S-adenosylmethionine; SAH-S-adenosylhomocysteine; FH4-tetrahydrofolate; MTX-methotrexate.


MTX
PURINES
DHFR
Tetrahydrofolate
(FH4)
MTX
DHFR
Dihydrofolate
dTMP
MTX
10 formyl -FH4
TS
Folate
dUMP
5.10- methenyl -FH4
MTHFR
5-methyl-FH4
5, 10 - methylene -FH4
Methionine
MS
BHMT
Serine
Homocysteine
Betaine
SAM
Choline
Cystathionine
SAH
α-ketobutyrate
Cysteine

I.6 MTHFR gene and MTHFR deficiency

Porcine complementary DNA isolation and sequencing by Goyette et al. (1993, 1994) allowed to define the structure of the human and mouse MTHFR genes (Goyette et al. 1998). It was found that the human gene is composed of 11 exons and maps to chromosome 1p36.3. Amino acid sequences of the human and mouse MTHFR enzyme were shown to be approximately 90% homologous (Goyette et al. 1998). Twenty-four rare mutations in the MTHFR gene have been identified thus far (Goyette et al. 1994, 1996; Sibany et al. 2000). In subjects with these rare mutations, specific activity of MTHFR enzyme is less than 20% of that in "normal" subjects (Goyette et al. 2000; Rosenblatt et al. 2004). The condition with very low enzyme activity, named severe MTHFR deficiency, was first described in 1972 by Freeman et al. Severe MTHFR deficiency is an autosomal recessive disorder and the most common inborn error of folate metabolism, although there have been only 68 patients reported worldwide (Tawari et al. 2002, Ogier de Baulny at al. 1998). These patients present with marked hyperhomocysteinemia and homocystinuria, severe defects of the nervous and vascular systems such as: mental retardation, developmental delay, gait abnormalities, incoordination, seizures, schizophrenia, stroke, and vascular thromboses (Schwahn et al. 2001).

The mild form of MTHFR deficiency, also with an autosomal recessive mode of inheritance, was described by Kang et al. in 1991. The mutated enzyme was found to be particularly sensitive to heat treatment.

Mild MTHFR deficiency results from a substitution at position 677(C→T) that converts an alanine to valine codon (A222V) (Frosst et al. 1995). The frequency of this polymorphism in the homozygous state is 10-15% among the North American population (Frosst et al. 1995). Specific activity of the enzyme in individuals with the T/T genotype is approximately 30% of that in individuals with the C/C genotype, while heterozygotes (the C/T genotype) have about 60% of normal enzyme activity (Ulrich et al. 2001). Individuals homozygous for the polymorphism develop mild heperhomocysteinemia when their dietary folate intake is low (Schwahn et al. 2001).

The 677 C→T polymorphism is associated with increased risk for premature cardiovascular disease and thromboembolism (Kang et al. 1988; Kluijtmans et al. 1996; Morita et al. 1997; Talmon et al. 1997; Eikelbom 1999; Ueland et al. 2000), neural tube defects (Mills et al. 1995; Christensen et al. 1999) pregnancy complications (Goddijn-Wessel et al. 1996; Ray et al. 1999, Sohda et al. 1997), Down syndrome (Hobbs et al. 2000), oral cleft (Mills et al. 1999) and some neuropsychiatric disorders (Regland et al. 1995, Bottigliery et al. 2000).

Interestingly, homozygotes for 677 C→T polymorphism were found to be at lower risk for development of several forms of cancer, in particular some forms of childhood and adult

leukemia (Skibola et al. 1999; Wiemels et al. 2001) and colon cancer (Chen et al. 1996, Ma et al. 1997)

The important role of MTHFR in providing reduced folate forms for nucleotide synthesis and methylation reaction explains a variety of pathological conditions associated with MTHFR deficiency. Reduced activity of MTHFR decreases the conversion rate of 5, 10-methylene-THF to 5-methyl-THF, which results in increased homocysteine, due to reduced methylation to methionine, and reduced conversion of methionine to SAM. SAM serves as a methyl donor for numerous methylation reactions, including methylation of DNA and proteins, phospholipid, myelin and neurotransmitter synthesis. Decreased SAM/SAH ratio leads to global DNA hypomethylation in several tissues (Chen et al. 2001). On the other hand, by increasing the pool of 5, 10-methylene-THF at the expense of the pool of 5-methyl-THF, MTHFR deficiency reduces misincorporation of uracil into DNA, which might otherwise result in strand breaks during the uracil excision repair process (Skibola et al. 1999).

I.7 Pathological consequences of hyperhomocysteinemia

Pathogenesis of Hcy toxicity has not been fully understood and is of great clinical interest. Understanding the mechanisms by which hyperhomocysteinemia exerts its toxic effects on cells

could lead to development of new methods of prevention and treatment of cardiovascular and other diseases.

Two main hypotheses that could explain molecular mechanisms of Hcy toxicity exist: the oxidative stress induction hypothesis and the "molecular targeting" hypothesis (Perna et al. 2003).

1.7.1 Oxidative stress

Hcy, like most of the other thiols, is capable of autooxidation in the presence of metal catalysts and molecular oxygen, with formation of reactive oxygen species (ROS), such as hydrogen peroxide and peroxynitrite (Starkebaum et al. 1986; Loscalzo et al. 1996).

ROS can impair endothelial cell function and stimulate lipid peroxidation, with initiation of an inflammatory response and formation of foam cells, which are atherosclerotic lesion components (Perna et al. 2003).

It has been found that high levels of Hcy are able to impair several antioxidative compensatory mechanisms in endothelial cells by suppressing nitric oxide synthase activity and reducing expression of glutathione peroxidase (Zhang et al. 2000). In addition, the concentration of extracellular superoxide dismutase, another important antioxidant in vascular tissue, was found to be correlated with Hcy levels (Wilcken et al. 2000).

Not all studies support this hypothesis. For instance, Zappacosta et al. (2001) found that Hcy

produces only negligible quantities of hydrogen peroxide (1/4000 mole of peroxide to 1 mole of Hcy). In normal individuals, the plasma Hcy concentration range from 5 to 12 μmol/L, in individuals with mild hyperhomocysteinemia – from 15 to 25 μmol/L, and in patients with homocystinuria – from 50 to 500 μmol/L (Majors et al. 2002; Kang et al. 1992). In all normal and pathological conditions, plasma the Hcy level is in micromolar range, and such concentrations are not high enough for Hcy to act as a pro-oxidant (Zappacosta et al. 2001).

1.7.2 Molecular targeting

According to this hypothesis, proposed by Jacobsen et al. (2001), molecular targeting of Hcy to specific plasma proteins may directly impair their function, thus promoting atherogenesis and the progression of cardiovascular disease. Protein homocysteinylation and global hypomethylation are considered to be the principal mechanisms of Hcy toxicity. The mechanism and consequences of hypomethylation were discussed above in the section I.6. It was suggested that protein homocysteinylation in hyperhomocysteinemia occurs through the following mechanisms:

1) Mediation of oxidation of thiol groups through formation of albumin-Cys 34 thiolate anion and Hcy-cysteine mixed disulfide (HCMD). Albumin-Cys 34- thiolate anion then attacks HCMD and Hcy to form albumin-Cys-S-S-Hcy. The binding of Hcy to cysteine residues may disrupt the normal protein structure and impair or alter its function
(Jacobsen et al. 2003).

2) Misincorporation of S-nitroso-Hcy in place of methionine during protein biosynthesis

(Jacobsen et al. 2003), and post-biosynthetic acylation of free amino groups (Jakubowski et al. 2000).

3) Formation of stable disulfide complexes with plasma fibronectin, which impairs its ability to interact with fibrin. Fibronectin–fibrin complexes are necessary for avoiding a delay in clot resolution. Such a delay could lead to further platelet accumulation and clot formation. (Majors et al. 2002).

Further studies are necessary for understanding the mechanisms of Hcy-induced toxicity.

1.8 Mthfr-knockout mice

Mthfr-deficient mice on a Balb/C background were recently generated by Chen et al. (2001). Homozygous knockout mice differ from their wild-type and heterozygous littermates in several important ways. They are significantly smaller, have abnormal faces, protrusive eyes, kyphosis, bent tail, gait and motor abnormalities, and severe tremors, and their fur is scarcer. Such mice usually die within the first weeks of life, with their 5-week survival rate being less that 25%.

Histopathological changes found in homozygotes include: laminar structure defects in the cerebellum, microvesicular steatosis in liver and lipid deposition in aortic sinus at 14 months of age (Chen et al. 2001).

Previous research in our lab has shown that administration of betaine to *Mthfr*-deficient mice can lead to beneficial effects (Schwahn et al. 2003). Dietary supplementation with 25 mmol/kg betaine reduced total plasma homocysteine level by 56% in wild type, 58% in heterozygous, and 50% in homozygous mice, and prevented severe fatty infiltration of the liver in the latter. Treatment of pregnant females with a 2% betaine solution, administered orally, was accompanied with a 70% survival rate, and diminished phenotypic abnormalities in the homozygous mutant pups.

Heterozygous mutant mice do not have any obvious phenotypic abnormalities. They grow normally and their body weight and life span are not different from those of their wild -type littermates.

Plasma total homocysteine levels in heterozygous and homozygous knockout mice are 1.6- and 10-fold higher, respectively, than those in wild type littermates. Both heterozygous and homozygous knockouts have either significantly decreased SAM levels or significantly increased SAH levels, or both, with global DNA hypomethylation. In mice, as in humans with mild MTHFR deficiency, the specific activity of the MTHFR enzyme in heterozygotes is decreased by approximately 40-50% (Chen et al. 2001).

Consequently, the *Mthfr*-heterozygous knockout and homozygous knockout mutant mice are a good model for the 677C→T polymorphism seen in most populations and severe MTHFR deficiency in homocystinuria, respectively. Please refer to the table below (Rozen, unpublished data).

MTHFR-deficient mice: a model for MTHFR deficiency in man

	mild MTHFR deficiency		severe MTHFR deficiency	
	Human	Mouse	Human	Mouse
Genetic disruption	677 T/T	*Mthfr +/-*	two path. mutations	*Mthfr -/-*
Residual MTHFR Activity	39 %	60 %	< 10 %	0 %
Plasma homocysteine	120-190 %	168 %	~ 1000%	~ 1000 %
5-Methyl-THF [% of folates]	71 %	58 %	low	4 %
DNA hypomethylation	161 %	208 %		224 %

CHAPTER II

THESIS PROPOSAL

II. THESIS PROPOSAL

Recently published pharmacogenetic studies suggest that individuals with the 677C→T polymorphism may respond differently to the treatment with several medications interfering with homocysteine, folate or vitamins B6 and B12 metabolic pathways. These medications include MTX, 5-fluorouracil, phenytoin, levodopa and fenofibrate (Yoo et al. 1999; Toffoli et al. 2000; Brattstrom et al. 2001), among others.

The first objective of this thesis project was to conduct a pharmacogenetic study of MTX, to learn about the influence of the MTHFR genotype on efficacy and toxicity of MTX, using *Mthfr*-heterozygous mice as a model of mild MTHFR deficiency in humans.

The second objective was to conduct a pharmacogenetic study of metafolin to assess the efficacy of this medication in the treatment of severe MTHFR deficiency, using *Mthfr*-homozygous knockout mice as a model.

II.1 Proposal for pharmacogenetic studies of MTX in a mouse model of mild MTHFR deficiency

This study was conducted with the purpose of evaluation of efficacy and toxicity of MTX in a

mouse model of MTHFR deficiency. The term efficacy of MTX therapy refers to the ability of the drug to achieve the goal of the therapy: induction of cell death in tissues characterized by a high proliferation rate. The term toxicity refers to the undesired ability of MTX therapy to cause cell death in tissues with a low proliferation rate.

We hypothesized that MTHFR deficiency aggravates choline deficiency and oxidative stress induced by MTX treatment. This should lead to increased levels of apoptosis in tissues and lipid accumulation in the liver. Thus, the toxicity of MTX should be higher in *Mthfr*-heterozygous (*Mthfr* +/-) mice than that in wild-type (*Mthfr* +/+) mice.

Confirmation of this hypothesis could imply that humans with mild hyperhomocysteinemia due to homozygosity for the 677C→T polymorphism may be more sensitive to MTX treatment and more likely to develop severe side effects than those without the polymorphism.

To evaluate the efficacy of MTX therapy in tissues with a high proliferation rate, we analyzed apoptosis in germinal centers of Peyer's patches of the small intestine, which are the sites of maturation and proliferation of lymphocytes.

To assess the toxicity of MTX therapy the following parameters were examined: 1) body weight and general appearance of the mice, which were assessed daily; 2) apoptosis rate in the liver and spleen, the tissues with low proliferation rate where induction of apoptosis by MTX therapy would be considered as a toxic effect; 3) bone marrow function, as indirectly evaluated by peripheral blood cell counts; 4) hepatotoxicity, as assessed by measuring the level of transaminases (ALT and AST) in serum, elevation of which indicates liver cell damage; 5) nephrotoxicity, as measured by serum creatinine.

To examine induction of choline deficiency by MTX therapy as a potential cause of fatty infiltration in the liver and apoptosis, choline compounds were measured in the liver of MTX treated animals and controls.

II.2 Proposal for pharmacogenetic studies of metafolin in a mouse model of severe and mild MTHFR deficiency

The objective of this study was to evaluate the ability of the synthetic form of 5-methyltetrahydrofolate (metafolin) to substitute for the natural 5-methyltetrahydrofolate - the product of the MTHFR reaction lacking in *Mthfr*-knockout mice. We hypothesized that metafolin would increase the survival of *Mthfr*-homozygous mutant mice, reduce the Hcy level, increase the SAM/SAH ratio and increase methylation capacity in homozygous and

heterozygous mutant mice.

We conducted two experiments with metafolin. The first one was designed to evaluate the effect of metafolin on survival of *Mthfr*-homozygous mutant mice and to test the ability of the drug to reduce plasma Hcy levels in both *Mthfr*-homozygous and heterozygous knockout mice. It was also of interest to compare the results of this experiment with those obtained with betaine treatment, which increased the survival of *Mthfr*-homozygous mutant mice and decreased Hcy levels in plasma (B.Schwahn, unpublished data).

In the second experiment, we compared the ability of metafolin and betaine (B.Schwahn, unpublished data) to reduce tissue Hcy levels, increase SAM/SAH ratio in tissues, and improve brain methylation capacity in *Mthfr*-homozygous and heterozygous mutant mice.

CHAPTER III

PHARMACOGENETIC STUDIES OF METHOTREXATE ON A MOUSE MODEL OF MILD MTHFR DEFICIENCY

III.1 INTRODUCTION

Methotrexate (MTX) is a folic acid analogue that is used in cancer therapy (leukemia, lymphoma, choriocarcinoma, squamous cell carcinomas of head and neck and osteosarcoma), for treatment of several autoimmune diseases (rheumatoid arthritis, psoriasis and systemic lupus erythematosus), and for prevention of graft-versus-host disease after transplantation (Jacob 1996).

III.1.1 Mechanism of action

MTX and its polyglutamates are competitive tight-binding inhibitors of DHFR. As a result of this inhibition, MTX depletes cells of reduced folates such as 5, 10-methylene-THF, 10-formyl-THF, the essential cofactors in the biosynthesis of thymidylate and purines, and also reduces the availability of 5-methyltetrahydrofolate for remethylation of Hcy to methionine (Guiseppe et al. 2001; Guttormsen et al. 1998; Quinin et al. 1996).

There are several other enzymes that are also inhibited by MTX polyglutamates: glycinamide ribonucleotide (GAR) transformylase, aminoimidazole carboxamide (AICAR), and thymidylate synthase. These are the enzymes involved in de novo synthesis of purines and pyrimidines (Guiseppe et al. 2001, Ranganathan et al. 2003).

Inhibition of these four enzymes leads to cell cycle arrest in the S-phase and apoptosis, in which DNA fragmentation is preceded by activation of caspases 9, 2 and 3 (Papaconstantinou 2001).

The main advantages of MTX therapy are its cost and experience in use, but there are also important limitations such as resistance to treatment and toxicity.

Several mechanisms responsible for drug resistance are known. These include: 1) impaired active transport, 2) decreased affinity of DHFR for MTX, 3) increased levels of DHFR resulting from DHFR gene amplification, 4) decreased polyglutamation, and 5) increased efflux due to overexpression of P-glycoprotein (Ranganathan et al. 2003; Giuseppe et al. 2001; Matherly et al.1996).

MTX toxicity often results in discontinuation of therapy (Alarson et al. 1989). Several known side effects are: gastrointestinal toxicity, myelosuppression, hepatitis with raised transaminases, renal dysfunction, hypersensitivity pneumonitis, nodulosis, central nervous system toxicity, rash, alopecia, and osteopathy (Ranganathan et al. 2003).

III.1.2 Methotrexate and MTHFR deficiency

Several recent studies found an association between the 677C→T polymorphism and increased MTX toxicity.

Rheumatoid arthritis patients homozygous for the 677 C→T polymorphism were found to be at risk for elevation of liver enzymes, particularly alanine aminotransferase (ALT), when they received MTX therapy (Aannelis et al. 2001).

Haagsma et al. (1999) studied the influence of MTX, sulfasalazine and combination of both treatments on the plasma Hcy level among rheumatoid arthritis patients. After one year of MTX administration and/or combined therapy, patients with the (CT) genotype had higher plasma Hcy levels when compared to baseline. Interestingly, patients with the (TT) genotype had higher Hcy level at baseline than patients without the polymorphism and the treatment did not further increase their Hcy level, probably due to the "ceiling effect". That study also found that patients suffering from gastrointestinal toxicity in both treatment groups had higher plasma Hcy levels than patients without the toxicity.

A retrospective study published by Urano et al. (2002) showed that efficacy and toxicity of MTX were correlated with presence of the 677C→T and 1298A→C polymorphisms. They reported that patients with the (TT) and (CT) genotypes experienced an increase in transaminases, stomatitis, nausea, vomiting, hair loss, fatigue and rash more often than (CC) patients. Interestingly, the 1298A→C polymorphism was associated with higher efficacy of MTX. Patients heterozygous or homozygous for this allele required lower doses and had lower C reactive protein levels and erythrocyte sedimentation rates than wild-type individuals (Urano et al. 2002).

Another study showed that patients with the (TT) genotype who had bone marrow transplantation and received MTX therapy for prevention of graft versus host disease had a higher incidence of oral mucositis and slower recovery of their platelet counts than patients with the (CC) genotype (Ulrich et al. 2001).

III.1.3 MTX, MTHFR deficiency and choline deficiency

A recently published study on *Mthfr*-knockout mice conducted in our lab by Schwahn et al. (2003) showed that there is "a strong interaction between Hcy and choline/betaine metabolism".

In that study, the levels of plasma betaine and dimethylglycine in homozygous knockout mice and of liver concentration of betaine, phosphocholine and glycerophosphocholine were observed to be lower in both heterozygous and homozygous *Mthfr*-knockout mice than in wild-type mice. The mechanism of induction of choline deficiency by MTHFR deficiency was shown to be through reduced availability of 5-methyltetrahydrofolate for the remethylation pathway resulting in low methionine and SAM levels. Low SAM levels in turn lead to induction of choline oxidase and BHMT activity and result in increased flow of choline and betaine through the remethylation pathway (Schwahn et al. 2003).

Several reports demonstrated a link between choline deficiency and apoptosis. Holmes-McNary et al. (2001) reported that choline deficiency induces apoptosis in fetal brain hippocampus and in several cell lines in vitro through up-regulation of the tumor suppressor protein p53 and concurrent up-regulation of the cyclin-dependent kinase-inhibitor $p21^{WAF/CIP1}$.

Yen CE et al. proposed that choline deficiency induces apoptosis through a decrease in membrane phosphatidylcholine (PC) concentration (Yen et al. 2001). PC is one of the choline derivatives needed for normal progression through the cell cycle (Jackowski 1994).

MTX treatment induces choline deficiency by increasing demand for betaine-mediated methylation of Hcy. Betaine deficiency leads to choline deficiency because choline is the dietary source of betaine (Schwahn et al. 2003). Decreases in the level of SAM and betaine in livers of MTX treated rats were previously reported (Barak et al. 1982).

Both choline deficiency and treatment with MTX have been shown to cause fatty infiltration of the liver (Pomfret et al. 1990). Choline deficiency is responsible for this side effect because choline is the precursor for the biosynthesis of PC, which is required for the synthesis of Very Low Density Lipoproteins (VLDL) from triacylglycerols and apolipoproteins in the liver. VLDL is a lipoprotein that exports triglycerides synthesized in the liver to other tissues. Reduced synthesis of VLDL results in accumulation of triglycerides in the liver.

III.2 MATERIALS AND METHODS

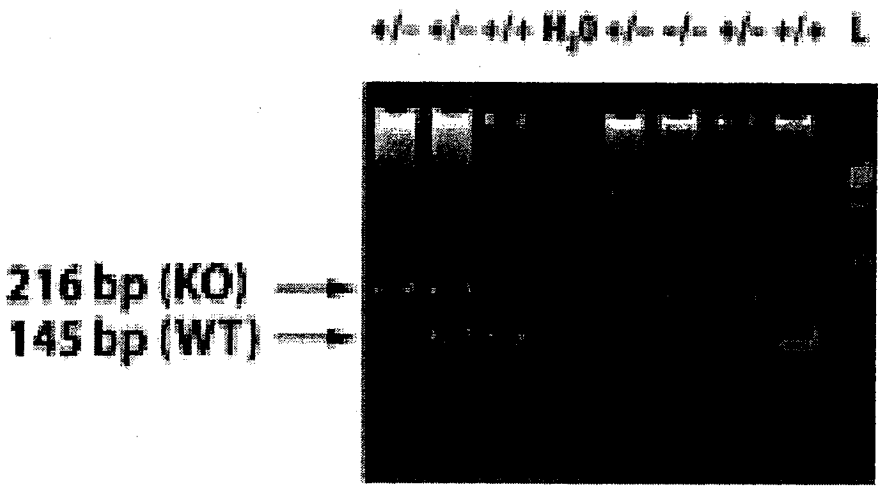
III.2.1 Mice

BALB/c mice were generated in a breeding facility at the Montreal Children's Hospital Research Institute and kept in shoebox cages with unlimited access to food and water. All the mice were fed standard rodent laboratory chow (the diet composition (lot number 5001) is shown in the appendix). Experiments were approved by the Animal Care Committee of the

III.1.2 Genotyping

Genotyping of MTHFR-deficient mice was performed by PCR with three primers in one reaction. The expected sizes of the bands were 216 bp for homozygous mutant mice, 145 bp for wild types and 216 bp with 145bp for heterozygous mice (Chen et al. 2001).

The picture of the gel below is of the polyacrylamide gel with the typical results of the PCR that is routinely used for allele specific genotyping of *Mthfr*-deficient and wildtype mice. Alleles are shown: +/+, wild-type, +/- heterozygous and -/- homozygous mutant (Chen et al. 2001).



III.1.3 Drug dose, regimen and experimental design

Two types of experiments were conducted, at MTX doses of 5 mg/kg and 10 mg/kg. The drug was obtained from Faulding (Canada). MTX was diluted in sterile isotonic saline immediately before the injections. Thirteen *Mthfr*-heterozygous (+/-) and 12 *Mthfr*-wild-type (+/+) mice at five weeks of age were injected with MTX at a dose of 5 mg/kg. Sixteen *Mthfr*-heterozygous (+/-) and 12 *Mthfr*-wild-type (+/+) mice of the same age were injected with MTX at a dose of 10 mg/kg.

As controls, five *Mthfr*-heterozygous (+/-) and eight *Mthfr*-wild-type (++) animals of the same age were injected with isotonic saline.

The regimen of MTX and saline administration was once every three days for two weeks intraperitoneally (four injections in total).

The doses of 5 and 10 mg/kg were in the range between LD1 and LD10 and were chosen as doses that would produce toxic effects but would not lead to death (Margolis et al. 1971).

The dose was adjusted according to body mass of the mice, which was measured every

morning before injection. Several symptoms of toxicity were evaluated, including general appearance and weight loss.

Twenty-four hours after the last injection, the mice were sacrificed in a CO_2 chamber. Blood, plasma or serum were collected in heparinized, potassium and EDTA pretreated tubes or tubes without pretreatment, respectively (Microvette Sarstedt). Blood for plasma collection was kept on ice, separated by centrifugation for 4 min at 4000g and frozen at -70 ^{0} C until the analysis. Blood for serum collection was left at room temperature for 30 minutes, centrifuged at 4000 g for 10 minutes and analyzed on the day of collection. Complete blood cell counts, ALT, AST and creatinine measurements were performed on an automatic blood cell counter and a clinical chemistry automatic analyzer by the Diagnostic and Research Support Service (Animal Care Facility at McGill University).

tHcy in plasma and choline compounds in the liver were measured with HPLC by collaborators (J.Cohn, Department of Hyperlipidemia and Atherosclerosis at the Institute of Clinical Research, Montreal; S.Zeisel, Department of Nutrition of the School of Public Health and School of Medicine, University of North Carolina at Chapel Hill).

III.2.4 Histomorphological and immunohistochemical analysis

Peyer's patches of the small intestine, spleen and liver were dissected, fixed in 10% formalin for 24 hours and kept in 70% ethanol until processing, which was performed according to standard histological protocols. Largest cross-sections of the Peyer's patches and longitudinal sections through the middle of the spleen and the right liver lobe were chosen for terminal deoxynucleotidyl transferase-mediated X-dUTP nick end labeling (TUNEL) staining for evaluation of apoptosis. (In Situ Cell Death Detection Kit, POD, Roche)

The sections were examined by light microscopy, and images of the light zone of the germinal centers of the Peyer's patches were made. At least five sections of the Peyer's patches and spleen per animal were examined and at least five germinal centers and four areas of the spleen per animal at magnification 400-X were photographed. The Northern Eclipse software program was used for quantification of labeled apoptotic cells. The area occupied by apoptotic cells was calculated by the program as a percentage of the total image area.

For liver, 23 and 28 sections obtained from wildtype and heterozygous 5mg/kg-MTX treated animals were TUNEL stained, and labeled hepatocytes and endothelial cells were counted manually. Counts were confirmed by a second researcher blinded to genotype status.

III.2.5 Statistical analysis

Statistical significance of the differences between mean values was examined with the two-sample t-test or ANOVA with the alpha level of 0.05. If the distribution of a variable was highly skewed, natural log transformation was applied.

III.3 RESULTS

No change in general appearance was observed in mice on MTX treatment. Mean body weight of mice increased during the experiment (t-test, $p< 0.01$) and was higher in males (t-test, $p<0.05$) but was similar across the genotypes and treatments (ANOVA, $p>0.05$) (Figures 1, 2, 3).

White blood cell counts (WBC) of the mice treated with 10 mg/kg MTX were lower than WBC of controls for both genotypes and genders, although the differences did not reach statistical significance (t-tests, $p>0.05$). This suggests that MTX treatment induced moderate leucopenia. No anemia or hypochromia (less dense staining of red blood cells due to lower hemoglobin concentration) was observed in the treatment or control groups (Table 1).

ALT, AST and creatinine levels were not elevated in the 10 mg/kg MTX treatment group compared to controls in either genotype group (t-tests, $p > 0.05$) (Table 1), and no difference was observed between the genotypes in either treatment group (t-tests, $p>0.05$).

High variation was observed in liver choline compound levels. No significant differences between the genotypes were detected among any of the choline compounds in the liver of 5 mg/kg MTX treated mice in either gender group (t-tests, $p > 0.05$) (Tables 2, 3). Among the controls, no statistically significant difference was observed in the levels of choline compounds between the genotypes across both genders except for phosphocholine level, which was significantly higher in heterozygous than in wildtype females (859 vs. 525 nmoles/g, t-test, $p<0.05$).

Gender differences were found for some of the choline compounds. In the MTX treatment group, heterozygous males had significantly higher choline levels than heterozygous females (893 vs.424 nmoles/g, t-test, $p < 0.05$), and wildtype males had significantly higher liver betaine and phosphocholine concentration than females of the same genotype (2636 vs. 1445 nmoles/g, T-test, $p<0.05$, and 625 vs. 307 nmoles/g, T-test, $p<0.05$, respectively).

Mean levels of tHcy were 10.5 μmol/L and 14.1μmol/L among heterozygotes, and 5.94

μmol/L and 6.1 μmol/L among wildtypes in the 5 mg/kg MTX and saline groups, respectively. tHcy levels were found to be statistically significantly associated with genotype (t-test, $p < 0.01$), in both treatment groups but were not associated with treatment (t-test, $p > 0.05$) (Fig.4).

Examination of TUNEL stained sections of the Peyer's patches and spleens by light microscopy revealed a difference in the number of lymphocytes undergoing apoptosis among *Mthfr*-heterozygous and wildtype animals in the MTX group (Fig. 5 and 6). Quantification of the apoptotic index (AI) as a percentage of the area occupied by apoptotic cells relative to the total area of the image confirmed this finding. The natural log-transformed AI was statistically significantly higher in heterozygous mice than in wild-type animals in both tissues (T-test, $p < 0.01$). In the control group, the AI was similar between the genotypes and was in a range of that of the wildtype MTX treated animals. Generally, fewer cells with apoptotic morphology were found in the spleens than in the Peyer's patches. (Fig. 5)

Single labeled hepatocytes and multiple labeled endothelial cells were found in the TUNEL stained liver sections (Fig.7). Quantification of apoptotic cells in the liver by two researchers independently showed that the number of hepatocytes undergoing apoptosis in the sections obtained from heterozygous animals was significantly higher than that in the sections obtained from wildtypes (t-test, $p < 0.05$) (Fig. 8). The same trend was observed for the number of

labeled endothelial cells but the difference failed to reach statistical significance (Fig.9).

III.4 DISCUSSION

MTX treatment at the dose 5 mg/kg was found to cause toxicity only in *Mthfr*-deficient animals, as revealed by the analysis of apoptosis rate in the Peyer's patches, the spleen, and the liver.

The number of apoptotic cells observed in proliferating zones of germinal centers of the Peyer's patches was higher than that in the liver and spleen, which are the tissues with a low proliferation rate. This finding is consistent with the fact that tissues with a high proliferation rate are the most susceptible to MTX therapy.

MTX treatment induced apoptosis in multiple endothelial cells in most hepatic sinusoids and single hepatocytes in *Mthfr*-heterozygous animals while the number of apoptotic cells in wildtypes was significantly lower. Hepatocytes are the cells performing most of the liver's functions. They form one-to-two cell plates that are separated from each other by large capillary spaces called sinusoids, lined by endothelial cells (Fox et al.1984, Jiman et al. 2000). The latter represent an important blood clearance system that removes macromolecular waste products from circulation (Nedredal et al. 2003).

Hcy toxicity with respect to endothelial cells is considered to be one of the mechanisms of atherosclerosis induction by hyperhomocysteinemia (Perna et al. 2003). Endothelial cells are more sensitive to high Hcy levels than other types of cells because they possess only one of the two pathways of Hcy metabolism. These cells are capable of remethylation of Hcy to methionine but the transsulfuration pathway does not function in those cells due to the absence of relevant enzymes (Jacobsen et al. 1995). *Mthfr*-deficiency could make endothelial cells more susceptible to MTX therapy because MTX treatment reduces the ability of these cells to detoxify Hcy by blocking remethylation – the only existing pathway of Hcy metabolism in those cells.

It is possible that the reason for not observing an elevation in the level of transaminases in plasma in MTX treated animals was because a two-week treatment with a mild dose may not have been sufficient to cause serious hepatocyte damage. In clinical studies, it had been observed that ALT and AST levels in humans increase after long-term and/or high-dose treatment with MTX (Kuijepers et al. 2000).

Plasma tHcy levels were strongly dependent on genotype, while no elevation of tHcy due to MTX treatment was detected. According to the experimental design, animals were sacrificed

24 hours after the last injection. By that time the administered medication could be expected to be almost completely eliminated by renal and biliary excretion. There is a possibility that there was a short increase of tHcy during the MTX peak plasma concentration period, but as MTX was being eliminated from the body, tHcy returned to initial levels. However, it is also possible that the dose and regimen were too mild to cause a detectable increase in Hcy level.

We did not observe the expected depletion of choline compounds in the liver; therefore, the hypothesis of increased cell death through choline deficiency induced by MTX therapy was not confirmed by our findings.

Previous studies in our lab showed that compared to wildtypes, *Mthfr*–deficient animals who were fed a choline deficient diet (TD00310) had significantly lower levels of betaine, choline, phosphocholine and glycerophosphocholine in the liver (Schwahn et al. 2003). According to that study, females had significantly higher levels of betaine, phosphocholine and glycerophosphocholine than males in all genotype groups.

The results of our study were not consistent with the results of the study by Schwahn et al. (2003). We did not observe genotype differences in any choline compound levels in MTX treatment group in either gender. In the saline group, a significant difference was found only in

phosphocholine concentration, which was higher in heterozygous females than in wildtype ones. We also found sexual dimorphism in the direction opposite from that found in the Schwahn study. In the MTX treatment group, heterozygous males had significantly higher choline levels, and wildtype males had significantly higher liver betaine and phosphocholine concentration than females of the corresponding genotype.

There could be several reasons for the described inconsistency of the results of the two studies. First, we observed a high variation in the levels of most of the choline compounds. Second, it is possible that MTX-treated animals were able to compensate for a choline deficiency from the mouse chow (AIN-93M), which is a diet sufficient in choline. Third, the treatment may not have been long enough to observe changes on choline compounds.

The hypothesis of cell death induction through choline deficiency induced by MTX therapy was not confirmed by our findings. However it would be premature to interpret our results as disproving the hypothesis before they are replicated in further studies or before higher doses or longer treatments are examined.

Another mechanism explaining a higher number of apoptotic cells in *Mthfr*-heterozygous than in wildtype mice might be through oxidative stress induced by hyperhomocysteinemia via

overproduction of reactive oxygen species and impairment of antioxidant enzymes such as glutathione peroxidase and superoxide dismutase (Chern et al. 2002; Massy et al. 2001). Oxidants are able to induce apoptosis through oxidative regulation of proteins (such as caspases, NF-κB and AP1) that directly modulate apoptosis (Gluckman et al.1996). Cell culture experiments carried out in our lab in several murine cell lines showed that cotransfection of NF-κB with the MTHFR downstream promoter led to two-fold activation of MTHFR gene expression. (Laura Pickell, personal communication). Upregulation of MTHFR should serve to lower homocysteine and correct hyperhomocysteinemia-induced stress. In MTHFR deficiency this correction cannot take place. The hypothesis of apoptosis induction through oxidative stress needs to be confirmed in future studies.

Figure 2. Body mass [g] of MTX- treated mice before injections 1 to 4 at the dose of 5 mg/kg. The bars represent mean +/- standard error. N= number of mice.

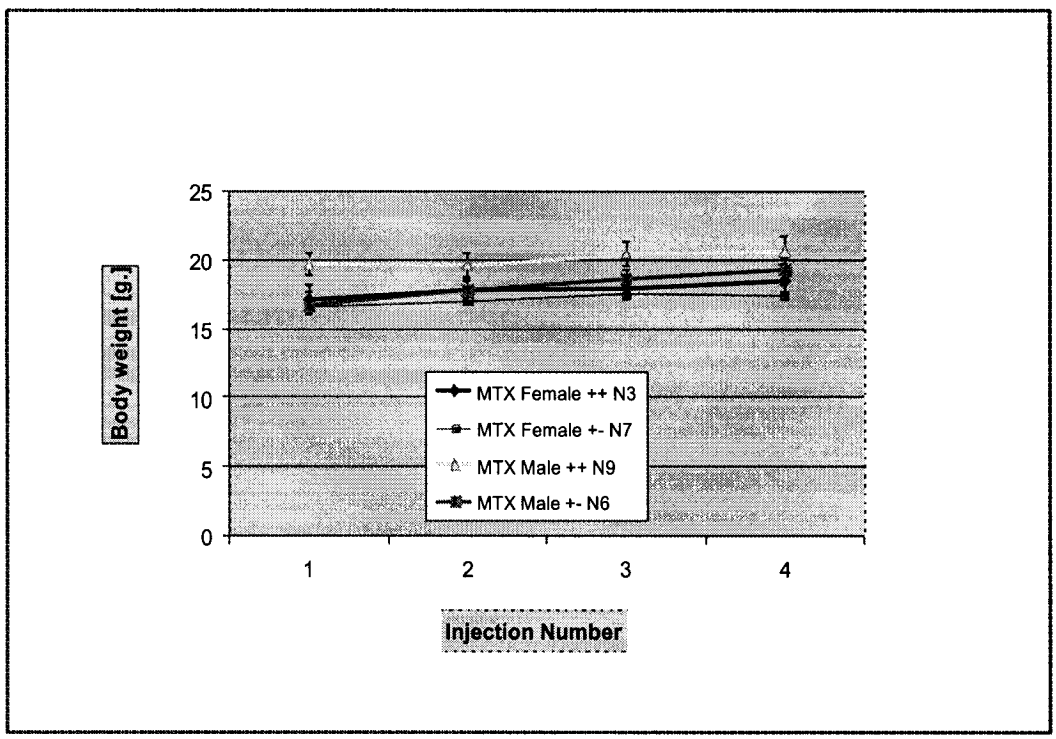

Body weight [g.]
25
20
15
10
5
0
1
2
3
4
Injection Number
MTX Female ++ N3
MTX Female +- N7
MTX Male ++ N9
MTX Male +- N6

Figure 3. Body mass [g] of MTX- treated mice before injections 1 to 4 at the dose of 10 mg/kg. The bars represent mean +/- standard error. N= number of mice.

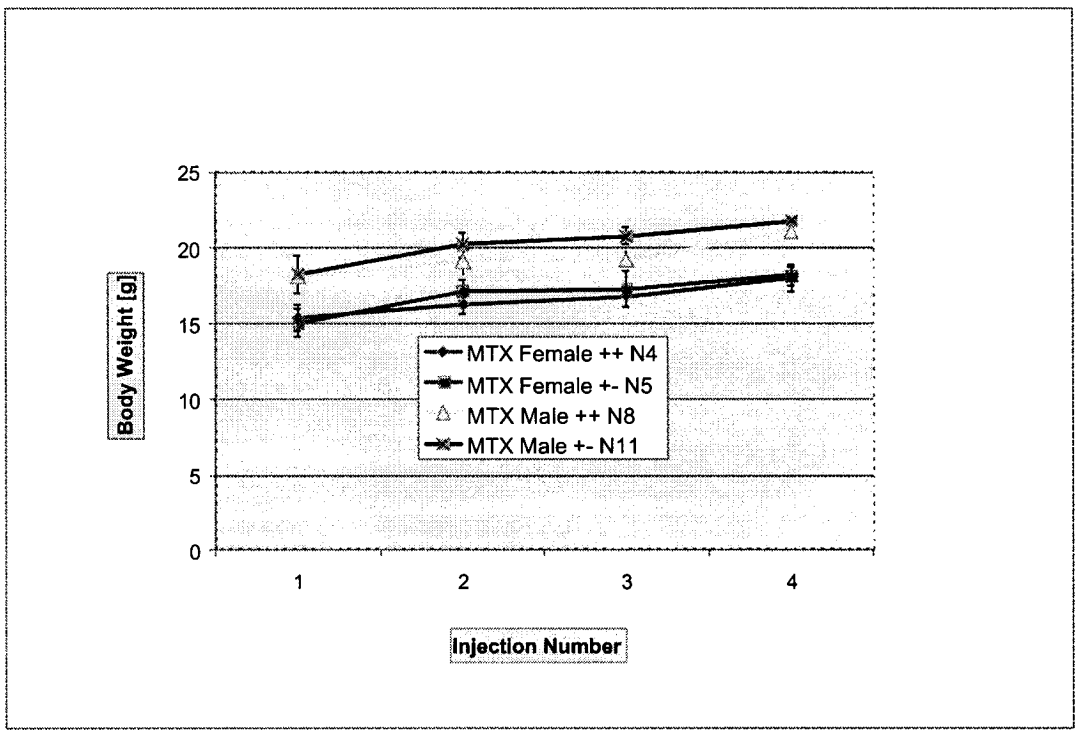

25
20
15
10
5
0
Body Weight [g]
1
2
3
4
Injection Number
MTX Female ++ N4
MTX Female +- N5
MTX Male ++ N8
MTX Male +- N11

Figure 4. Body mass [g] of saline treated mice before injections 1 to 4. The bars represent mean +/- standard error. N= number of mice.

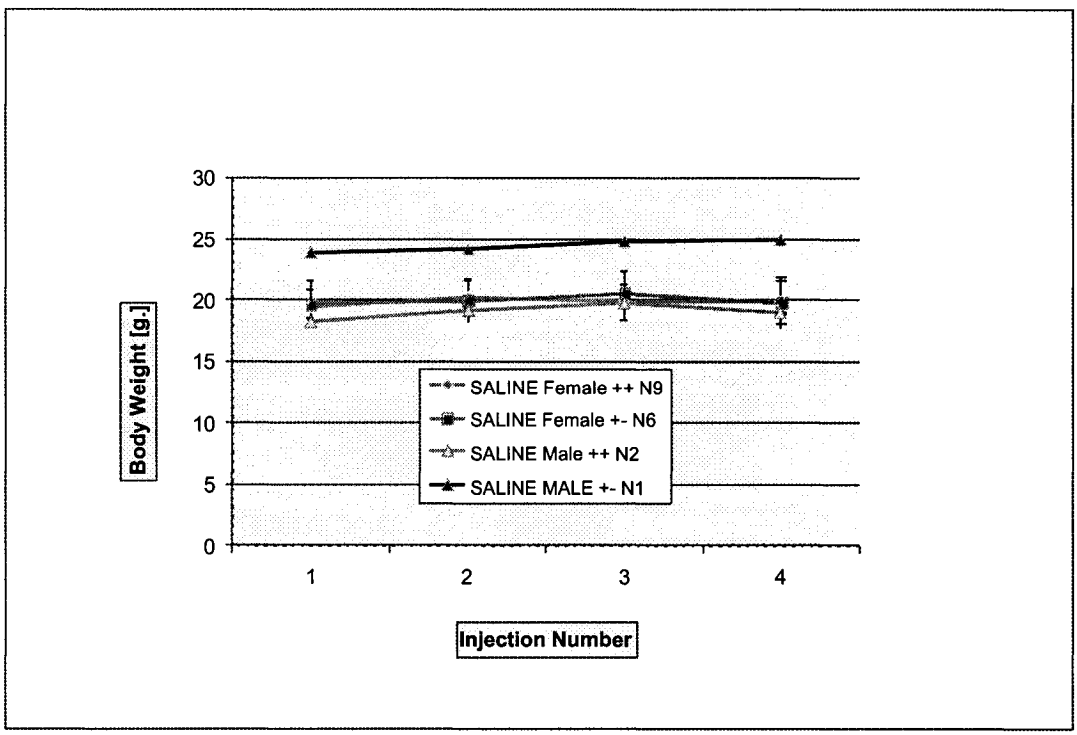

30
25
20
15
10
5
0
Body Weight [g.]
1
2
3
4
Injection Number
SALINE Female ++ N9
SALINE Female +- N6
SALINE Male ++ N2
SALINE MALE +- N1

Table 1. Blood cell counts and biochemical values of MTX-treated mice at the dose of 10 mg/kg and saline treated mice. Abbreviations: HCT –hematocrit, HB-hemoglobin, RBC-red blood cells, WBC-white blood cells, CRT- creatinine, ALT- alanine aminotransferase, AST-aspartate aminotransferase. The first number under N refers to the number of animals in MTX group and the second number under N refers to the number of animals in saline group.

Parameter	Gender	Genotype	N	MTX 10mg/kg		Saline	
				Mean	Std. Dev	Mean	Std. dev.
HCTL/L	Combined	++	7/5	0.42	0.02	0.42	0.04
HCTL/L	Combined	+-	7/1	0.43	0.02	0.47	-
HB (g/L)	Combined	++	7/5	141.00	6.50	149.80	11.90
HB (g/L)	Combined	+-	7/1	147.00	5.40	184.00	-
RBCX10^{12}/L	Combined	++	7/5	8.70	0.40	8.50	0.80
RBCX10^{12}/L	Combined	+-	7/1	8.90	0.40	9.50	-
WBCX 10^9/L	Combined	++	7/5	5.00	1.50	6.70	2.60
WBCX 10^9/L	Combined	+-	7/1	5.60	1.60	6.00	-
CRT (mmol/L)	Combined	++	7/5	32.00	2.70	34.70	2.90
CRT (mmol/L)	Combined	+-	7/1	33.00	2.40	33.00	-
ALT(U/L)	Female	++	2/2	28.00	1.40	251.00	278.00
ALT(U/L)	Female	+-	1/0	34.00	-	-	-
ALT(U/L)	Male	++	5/3	44.00	16.60	117.00	95.50
ALT(U/L)	Male	+-	6/1	39.00	6.80	47.00	-
AST(U/L)	Female	++	2/2	58.00	1.40	500.50	317.50
AST(U/L)	Female	+-	1/0	67.00	-	-	-
AST(U/L)	Male	++	5/3	53.00	10.50	121.00	0
ASTU/L)	Male	+-	6/1	62.00	3.70	163.00	-

Tables 2/3. Choline compounds (nmoles/g) in the liver of 5mg/kg MTX-treated and saline-treted mice: betaine, choline, glycerophosphocholine, phosphocholine (p.49) phosphatidylcholine, sphingomyelin (p.50).

Treatment	MTX 5mg/kg				Saline		
Compound	Betaine						
Genotype	++	+-	++	+-	++	+-	++
# Obs.	3	4	6	4	4	2	2
Mean	592	712	1880	1511	847	854	554
Std Dev	343	462	721	729	305	332	11
Min	196	187	984	933	542	620	547
Max	806	1313	3075	2559	1208	1089	562
Treatment	MTX 5mg/kg				Saline		
Compound	Choline						
Genotype	++	+-	++	+-	++	+-	++
# Obs.	3	4	6	4	4	2	2
Mean	510	424	574	893	457	501	617
Std Dev	363	210	135	172	111	107	0
Min	198	181	389	723	322	426	617
Max	908	655	724	1065	576	576	617
Treatment	MTX 5mg/kg				Saline		
Compound	Glycerophosphocholine						
Genotype	++	+-	++	+-	++	+-	++
# Obs.	3	4	6	4	4	2	2
Mean	463	325	271	276	299	276	295
Std Dev	284	178	111	225	51	52	76
Min	206	150	139	148	260	240	242
Max	768	573	460	684	373	313	349

Treatment	MTX 5 mg/kg				Saline		
Compound	Phosphocholine						
Genotype	++	+-	++	+-	++	+-	++
# Obs.	3	4	6	4	4	2	2
Mean	307	420	625	602	525	859	398
Std Dev	157	277	89	255	107	122	10
Min	130	114	526	403	408	773	391
Max	430	699	730	960	648	945	405

Treatment	MTX 5mg/kg				Saline		
Compound	Phosphatidylcholine						
Genotype	++	+-	++	+-	++	+-	++
# Obs.	3	4	6	4	4	2	2
Mean	19236	20406	21744	21700	21546	20642	20894
Std Dev	1421	1792	1627	789	899	1323	1010
Min	17760	18406	19722	20791	20272	19707	20180
Max	20595	22267	23449	22717	22460	19707	21578
Treatment	MTX 5mg/kg				Saline		
Compound	Sphingomyelin						
Genotype	++	+-	++	+-	++	+-	++
# Obs.	3	4	6	4	4	2	2
Mean	2252	2574	2092	2208	2173	2773	2424
Std Dev	452	500	260	391	412	203	14
Min	1731	2006	1793	1629	1860	2630	2414
Max	2543	3120	2506	2475	2746	2917	2434

Figure 5. Total plasma Hcy levels [µmol/L] based on treatment and genotype. The bars represent mean +/- standard error.

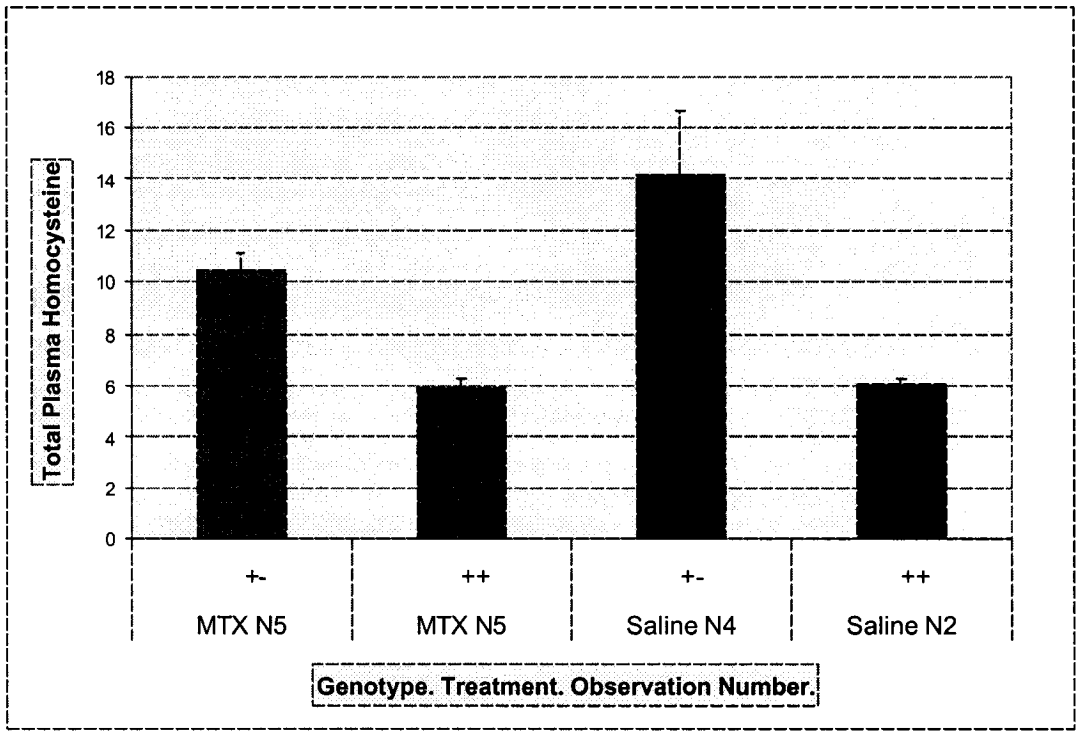
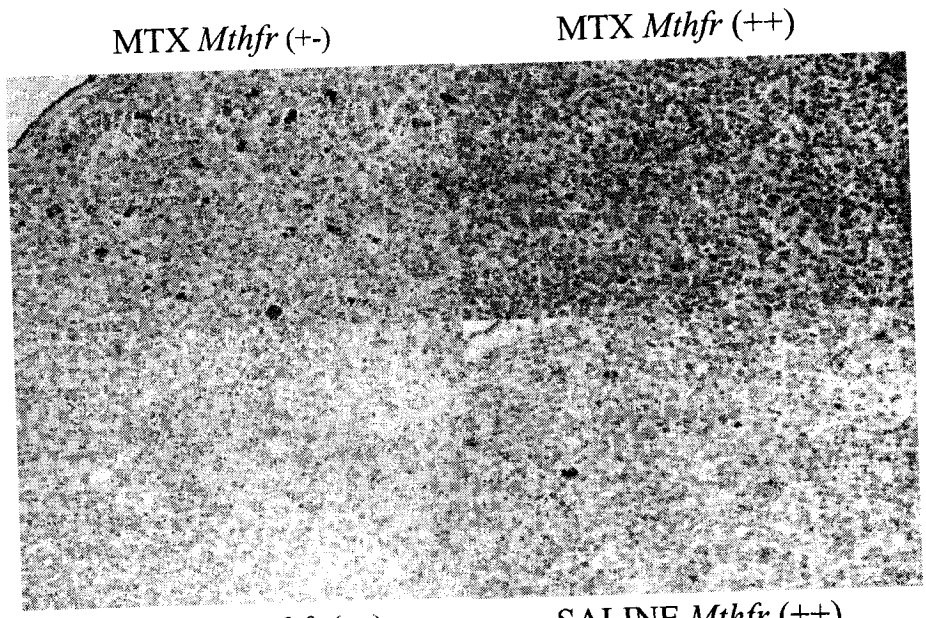
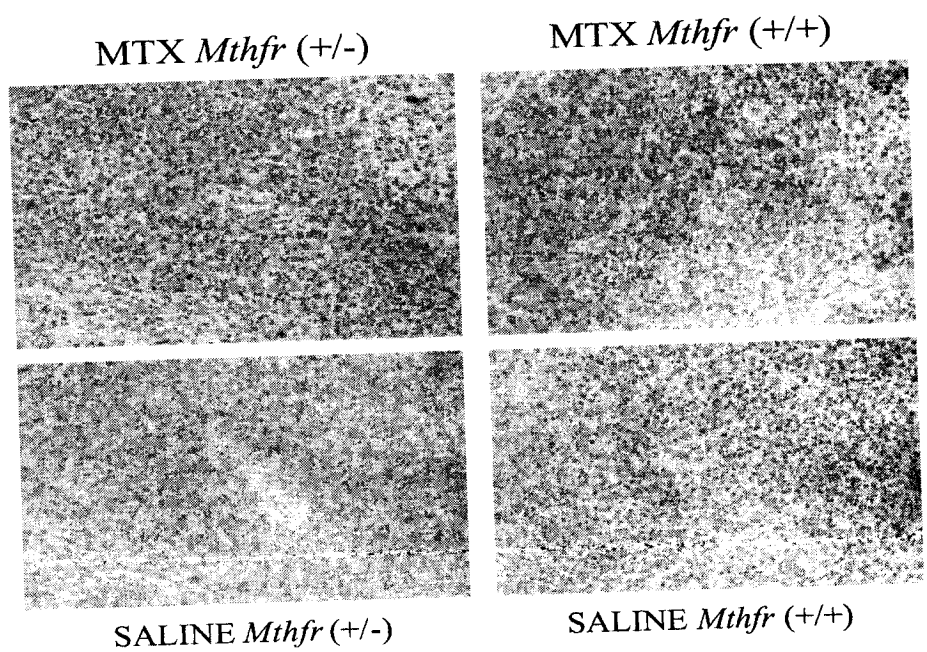

Total Plasma Homocysteine
18
16
14
12
10
8
6
4
2
0
+-
++
+-
++
MTX N5
MTX N5
Saline N4
Saline N2
Genotype. Treatment. Observation Number.

Figure 6. Photograph of the TUNEL-stained sections of germinal centers of Peyer's patches in the small intestine (A) and spleens (B). Magnification 4000X. Apoptotic cells are stained brown.


MTX *Mthfr* (+-)
MTX *Mthfr* (++)
SALINE *Mthfr* (+-)
SALINE *Mthfr* (++)

A


MTX *Mthfr* (+/-)
MTX *Mthfr* (+/+)
SALINE *Mthfr* (+/-)
SALINE *Mthfr* (+/+)

B

Figure 7. Quantification of results of TUNEL staining of Peyer's patches in the small intestine and spleen. Apoptotic Index in Tissues of MTX Treated Mice and Controls - 5mg/kg Experiment. The bars represent mean +/- standard error.

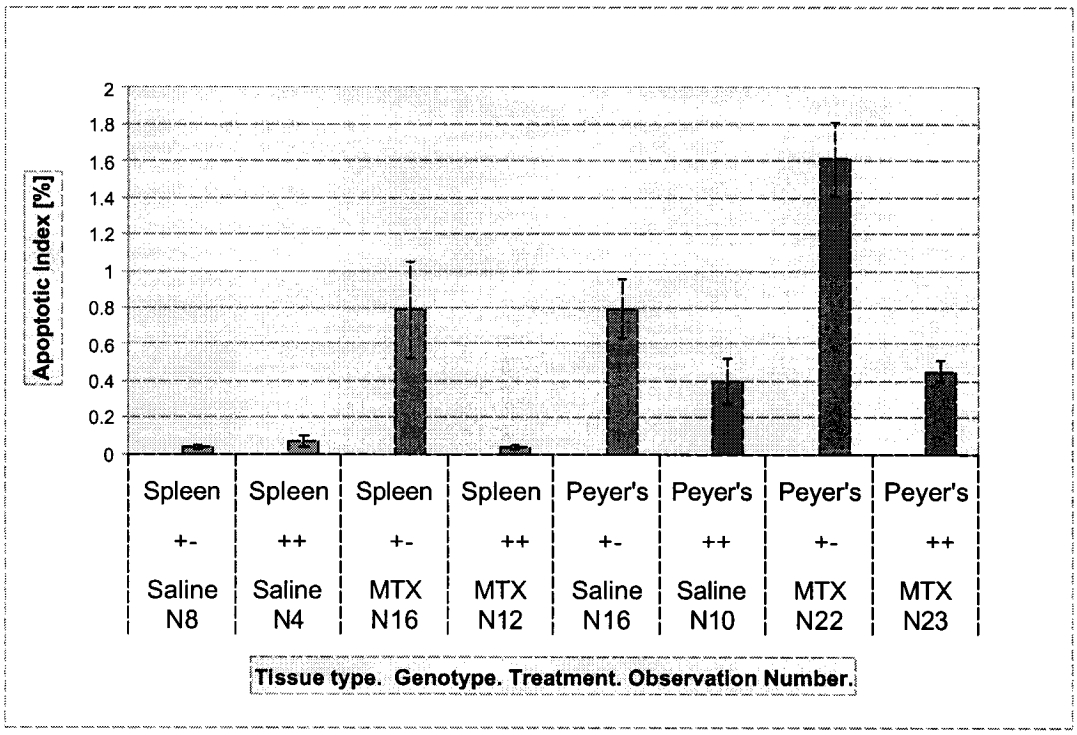

Apoptotic Index [%]
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Spleen +- Saline N8
Spleen ++ Saline N4
Spleen +- MTX N16
Spleen ++ MTX N12
Peyer's +- Saline N16
Peyer's ++ Saline N10
Peyer's +- MTX N22
Peyer's ++ MTX N23
Tissue type. Genotype. Treatment. Observation Number.

Figure 8. Apoptotic hepatocytes and small endothelial cells in the TUNEL-stained liver section of MTX treated animal. Apoptotic cells are stained dark brown.

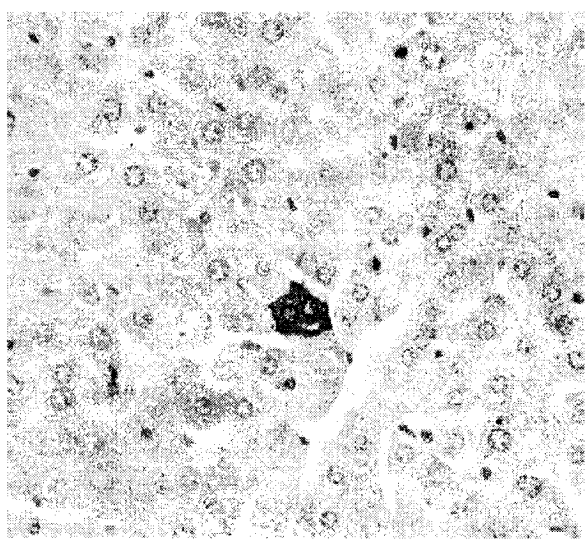


Figure 9. Graphic representation of the number of apoptotic hepatocytes per section in the TUNEL stained livers of 5mg/kg MTX treated animals and saline treated controls. The bars represent mean +/- standard error.

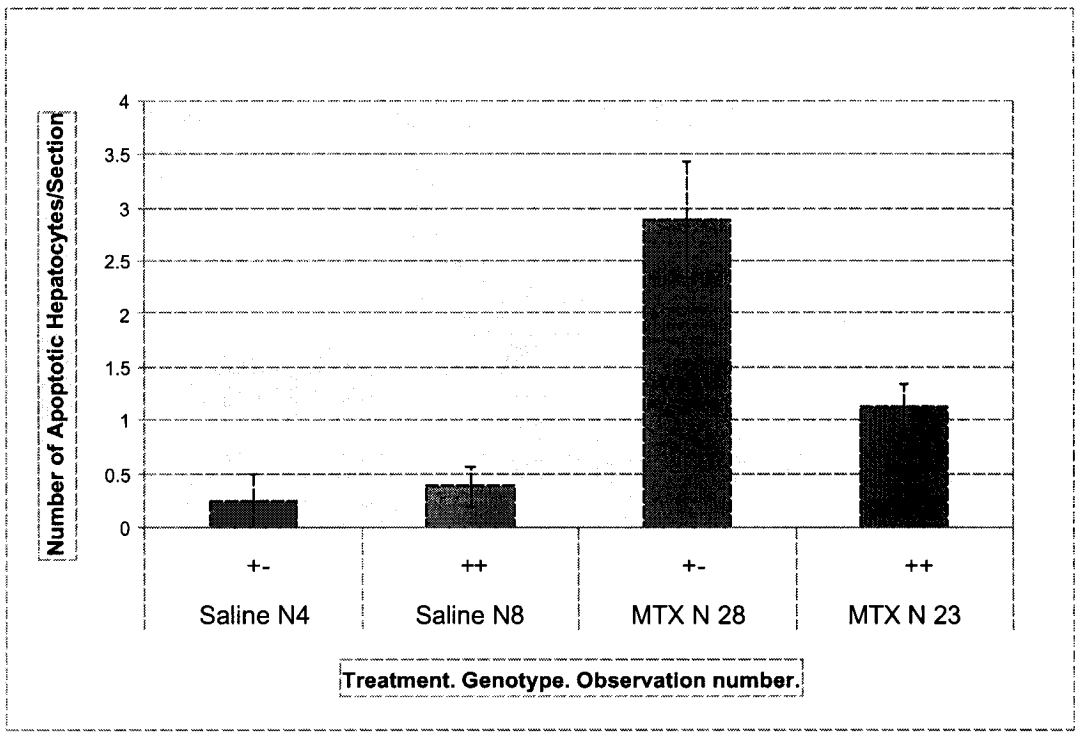
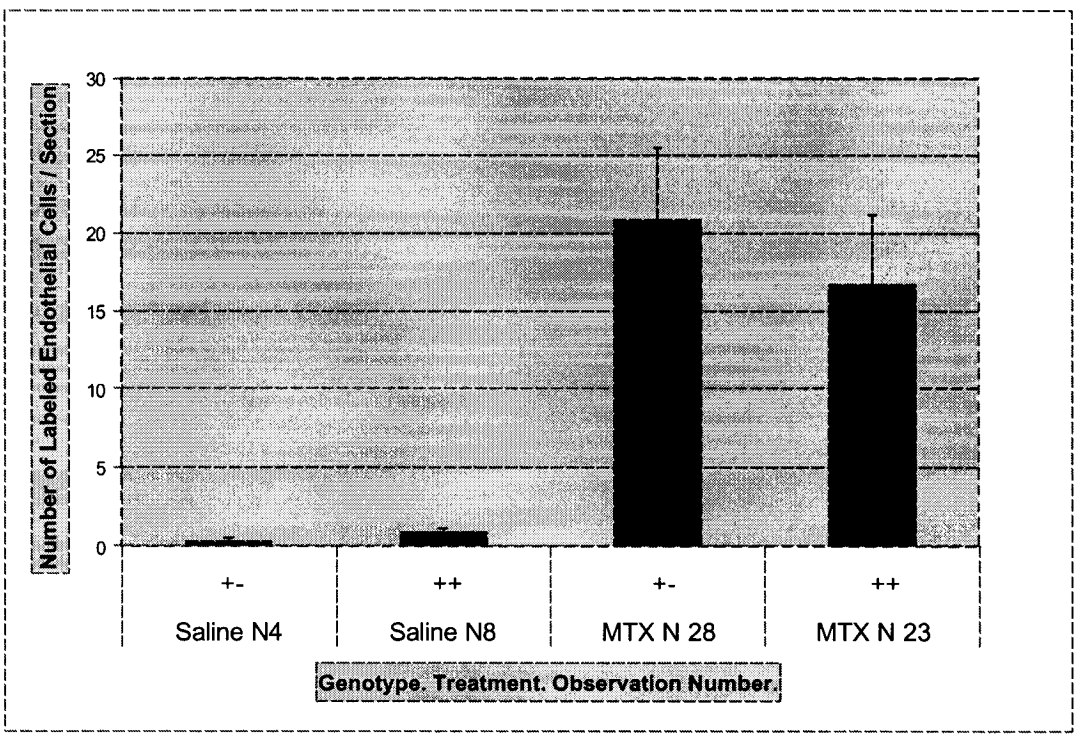

Number of Apoptotic Hepatocytes/Section
4
3.5
3
2.5
2
1.5
1
0.5
0
+-
++
+-
++
Saline N4
Saline N8
MTX N 28
MTX N 23
Treatment. Genotype. Observation number.

Figure 10. Graphical representation of the number of apoptotic endothelial cells per section in the TUNEL stained livers of 5mg/kg MTX treated animals and saline treated controls. The bars represent mean +/- standard error.


Number of Labeled Endothelial Cells / Section
30
25
20
15
10
5
0
+-
++
+-
++
Saline N4
Saline N8
MTX N 28
MTX N 23
Genotype. Treatment. Observation Number.

CHAPTER IV

PHARMACOGENETIC STUDIES OF 5-METHYLTETRAHYDROFOLATE (METAFOLIN) IN A MOUSE MODEL OF SEVERE AND MILD MTHFR DEFICIENCY

IV.1 INTRODUCTION

5-methyl-THF is a product of the MTHFR reaction, the predominant form of folate in serum and in tissues of humans, and the only form of folate that can cross the blood-brain barrier (Rosenblatt et al. 2004; Spector et al. 1995).

A synthetic 6S-5-methyltetrahydrofolic acid monoglutamate (metafolin) has recently become commercially available (Eprova, Switzerland). This medication has some benefits over natural folates, which can be obtained from food. Folate polyglutamates found in food require the activity of the intestinal conjugase enzyme to be absorbed and released into the circulation (Rosenblatt et al. 2004), while metafolin, being a monoglutamate, does not need to be hydrolyzed by the enzyme and is more easily absorbed (Venn et al.2002).

A suggestion has been made that metafolin supplements might be more effective than those of folic acid (Fohr et al. 2002; Venn et al. 2002). To become biologically active, folic acid has to be converted to the 5-methyltetrahydrofolate form (Fohr et al. 2002). Moreover, folic acid supplementation might mask hematological signs of vitamin B-12 deficiency because it is readily converted to THF, while metafolin requires the vitamin B-12-dependent enzyme methionine synthase for this conversion and is less likely to mask the deficiency (Venn et al. 2002).

However, clinical studies thus far have not shown higher efficacy of metafolin versus FA in reducing homocysteine levels or increasing plasma and red blood cell folate levels (Fohr et al. 2002; Venn et al. 2002). Absorption characteristics of metafolin were found to be similar to those of folic acid (Prinz-Langenohl et al. 2001).

Fohr et al. (2002) treated young healthy women with equimolar amounts of folic acid (400 μg a day) and 5-methyl-THF (480 μg. a day). After a month of supplementation, women who were homozygous for the 677C→T polymorphism had a 20% reduction of tHcy levels in the folic acid group and a 15% reduction in the 5-methyl-THF group. In heterozygous and wild type women, folic acid was shown to be more effective than 5-methyl-THF in reducing the homocysteine level.

Venn et al. (2002) showed that supplementation of women of childbearing age with (6S)-5-methyltetrahydrofolate (113 mg/day) and folic acid (100 mg/day) increased blood folate indices to the same extent.

IV.2 METHODS AND MATERIALS

IV.2.1 Mice

BALB/c mice were generated in a breeding facility at the Montreal Children's Hospital Research Institute and kept in shoebox cages with unlimited access to food and water bottles which contained dissolved medication in the treatment group and tap water in the control group. All the mice were fed standard laboratory mouse chow. Experiments were approved by the Animal Care Committee of the Montreal Children's Hospital and complied with the guidelines of the Canadian Council for Animal Care.

IV.2.2 Genotyping (described on page 28)

IV.2.3 Drug dose, regimen and experimental design

Twenty-four and seven pairs of heterozygous *Mthfr*-deficient female and male mice were bred to obtain homozygous mutant pups in the first and second experiments, respectively. The metafolin treatment was started at the moment each pair of mice was placed into the same cage. Metafolin was used in 1% concentration dissolved in distilled and deionized water, prepared fresh every day and given to mice as drinking water. The same number of heterozygous males and females was used as controls; they were given tap water.

The dose was chosen according to recommendations of the manufacturer as the highest dose tolerated by mice when given orally. The stability of the medication in distilled and deionized water was good during 24 hours and was specifically tested by the company (R.Moser, Eprova, personal communication).

It was calculated that if a mouse weighs 30 g and drinks 1 ml of 1% (0.02 M) solution of metafolin per day, it would receive approximately 10 mg of the medication per day (300 mg/kg).

In the first experiment, weights of the homozygous mutant pups and their littermates were measured at birth, every day during the first week of life, and once per week during weeks 2-5. The treatment continued throughout pregnancy and lactation until the age of five weeks. Heterozygous mutant and wildtype pups were weaned at the age of three weeks and the treatment with metafolin continued as before. Homozygous mutant pups were not weaned and were left in the same cage with their mothers for two weeks longer than heterozygotes and wildtypes. When pups reached the age of five weeks, they and their mothers were sacrificed in a CO_2 chamber after overnight fasting. Blood was kept on ice after collection, and plasma was separated by centrifugation for four minutes at 4,000g and frozen at -70 ^{0} C until analysis. The

livers were fixed in 10% buffered formalin. Brains of six homozygous mutant knockout pups in metafolin and control groups each were weighed, dissected and fixed in 10% formalin.

In the second experiment, the mothers were treated throughout the pregnancy and until the pups reached the age of six days. At this age homozygous mutant pups and their littermates were euthanized by decapitation. The brains were dissected, weighed, immediately frozen in liquid nitrogen, and kept at -70 ^{0} C until the analysis. Four livers were formalin fixed for morphological evaluation. The rest of the livers were frozen by the same procedure as the brains.

IV.2.4 Histomorphological analysis

Formalin fixed livers and brains were processed according to standard histological protocols and hematoxylin and eosin stained. The level of lipid infiltration in the livers was evaluated according to the classification of Schwahn et al. (2003). When microvesicular lipid droplets were present in fewer than 50% of the cells, steatosis was graded as mild; if microvesicular lipid droplets were found in more than 50% of the cells, steatosis was graded as moderate; and if macrovesicular droplets were present, steatosis was graded as severe (Schwahn et al. 2003). Brain morphology was assessed by light microscopy.

tHcy levels were measured by HPLC analysis (J Cohn, Department of Hyperlipidemia and Atherosclerosis at the Institute of Clinical Research, Montreal). Tissue thiol levels and DNA methylation capacity will be measured by HPLC and cytosine-extension assay, respectively, by another collaborator (S.J. James, Arkansas Children's Hospital Research Institute).

IV.2.5 Statistical analysis

For continuous variables, statistical significance of the differences between mean values was examined with the two-sample t-test or ANOVA with the alpha level of 0.05. For binary variables, statistical significance of the differences between proportions was examined with the chi-square test with the alpha level of 0.05. For survival analysis, survival rates were estimated with the Kaplan-Meier method and the differences were examined with the log-rank test.

IV.3 RESULTS

In the first experiment, 22 and 15 *Mthfr*-homozygous mutant pups were obtained in the metafolin and the control groups, respectively. Twelve pups survived until the age of five weeks in the treatment group, and three pups in the control group. The cumulative survival rate at the age of five weeks was 54 % in the treatment group and 20 % in the control group (Fig. 11). Chi-square analysis showed that the difference of 34 % in the cumulative survival rate between the two groups was statistically significant (p-value < 0.05).

When we considered the timing of death of those mutants who died, we found that in the mice in the metafolin group were dying at a much slower rate (log-rank test, p-value < 0.05). The median survival time was seven days for the control mice, while it was inestimable for the metafolin-treated mice, the majority of which were still alive by 35 days.

Weights of the mutant pups who died and survived throughout the treatment period were not different between the treatment groups (t-test, $p>0.05$), but were significantly lower than those of their heterozygous and wild-type littermates (t-tests, $p< 0.05$) (Fig.12).

The mean brain weight at the time of sacrifice (at the ages of six days and five weeks) showed the same trend as the mean body weight (Fig.13, 14). In both age groups, the mean brain weights of the surviving homozygous mutant pups in the metafolin group were not significantly different from those of surviving controls of the same genotype (t-tests, $p>0.05$).

Heterozygous and wildtype littermates at 6 days and 5 weeks of age had a statistically significantly higher mean weight of the brain than homozygous mutant mice (t-tests, $p<0.01$)

Histomorphological analysis of the brains in the survived homozygous mutant pups from at least 6 animals in both metafolin treatment and control groups showed characteristic laminar structure defects in the cerebella (Chen et al. 2001). No visible improvement in morphology of the cerebella was found in the treatment group. The brains of heterozygous mutants were histomorphologically normal in both treatment and control groups (Fig. 15).

Histomorphological analysis of the livers of homozygous mutant pups did not show any decrease in lipid infiltration with the treatment. In the metafolin group, among 11 mice with evaluated sections, five had severe steatosis, four presented with moderate and two with mild steatosis. In the control group (n=3), two mice had severe steatosis and one showed mild steatosis (Fig. 16).

THcy levels among 5-week-old pups were strongly dependent on genotype within each treatment group (ANOVA, $p < 0.01$), but were not influenced by the treatment (t-test, $p>0.05$) (Figure 17). The tHcy levels of the mothers of the pups were not decreased by metafolin treatment when compared to controls (T-test, $p>0.05$) (Fig.18).

The measurements of Hcy levels, SAM and SAH concentrations in the liver as well as evaluation of DNA methylation in the brain are in progress.

IV.5 DISCUSSION

Our study showed that metafolin treatment increased the survival of *Mthfr*-homozygous mutant pups by approximately 34% in comparison with untreated mice of the same genotype. However, it is unclear which parameters exactly were improved by the treatment that allowed these animals to survive at a higher rate.

Several abnormalities of *Mthfr*-deficient homozygous mutants could contribute to their increased death rate, for example, reduction in the cerebellum size with laminar structure defects and macrovesicular steatosis in the liver (Chen et al. 2001). It is possible that these mice have other abnormalities incompatible with life that have not been yet described.

The increased death rate in the homozygous mutant pups could be caused by demyelination or thrombosis in the respiratory centers of the brain stem. Bianchi et al. (1999) postulated that availability of methyl donors was closely related to the formation of myelin components and showed that inhibitors of the methyl transfer enzymes produce less compact myelin in oligodendroglial cells. In support of this hypothesis are electron microscopy data that revealed thinner myelin sheaths in axons of the nerves in *Mthfr*-homozygous mutant knockout mice compare to those of *Mthfr*-heterozygous mutant knockout and wildtype mice (B.Shwahn,

personal communication).

There are several inherited diseases with altered myelination such as metachromatic leukodystrophy, Krabbe disease and adrenoleukodystrophy. Signs in the patients with these diseases are similar to those of *Mthfr*-deficient knockout mice. These signs include: progressive hypotension, motor weakness, mental regression, ataxia, dysphagia, seizures and several other signs. It is possible that demyelination is one of the mechanisms responsible for neurological abnormalities of *Mthfr*-knockout homozygous mutant mice.

Another potential mechanism that could be responsible for higher lethality in such mice could be thrombosis of the respiratory centers of the brain stem or pulmonary artery. Patients with severe MTHFR deficiency often experience thrombosis and stroke (Rosenblatt et al. 2004) and individuals with mild MTHFR deficiency due to the 677C→T polymorphism are also predisposed to thrombosis (Kang et al. 1988; Kluijtmans et al. 1996; Morita et al. 1997; Talmon et al.1997; Eikelbom et al.1999; Ueland et al. 2000).

Metafolin treatment possibly was able to increase myelination to some extent or reduce the risk of thrombosis. It is possible that since it is the only form of folate able to cross the blood-brain barrier, 5-methyl-THF provided methyl groups that were used by the brain at the expense of

other tissues. Measurement of DNA methylation in the brain of homozygous mutant mice and their littermates could confirm this hypothesis. However, the measurements are being performed by collaborators and the results were not available at the time of thesis submission.

Morphological analysis of the brains of the survived homozygous mutant mice in comparison to survived controls did not show any improvement in gross brain morphology with the treatment. The sizes of the cerebella were similar to those of untreated mice. This could be a result of selective survival: some mice with small brain size in the metafolin group may have been able to survive only due to the treatment, while the mice with the same brain size in the untreated group died out, thus leading to survival of only mice with relatively large brain size among them.

No decrease in tHcy level was observed either in 5-week-old pups or in their mothers. Thus, the metafolin treatment increased survival without decreasing tHcy levels in plasma. Results of measurements of Hcy and SAM/SAH in the tissues were not available at the time of thesis submission.

It was of interest to compare similar experiments conducted with betaine to those with metafolin treatment. The experiments with betaine showed an increase in survival rate, and improvements in liver morphology, brain mass and structure (B.Schwahn, unpublished data). However,

betaine was used in a higher concentration (2%) and molarity (0.17 M), while the molarity of the 1% metafolin solution used in our study was only 0.02 M. According to the information provided by the manufacturer, previous experiments with metafolin showed that mice refused to drink metafolin solutions with concentrations higher than 1% (R.Moser, Eprova, personal communication). Therefore, we could not test the dose equimolar to that of betaine used in the study of B.Schwahn without changing the oral route of administration, which was chosen as the least stressful for pregnant mothers during the long treatment period. Because the concentrations of betaine and metafolin solutions were not equimolar, we could not compare these two experiments directly. However, the increase in survival following metafolin treatment was not associated with an improvement in plasma homocysteine or in gross liver or brain morphology, suggesting that other marker systems must reflect the mortality in severe MTHFR deficiency.

Figure 11. Survival curves of metafolin treated and untreated homozygous mutant pups and betaine-treated homozygous mutant pups (Schwahn, unpublished data).

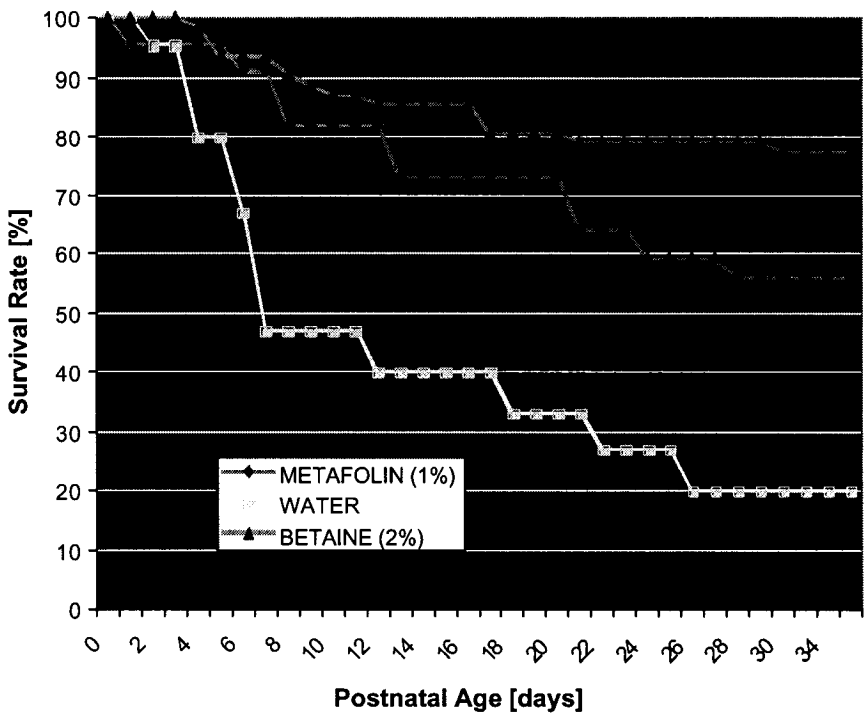

Survival Rate [%]
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
34
METAFOLIN (1%)
WATER
BETAINE (2%)
Postnatal Age [days]

Figure 12. Growth curves of homozygous (--) and heterozygous (+-) mutant pups and wildtypes (++) on metafolin treatment and water. The bars represent mean +/- standard deviation.

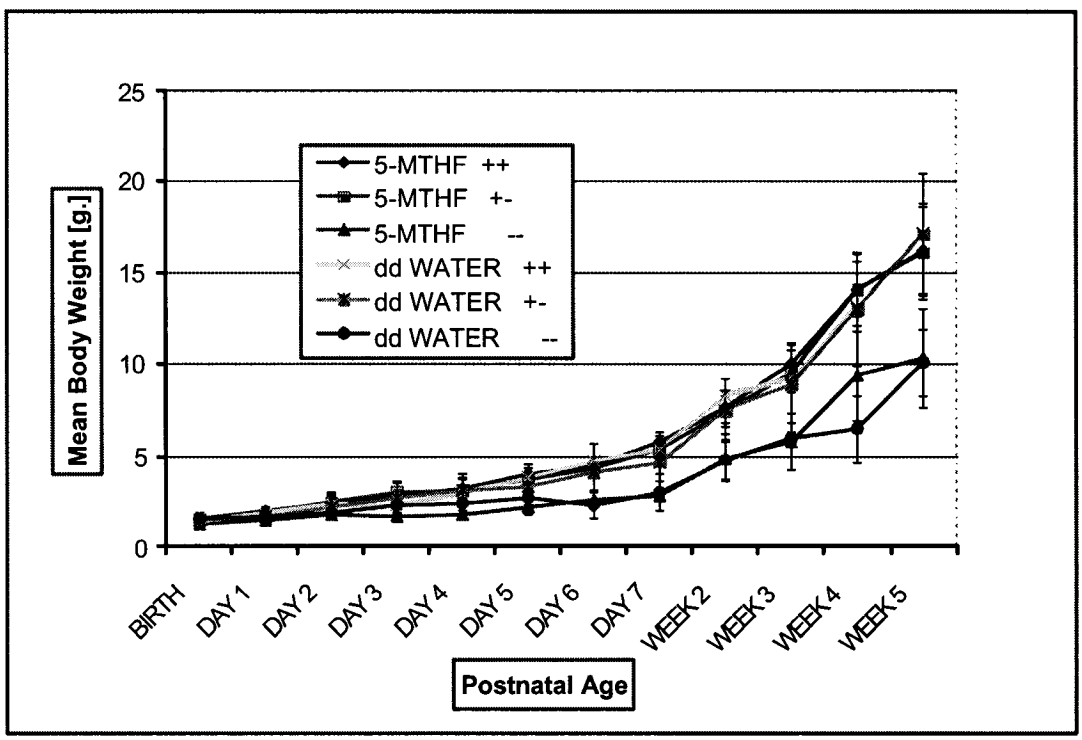

Mean Body Weight [g.]
25
20
15
10
5
0
5-MTHF ++
5-MTHF +-
5-MTHF --
dd WATER ++
dd WATER +-
dd WATER --
BIRTH
DAY 1
DAY 2
DAY 3
DAY 4
DAY 5
DAY 6
DAY 7
WEEK 2
WEEK 3
WEEK 4
WEEK 5
Postnatal Age

Figure 13. Mean brain weight of *Mthfr*-homozygous mutants (--) and their heterozygous mutant (+-) and wildtype (++) littermates at the age of 5 weeks. The bars represent mean +/- standard deviation.

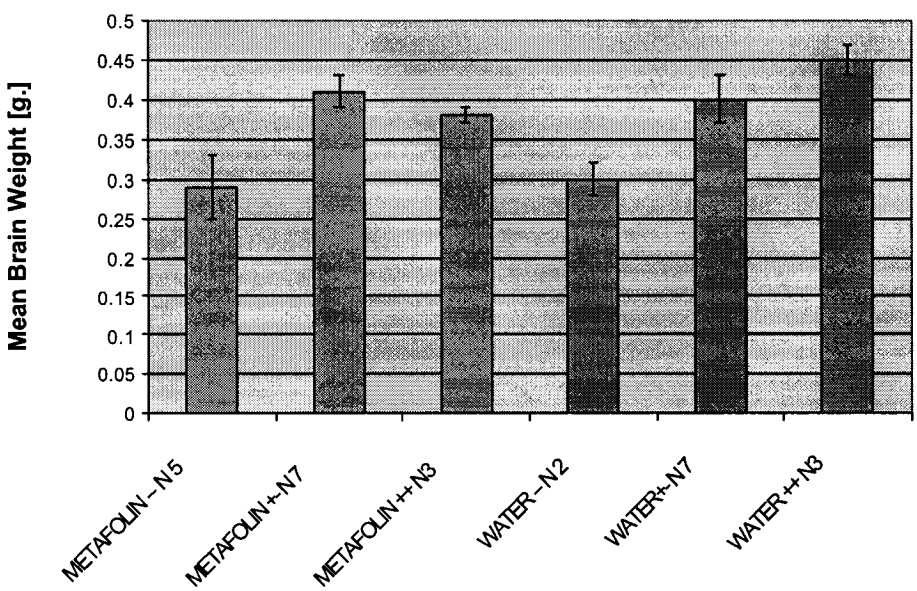

Mean Brain Weight [g.]
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
METAFOLIN – N5
METAFOLIN+-N7
METAFOLIN++N3
WATER – N2
WATER+-N7
WATER++N3
Treatment. Genotype. Observation Number.

Figure 14. Mean brain weight of 6-day-old homozygous mutant pups (--) and their heterozygous mutant (+/-) and wildtype(++) littermates. The bars represent mean +/- standard error.

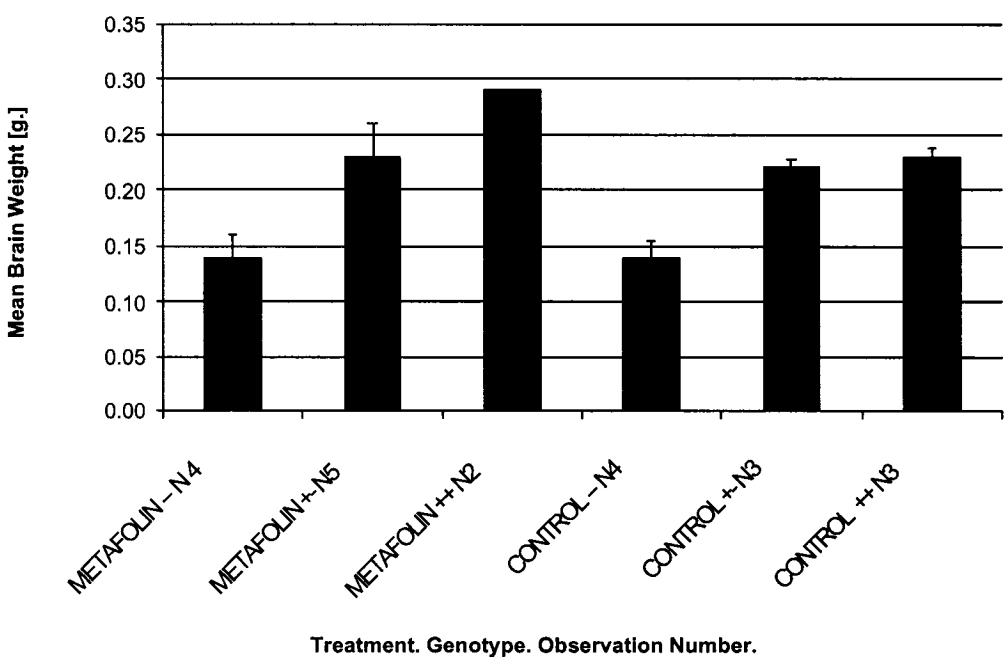
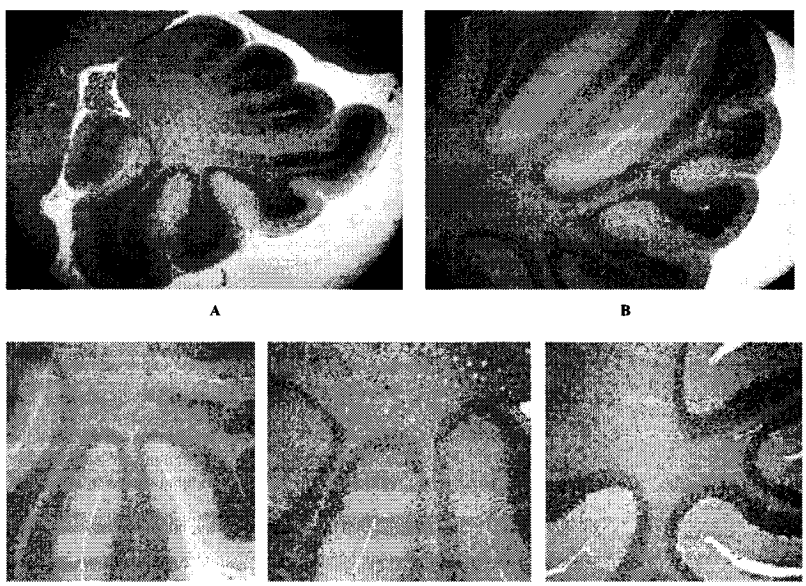
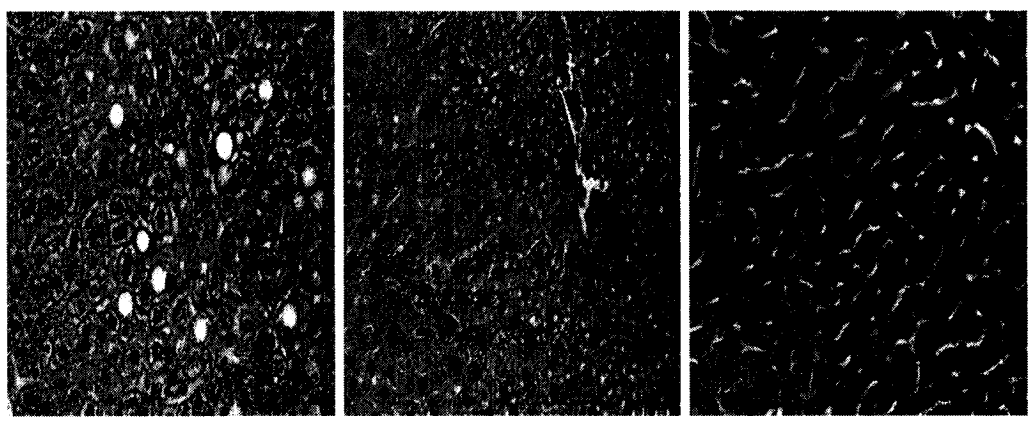

Mean Brain Weight [g.]
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
METAFOLIN – N4
METAFOLIN +- N5
METAFOLIN ++ N2
CONTROL – N4
CONTROL +- N3
CONTROL ++ N3
Treatment. Genotype. Observation Number.

Figure 15. Photographs of hematoxylin-eosin stained cerebellum sections of 9-day-old *Mthfr*-homozygous mutant (A) and heterozygous mutant (B) metafolin treated mice (magnification 400 X) and hematoxylin-eosin stained cerebellum sections of 5-week-old metafolin-treated (C) and untreated (D) *Mthfr*-homozygous mutant pups and metafolin-treated heterozygous mutant pup (E) (magnification 4000X).



A B C D E

Figure 16. Photographs demonstrating lipid infiltration in liver sections of 5-week-old metafolin treated *Mthfr*-homozygous mutant pups. Severe steatosis is shown in photograph A, moderate steatosis is shown in photograph B, and mild steatosis is shown in photograph C.



A B C

Figure 17. Total plasma homocysteine (tHcy) [μmol/L] levels among homozygous mutant (-/-), heterozygous mutant (+/-) and wildtype (+/+) pups based on the treatment. The bars represent mean +/- standard errors.

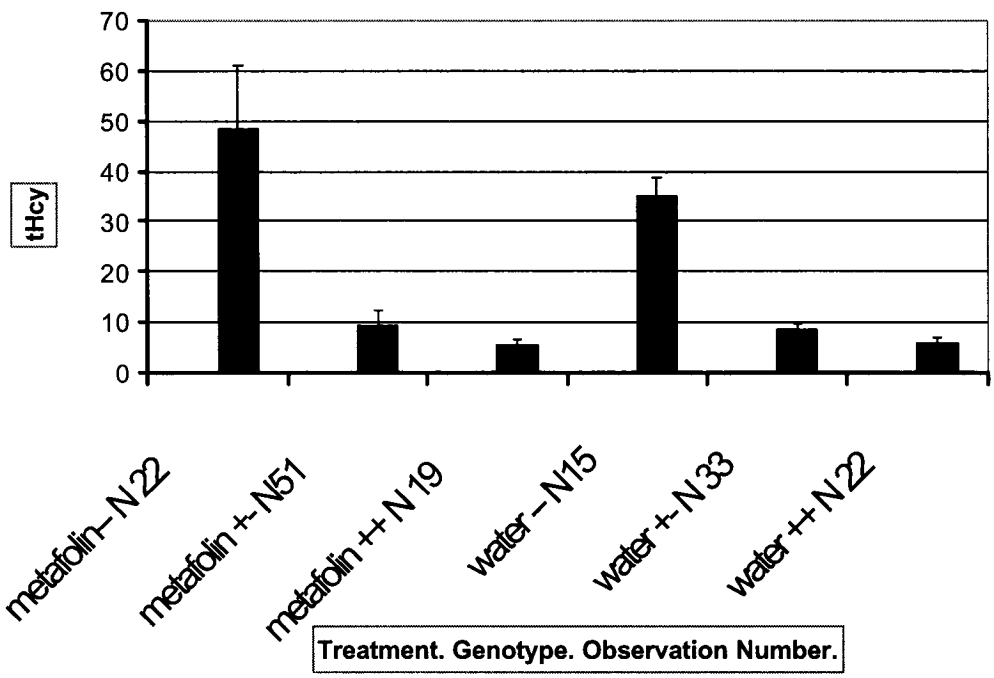
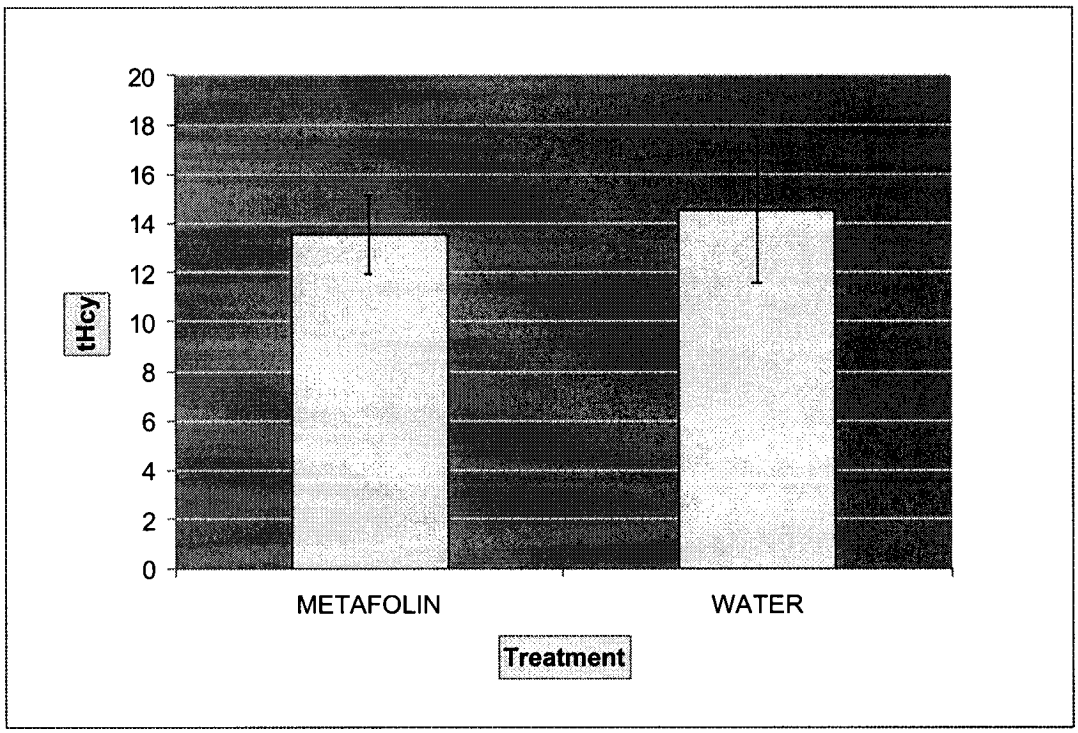

70
60
50
40
30
20
10
0
tHcy
metafolin– N22
metafolin +- N51
metafolin ++ N19
water – N15
water +- N33
water ++ N22
Treatment. Genotype. Observation Number.

Figure 18. Total plasma homocysteine [μmol/L] levels among mothers in the metafolin and the control group. The bars represent standard error


20
18
16
14
12
10
8
6
4
2
0
tHcy
METAFOLIN
WATER
Treatment

CHAPTER V

CONCLUSIONS

Conclusion for pharmacogenetic studies of MTX in a mouse model of mild MTHFR deficiency

In this study, an elevated apoptosis rate was found in the tissues *of Mthfr*-heterozygous animals as compared to wildtype and saline-treated controls. Our results indicate increased toxicity of MTX in M*thfr*-deficient heterozygous mice, serving as a model of mild hyperhomocysteinemia in humans due to the 677C→T polymorphism in the MTHFR gene. This suggests that such individuals could be more sensitive to MTX treatment and are more likely to develop severe side effects than persons without the polymorphism. Genotyping for 677C→T polymorphism prior to prescription of MTX chemotherapy may ultimately help prevent severe side effects in patients who are homozygous for the polymorphism.

Conclusion for pharmacogenetic studies of metafolin in a mouse model of severe and mild MHFR deficiency

Metafolin treatment increased the survival of *Mthfr*-knockout homozygous mutant mice. Additional data are needed to explain the mechanisms of the increased survival. The results of the study suggest that metafolin could be useful in the treatment of severe MTHFR deficiency in humans.

FUTURE DIRECTIONS

There are a number of other medications of potential interest for pharmacogenetic studies of MTHFR deficiency in a mouse model. It was not possible to test all these medications within the time frames of the Master's degree program.

PHENYTOIN

(PHE) is a medication used for treatment of epilepsy and arrhythmias. Many studies have found an association between phenytoin and folic acid deficiency. A decrease in plasma and erythrocyte folate concentration in patients receiving phenytoin therapy has been observed and reported frequently (Reynolds et al. 1975; Froscher et al. 1995; Ono et al. 1997; Tamura et al. 2000; Schwaninger et al. 1999).

Several mechanisms have been proposed to explain folate deficiency during phenytoin therapy. Billings et al. (1984) observed a decrease in hepatic MTHFR activity in mice after chronic phenytoin treatment and suggested that phenytoin may directly interfere with homocysteine metabolism by blocking MTHFR. There is also a report that homozygosity for the 677C→T mutation is associated with elevated tHcy and low folate level during phenytoin therapy (Yoo et al. 1999).

5-FLUOROURACIL

(5-FU) is a fluorine-substituted analogue of uracil that is used in cancer chemotherapy. 5-FU blocks the enzyme thymidylate synthase, which methylates deoxyuridinemonophosphate (dUMP) to form deoxythymidinemonophosphate (dTMP). By inhibiting thymidylate synthase, 5-FU reduces the conversion of 5-10-methylene-THF to DHF. MTHFR deficiency results in reduced folate recycling because of decreased conversion of 5-methyl-THF to THF. Thus MTHFR deficiency in combination with 5-FU treatment may lead to folate deficiency, especially when dietary folate intake is low. This may result in increased toxicity of 5-FU in MTHFR deficient individuals.

LEVODOPA (LD)

LD is the precursor to dopamine. It is used for treatment of Parkinson's disease (PD). Many studies documented an increase in tHcy levels among PD patients treated with LD (Allain et al. 1995; Kuhn et al. 1998; Muller et al. 1999). There are several reports on association of homozygosity for the 677C→T polymorphism and higher elevation of tHcy, when compared to wild-type or heterozygous subjects (Yasui et al. 2000; Brattstrom et al. 2001; Kuhn et al. 2001). High demand for methylation of LD, along with decreased SAM synthesis due to folate or vitamin B12 deficiency or homozygosity for 677 C→T polymorphism, results in SAM-deficiency and subsequent hyperhomocysteinemia (Brattstrom et al. 2001).

FENOFIBRATE (FEN)

FEN is a fibric acid derivative used in the treatment of hypertriglyceridemia, mixed hyperlipidemia, and diabetic dyslipidemia.

FEN can induce hyperhomocysteinemia (Dierkers et al. 2003; Bissonnete et al. 2001; Dierkes et al. 2001) by the mechanism which has not been fully explained yet. One of the proposed explanations is through an interaction of FEN with the metabolism of homocysteine and vitamins involved.

Pharmacogenetic studies of the described drugs in a mouse model of MTHFR deficiency will further explore and explain the MTHFR gene-drug interaction.

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APPENDIX

Project # 3132
Dr. Rima Rozen

Continuation of #7C:

	Sp/strain 7	**Sp/strain 8**	**Sp/strain 9**
Species	Mouse	Mouse	Mouse
Supplier/Source	Jackson Laboratory	Charles River	Generated in-house
Strain	ApoE KO	C57	Mthfr normal
Sex	M/F	M/F	M/F
Age/Wt	up to 1 yr/wt as per age	up to 1 yr/wt as per age	up to 2 yr/wt as per age
# To be purchased	4	4	0
# Produced by in-house breeding	50	16	69
# Other (e.g. field studies)	n/a	n/a	n/a
# needed at one time	27	4	35
# per cage	4-5	4-5	4-5
Total # per year	54	20	69

- Breeding colony, dietary manipulations, blood collection, euthanasia, tissue collection, transgenic breeding, knockout/in generation, oral drug administration.

6. Animals Use data for CCAC

6 a) Purpose of Animal Use (Check one):

1. ☒ **Studies of a fundamental nature/basic research**
2. ☐ **Studies for medical purposes relating to human/animal diseases/disorders**
3. ☐ **Regulatory testing**
4. ☐ **Development of products/appliances for human/veterinary medicine**
5. **If for Teaching, use the Animal Use Protocol form for Teaching (www.mcgill.ca/rgo/animal)**

6 b) Will field studies be conducted? NO ☒ YES ☐ If yes, complete "Field Study Form"
Will the project involve the generation of genetically altered animals? NO ☐ YES ☒ If yes, complete SOP #5
Will the project involve breeding animals? NO☐ YES ☒ If breeding transgenics/knockouts, complete SOP #4

7. Animal Data

7 a) Please justify the need for live animals versus alternate methods (e.g. tissue culture, computer simulation)

- the mice have naturally occurring mutations (ct/ct and Min) or generated mutations in MTHFR which allow us to examine diseases in vivo.

7 b) Describe the characteristics of the animal species selected that justifies its use in the proposed study (consider characteristics such as body size, species, strain, data from previous studies or unique anatomic/physiological features)

- The ct/ct mice are inbred stains with a high incidence of neural tube defects. The Min mice are inbred strains that develop intestinal tumors.

7 c) Description of animals

Quality Control Assurance: To prevent introduction of infectious diseases into animal facilities, a health status report or veterinary inspection certificate may be required prior to receiving animals from all non-commercial sources or from commercial sources whose animal health status is unknown or questionable. Quarantine and further testing may be required for these animals.

If more than 6 columns are needed, please attach another page

	Sp/strain 1	Sp/strain 2	Sp/strain 3	Sp/strain 4	Sp/strain 5	Sp/strain 6
Species	Mouse	Mouse	Mouse	Mouse	Mouse	Mouse
Supplier/Source	Jackson Laboratory	Jackson Laboratory	Jackson Laboratory	Generated in-house	Generated in-house	Jackson Laboratory
Strain	ct/ct	Splotch	Min	Mthfr KO	Mthfr transgenic	CBS
Sex	M/F	M/F	M/F	M/F	M/F	M/F
Age/Wt	up to 1 yr/wt as per age	up to 1 yr/wt as per age	up to 6 months/wt as per age	up to 2 yr/wt as per age	up to 2 yr/wt as per age	up to 1 yr/wt as per age
# To be purchased	4	4	4	0	0	0
# Produced by in-house breeding	104	104	68	63	30	36
# Other (e.g.field studies)	n/a	n/a	n/a	n/a	n/a	n/a
#needed at one time	54	54	18	21	10	12
# per cage	4-5	4-5	4-5	4-5	4-5	4-5
TOTAL# /YEAR	108	108	72	63	30	36

7 d) Justification of Animal Usage: BASED ON THE EXPERIMENTAL OBJECTIVES OF THE PROJECT, describe the number of animals required for one year. Include information on experimental and control groups, # per

7 d) Justification of Animal Usage: BASED ON THE EXPERIMENTAL OBJECTIVES OF THE PROJECT, describe the number of animals required for one year. Include information on experimental and control groups, # per group, and failure rates. For breeding, specify how many adults are used, number of offspring produced, and how many offspring are used in experimental procedures. Use the table below when applicable. The arithmetic explaining how the total of animals for each column in the table above is calculated should be made clear. ***Space will expand as needed.***

All calculations in the table below assume an average of 8 offspring per litter. In experiments where the Mthfr strain is crossed with a different strain, the number of offspring are assigned to the non-Mthfr strain in Table 7c.
The ct/ct (curly tail) mice and splotch mice are good animal models for neural tube defects. Each of these strains will be crossed with the Mthfr knockout strain, under 3 dietary conditions (control diet, low folate and high folate diets) to examine the incidence of neural tube defects below.
The Min mice are a good animal model for colorectal cancer. The Min mice will be crossed with Mthfr knockout mice, as well as mice containing a normal Mthfr gene, to assess tumor numbers and sizes.
CBS knockout mice (mouse model for hyperhomocysteinemia and vascular disease) and ApoE knockout mice (model model for hypercholesterolemia and vascular disease) will be crossed with Mthfr knockout mice to examine lipid accumulation.
Mthfr knockout mice will be treated with saline, betaine (an alternate methyl donor that can replace folate in homocysteine remethylation to methionine), or 5-methyltetrahydrofolic acid to examine metabolites and clinical parameters.
We are working on generating Mthfr transgenic mice, that overexpress Mthfr, through a collaboration with Dr. Alan Peterson (McGill). These mice, when available, as well as control Mthfr mice, will be treated with control diets, low folate and high folate diets, to examine metabolites and clinical parameters.
C57Bl mice are the background strain for the Min, Mthfr KO and Mthfr transgenic mice and will occasionally be required to maintain colony.

The following table may help you explain the animal numbers listed in the 7c table:

Test Agents or Procedures *e.g. 2 Drugs*	**# of Animals and Species Per Group** *e.g. 6 rats*	**Dosage and/or Route of Administration** *e.g. .03, .05 mg/kg – IM, IP (4 variables)*	**# of endpoints** *e.g. 1, 7, 10 days (3 variables)*	**Other variables (i.e. sex weight, genotypes,etc.)** *e.g. Male, Female groups (2 variables)*	**Total number of animals per year** *e.g. 2 x 6 x 4 x 3 x 2 = 288*
See attached Table					

8. Animal Husbandry and Care

8 a) If projects involves non-standard cages, diet and/or handling, please specify

Low folate and high folate diets have been used and will continue to be used for some experiments as indicated in the above table. These diets did not affect weight or other obvious health parameters.

8 b) Is there any component to the proposed procedures which will result in immunosuppression or decreased immune function (e.g. stress, radiation, steroids, chemotherapeutics, genetic modification of the immune system)?

NO ☒ YES ☐ **if yes, specify:**

8 c) Indicate area(s) where animal use procedures will be conducted:

Building: Place Toulon **Room:** Animal Facility

Indicate area(s) all facilities where animals will be housed:

Building: Place Toulon **Room:** Animal Facility

If animal housing and animal use are in different locations, briefly describe procedures for transporting animals:
n/a

9. Standard Operating Procedures (SOPs)

Complete this section if you plan to use any of the UACC SOPs listed below. IT IS UACC POLICY THAT THESE SOPS BE USED WHEN APPLICABLE. Any proposed variation of the SOPs must be described and justified. The Standard Operating Procedures can be found at the UACC website at www.mcgill.ca/rgo/animal . The completed and signed SOP from must be attached to the protocol.

Check all SOPs that will be used:

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7 d) Justification of Animal Usage

Test Agents or procedures e.g.: Drugs	**# of Animals and Species per Group e.g.: 6 rats**	**Dosage and/or Route of Administration e.g. .03, .05 mg/kg-IM, IP (4 variables)**	**# of endpoints e.g. 1, 7, 10 days (3 variables)**	**Other variables (i.e. sex, weight, genotypes, etc.) e.g. Male, Female groups (2 variables)**	**Total number of animals per year e.g. 2 x6 x4 x3 x2 = 288**
3 diets (control, high and low folate) for ct/ct crossed with Mthfr KO	ct/ct 4 mice/diet = 12 mice Mthfr KO 4 mice/diet = 12 mice		2 weeks for offspring		24 adult mice (12 pairs) + 96 offspring Total = 120
3 diets (control, high and low folate) for splotch crossed with Mthfr KO	Splotch 4 mice/diet = 12 mice Mthfr KO 4 mice/diet = 12 mice		2 weeks for offspring		24 adult mice (12 pairs) + 96 offspring Total = 120
2 Mthfr groups for crossing with Min	Min 8 mice Mthfr normal 4 mice Mthfr KO 4 mice		12 weeks for offspring		16 adult mice (8 pairs) + 64 offspring Total =80
2 Mthfr groups for crossing with CBS	CBS 4 mice Mthfr normal 2 mice Mthfr KO 2 mice		6 months for offspring		8 adult mice (4 pairs) + 32 offspring Total = 40
2 Mthfr groups for crossing with apoE KO	ApoE KO 6 mice Mthfr normal 3 mice Mthfr KO 3 mice		6 months for offspring		12 adult mice (6 pairs) = 48 offspring Total = 60
3 treatments (saline, betaine, 5-methyltetrahydrofolic acid)	Mthfr normal 10 mice per group = 30 mice Mthfr KO 10 mice per group = 30 mice		2 months		30 30 Total - 60
3 diets (control, high and low folate)	Mthfr normal 10 mice per group = 30 mice Mthfr transgenic 10 mice per group = 30 mice		6 months		30 30 Total - 60
colony maintenance	C57Bl for breeding with Min, Mthfr transgenic or Mthfr KO to maintain colony				20 Total = 20

Anaesthesia in rodents UACC#2	☐	Rodent Survival Surgery UACC#10	☐
Analgesia in rodents UACC#3	☐	Anaesthesia & Analgesia Neonatal Rodents UACC#11	☐
Breeding transgenics/knockouts UACC#4	☒	Stereotaxic Survival Surgery in Rodents UACC#12	☐
Transgenic Generation UACC#5	☒	Euthanasia of Adult & Neonatal Rodents UACC#13	☐
Knockout/in Generation UACC#6	☐	Field Studies Form	☐
Production of Monoclonal Antibodies UACC#7	☐	Phenotype Disclosure Form	☐
Production of Polyclonal Antibodies UACC#8	☐	Other, specify:	☐

10. Description of Procedures

10 a) . IF A PROCEDURE IS COVERED BY AN SOP, WRITE "AS PER SOP", NO FURTHER DETAIL IS REQUIRED.

FOR EACH EXPERIMENTAL GROUP, DESCRIBE ALL PROCEDURES AND TECHNIQUES, WHICH ARE NOT PART OF THE SOPs, IN THE ORDER IN WHICH THEY WILL BE PERFORMED – surgical procedures, immunizations, behavioural tests, immobilization and restraint, food/water deprivation, requirements for post-operative care, sample collection, substance administration, special monitoring, etcAppendix 2 of the Guidelines (www.mcgill.ca/rgo/animal) provides a sample list of points that should be addressed in this section.

Blood Collection: See SOP.

-To determinee if animals have disrupted gene or genetic mutation, the mice will be evaluated for genotypes at 8-10 days of age by removing approximately 1.0 cm of tail for DNA analysis. Mice under general anaesthesia will be perfused through the heart with paraformaldehyde, paraformaldehyde/glutaraldehyde or Bouin's. They will then be dissected and tissues prepared for histopathologic analysis. For some experiments, mice will be placed on special diets, mated and offspring will be analyzed as described.

For some experiments, mice will be sacrificed using a C02 chamber and dissected, and tissues will be snap frozen.

Sub-project #1: To generate and characterize mice overexpressing MTHFR. The goal is to determine if overexpression of MTHFR affects metabolites and phenotype of mice. These mice will be generated at the McGill/Royal Victoria Hospital facility by Dr. Alan Peterson and placed on 3 diets (control, high and low folate). Initial studies will examine weight (weekly measurements), liver and kidney function and blood counts (all performed by blood sampling through Animal Resource Center, McGill; samples taken every 2 months). Animals will be sacrificed at 6 months and tissues collected for histological and biochemical analyses.

Sub-project #2: To treat mice with MTHFR deficiency. Two therapeutics (and drinking water as a control) already in use for the treatment of the human disease associated with MTHFR deficiency (betaine and 5-methyltetrahydrofolic acid) will be used to improve survival in Mthfr KO mice. Dosage and route: betaine, 2% in drinking water; 5-methyltetrahydrofolic acid, 1% in drinking water for 8 weeks; control group: Drinking water for 8 weeks. Ten mice in each group will be used. After treatment, mice will be sacrified and blood and tissues will be collected for biochemical and histological analyses.

Sub-project #3: Two strains (CBS knockout and ApoE knockout) have increased risk of cardiovascular disease. These strains will be crossed with Mthfr-deficient mice to evaluate whether there is increased lipid deposition at 6 months in the offspring of the cross.Two strains (curly tail (ct) and splotch) have increased risk of neural tube defects.
These strains will also be crossed with Mthfr-deficient mice to evaluate whether there is an increase in neural tube defects in the offspring of the cross.

Sub-project #4 – Min mice develop numerous colorectal adenomas from 1 to 6 months of age. Min mice will be crossed with Mthfr-deficient mice to evaluate numbers and sizesof adenomas in the offspring, when

10 b) Experimental endpoint – for each experimental group indicate survival time

Endpoints for experiments are provided in the table.

10 c) Clinical endpoint – describe the conditions, complications, and criteria (e.g. >20% weight loss, maximum tumour size, vocalizing, lack of grooming) that would lead to euthanasia of an animal before the expected completion of the experiment (specify per species and project if multiple projects involved)

Cardiovascular complications are not expected, since our endpoint is lipid deposition at 6 months of age.
For the Min mice with colorectal adenomas, animals will be monitored twice weekly and will be sacrificed if they exhibit 15% - 20% weight loss, dehydration and tenesmus. However, the usual life expectancy of these mice is 20 weeks, due to the intestinal tumors; consequently we do not anticipate euthanasia in our experiments since decreased Mthfr expression is expected to decrease tumor numbers or sizes (based on the clinical literature on humans with MTHFR deficiency).

10 d) Specify person(s) who will be responsible for animal monitoring and post-operative care ***(must also be listed in section 4)***

Name: Qing Wu	**Phone #:** (514) 412-4400, ext. 23281

10 e) Pre-Anesthetic/Anaesthetic/Analgesic Agents: List all drugs that will be used to minimize pain, distress or discomfort. ***Table will expand as needed.***

Species	Agent	Dosage (mg/kg)	Total volume(ml) per administration	Route	Frequency
Ketamine (200 mg/kg) mixed with xylazine (10 mg/kg) administered intramuscularly to an average 25g mouse					

10 f) Administration of ALL other substances: List all non-anaesthetic agents under study in the experimental component of the protocol, including but not limited to drugs, infectious agents, viruses. ***Table will expand as needed.***

Species	Agent	Dosage (mg/kg)	Total volume(ml) per administration	Route	Frequency
Mouse	Betaine	2% in drinking water		enteral	daily
Mouse	5-methyltetrahydrofolic acid	1% in drinking water		enteral	daily

10 g) Method of Euthanasia

Specify Species	
	☐ **Anaesthetic overdose, list agent/dose/route:**
	☒ **Exsanguination with anaesthesia, list agent/dose/route:** Ketamine/Xylazine, route = IM, dose = 200/10 mg/kg
	☐ **Decapitation without anaesthesia *** ☐ **Decapitation with anesthesia, list agent/dose/route:**
	☐ **Cervical dislocation without anaesthesia *** ☐ **Cervical dislocation with anaesthesia, list agent/dose/route:**
Mouse	☒ **CO_2 chamber**
	☐ **Other, specify:**
	☐ **Not applicable, explain:**

*** For physical method of euthanasia without anaesthesia, please justify:**

11. Category of Invasiveness: B ☐ C ☒ D ☐ E ☐

Categories of Invasiveness (from the CCAC *Categories of Invasiveness in Animal Experiments*). Please refer to this document for a more detailed description of categories.
Category A: Studies or experiments on most invertebrates or no entire living material.
Category B: Studies or experiments causing little or no discomfort or stress. *These might include holding animals captive, injecting*

percutaneous blood sampling, accepted euthanasia for tissue harvest, acute non-survival experiments in which the animals are completely anaesthetized.
Category C: Studies or experiments involving minor stress or pain of short duration. *These might include cannulation or catheterizations of blood vessels or body cavities under anaesthesia, minor surgery under anaesthesia, such as biopsy; short periods of restraint, overnight food and/or water deprivation which exceed periods of abstinence in nature; behavioural experiments on conscious animals that involve short-term stressful restraint.*
Category D: Studies or experiments that involve moderate to severe distress or discomfort. *These might include major surgery under anaesthesia with subsequent recovery, prolonged (several hours or more) periods of physical restraint; induction of behavioural stresses, immunization with complete Freund's adjuvant, application of noxious stimuli, procedures that produce pain, production of transgenics (in accordance with University policy).*
Category E: Procedures that involve inflicting severe pain, near, at or above the pain threshold of unanaesthetized, conscious animals. *Not confined to but may include exposure to noxious stimuli or agents whose effects are unknown; exposure to drugs or chemicals at levels that (may) markedly impair physiological systems and which cause death, severe pain or extreme distress or physical trauma on unanaesthetized animals.* According to University policy, E level studies are not permitted.

12. Potential Hazards to Personnel and Animals **It is the responsibility of the investigator to obtain the necessary Biohazard and/or Radiation Safety permits before this protocol is submitted for review.**
A copy of these certificates must be attached, if applicable.

No hazardous materials will be used in this study: ☒

12 a) Indicate which of the following will be used in animals:

☐ **Toxic chemicals** ☐ **Radioisotopes** ☐ **Carcinogens** ☐ **Infectious agents** ☐ **Transplantable tumours**

12 b) Complete the following table for each agent to be used (use additional page as required):

Agent name			
Dosage			
Route of administration			
Frequency of administration			
Duration of administration			
Number of animals involved			
Survival time after administration			

12 c) After administration the animals will be housed in:

☒ **the animal care facility** ☐ **laboratory under supervision of laboratory personnel**

Please note that cages must be appropriately labeled at all times.

12 d) Describe potential health risk (s) to humans or animals:

n/a

12 e) Describe measures that will be used to reduce risk to the environment and all project and animal facility personnel:

n/a

13. Reviewer's Modifications **(to be completed by ACC only): The Animal Care Committee has made the following modification(s) to this animal use procedure protocol during the review process. Please make these changes to your copy and comply with the recommended changes as a condition of approval.**

Rodent Laboratory Chow 5001

Chemical Composition[1]

Nutrients[2]

General Constituents

Crude Protein, %	23.4
Crude Fat, %	4.5
Crude Fibre, %	5.8
Ash, %	7.3
Nitrogen Free Extract (by difference), %	49.0

Chemical Constituents

Neutral Detergent Fibre (NDF), %	16.0
Acid Detergent Fibre (ADF), %	8.2
Cholesterol, mg/kg	270.0
Carotene, mg/kg	4.5

Energy

Total Digestible Nutrients (TDN), %	76.0
Gross energy (GE), kg/g	4.3
Physiological Fuel Value[3] (PFV), kcal/g	3.3

Amino Acids

Arginine, %	1.38
Cysteine, %	0.32
Glycine, %	1.20
Histidine, %	0.55
Isoleucine, %	1.18
Leucine, %	1.70
Lysine, %	1.42
Methionine, %	0.43
Phenylalanine, %	1.03
Tyrosine, %	0.68
Threonine, %	0.91
Tryptophan, %	0.29
Valine, %	1.21

Minerals

Calcium, %	1.00
Phosphorus, %	0.61
Potassium, %	1.10
Magnesium, %	0.21
Sodium, %	0.40
Chloride, %	0.56
Fluorine (mas.), mg/kg	35
Iron, mg/kg	198
Zinc, mg/kg	70
Manganese, mg/kg	64
Copper, mg/kg	18
Cobalt, mg/kg	0.60
Iodine, mg/kg	0.70
Selenium, mg/kg	0.20

Vitamins

Menadione (added), mg/kg	1.0
Thiamin, mg/kg	15
Riboflavin, mg/kg	8
Niacin, mg/kg	95
Pantothenic Acid, mg/kg	24
Choline, mg/g	2.3
Folic Acid, mg/kg	6
Pyridoxine, mg/kg	6
Biotin, mcg/kg	100
Vitamin B_{12}, mcg/kg	22
Vitamin A, IU/g	15.0
Vitamin D_3, IU/g	4.5
Vitamin E, IU/kg	40
Ascorbic Acid, mg/g	-

[1] The chemical composition data is based upon ingredient analysis information. Nutrient composition may vary due to the natural variation in ingredient composition.

[2] Nutrients have been calculated on a 90% dry matter basis.

[3] PFV (kcal/g) = ((Protein, % x 4) + (Fat, % x 9) + (NFE, % x 4)) ÷ 100