The Impact of Maternal Genetic and Dietary Disturbances in One-Carbon Metabolism on Embryonic Development

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Dedication

This thesis is dedicated to Ammi, Papa, Taimoor, and to my dear friends for their unwavering support and love throughout my academic endeavours. With each passing day, I learn more from all of you.

Abstract

The vitamin folate is involved in one-carbon metabolism and the remethylation of homocysteine to methionine. Disturbances in folate metabolism can result in an increased risk of neural tube defects (NTD), other birth defects or pregnancy complications. The most common cause of genetic folate deficiency is the *MTHFR* 677C>T single nucleotide polymorphism (SNP), which results in elevated levels of homocysteine and a thermolabile methylenetetrahydrofolate reductase (MTHFR) enzyme with reduced activity. Another methyl donor for homocysteine remethylation is betaine, which is derived from the nutrient choline. The choline-betaine remethylation pathway is upregulated when folate metabolism is disturbed. While folate is present in pregnancy supplements, choline is absent. Choline is used to produce metabolites required for neurodevelopment. However, there are few studies on the importance of choline when folate metabolism is disturbed, such as in the case of MTHFR deficiency.

A mouse model for MTHFR deficiency, the heterozygous Mthfr knockout mouse (*Mthfr*+/-), was generated by the Rozen laboratory and has been used as a model for mild MTHFR deficiency due to the *MTHFR* 677 TT genotype, since it has similar enzyme activity. Dietary studies previously performed on the *Mthfr*+/- mouse showed that low maternal folate increased the risk of embryonic defects and pregnancy complications, and low maternal choline impaired memory and neurodevelopment in pups. The first objective of this thesis was to investigate if low dietary choline and MTHFR deficiency results in increased risk of pregnancy complications, embryonic delays, or embryonic defects in the *Mthfr*+/- mouse model. When fed a low choline diet (3-fold lower than recommended levels for mice), pregnant *Mthfr*+/- females had lower brain weights than *Mthfr*+/+ females. An increased risk of embryonic defects, pregnancy complications, or embryonic growth restriction was not observed, suggesting a compensatory mechanism or insufficient reduction of dietary choline.

A separate model, the novel *Mthfr* 677 TT mouse, was generated recently by collaborators at the Jackson Laboratory. While it is known that human *MTHFR* 677C>T SNP results in a thermolabile enzyme with reduced activity, this is not the case in the *Mthfr*+/- knockout mouse. A model with the exact SNP seen in humans may be more suitable for understanding reproductive outcomes when dietary folate levels are altered. The second objective of this thesis was to perform preliminary analysis of reproductive outcomes, such as risk of embryonic defects and pregnancy complications, in the *Mthfr* 677 TT mouse when folate

is either decreased or supplemented in the maternal diet. Measurements of relevant metabolites and MTHFR protein levels will also be assessed. Preliminary results in the reproductive studies suggest that there may be an increase in % resorbed embryos between *Mthfr* 677 CC (8.7%) and *Mthfr* 677 TT (31.3%) mothers on control diet. However, due to low sample size, additional sampling is necessary for proper statistical analysis. Metabolite analyses showed that non-pregnant *Mthfr* 677 TT females had elevated homocysteine and decreased methionine, betaine, and choline in plasma, with decreased hepatic MTHFR protein levels, compared to *Mthfr* 677 CC females.

These murine models are important to study the role of dietary and genetic variation in folate and choline metabolism. Approximately 85-90% of women of reproductive age do not acquire enough choline in the diet, and its supplementation may be important, particularly for individuals with MTHFR deficiency. Characterizing models to study methyl group deficiencies can help to elucidate the role of folate and choline in humans.

Résumé

Le folate est une vitamine impliquée dans le métabolisme des monocarbones et permet la reméthylation de l'homocystéine en méthionine. Des perturbations du métabolisme du folate peuvent augmenter le risque de malformations du tube neural (MTN), d'autres malformations congénitales ou de complications de la grossesse. La cause génétique la plus fréquente d'un déficit en folate est le polymorphisme *MTHFR* 677C>T, qui est caractérisé par des niveaux élevés d'homocystéine et une enzyme méthylènetétrahydrofolate réductase (MTHFR) thermolabile dont l'activité est moindre. La bétaïne, qui est dérivée de la choline, constitue un donneur distinct de méthyle pour la reméthylation de l'homocystéine. La voie de reméthylation choline-bétaïne est régulée à la hausse lors de perturbations du métabolisme du folate. Bien que le folate fasse partie intégrante des suppléments de grossesse, la choline en est absente. Par surcroît, la choline est impliquée dans la production de métabolites importants lors du neurodéveloppement. Malgré tout, peu de travaux se sont intéressés au rôle que joue la choline lorsque le métabolisme des folates est perturbé, comme c'est le cas lors d'une déficience en MTHFR.

La souris hétérozygote *Mthfr*+/- s'est avérée utile pour étudier le déficit en MTHFR. Cette souris a été générée dans le laboratoire de la Dre Rozen et a été beaucoup utilisée comme modèle de la déficience légère en MTHFR observée en présence du génotype *MTHFR* 677 TT parce qu'elle résulte en une activité enzymatique résiduelle d'un niveau similaire. En étudiant l'effet de l'apport alimentaire en folate des souris *Mthfr*+/-, il a été démontré que de faibles niveaux de folate maternel augmentaient le risque de défauts embryonnaires et de complications de la grossesse. De plus, de faibles niveaux de choline des mères altéraient la mémoire et le neurodéveloppement chez les souriceaux. Le premier objectif de cette thèse était d'étudier si une légère carence en choline et en MTHFR entraîne un risque accru de complications de la grossesse, de retards embryonnaires ou de défauts embryonnaires en utilisant le modèle de souris *Mthfr*+/-. Lorsqu'elles étaient nourries avec un régime appauvri en choline (niveaux 3 fois inférieurs aux niveaux recommandés pour les souris), la masse du cerveau des femelles *Mthfr*+/- enceintes était inférieure à celle observée pour les femelles *Mthfr*+/+. Les risques de défauts embryonnaires, de complications de grossesse ou de restriction de croissance embryonnaire n'étaient pas augmentés, suggérant un mécanisme compensatoire ou une réduction insuffisante de la choline dans l'alimentation des souris.

Un tout nouveau modèle animal, la souris *Mthfr* 677 TT, a récemment été créée par des collaborateurs du laboratoire Jackson. Bien qu'il soit connu que le polymorphisme humain *MTHFR* 677C>T résulte en une enzyme qui est thermolabile en plus de posséder une activité réduite, ce n'est pas le cas pour la souris *Mthfr*+/- dont l'enzyme MTHFR ne présente pas de thermolabilité. Pour cette raison, un modèle animal reproduisant plus fidèlement le polymorphisme humain peut être préférable pour étudier les effets d'une altération des niveaux de folate alimentaire sur la reproduction des souris. Le second objectif de cette thèse était d'effectuer des analyses préliminaires en utilisant la souris *Mthfr* 677 TT, comme la détermination du risque de défauts embryonnaires et de complications de la grossesse, lorsque le folate est diminué ou augmenté dans l'alimentation maternelle. La mesure des métabolites pertinents et l'évaluation des niveaux de la protéine MTHFR seront également effectuées. Notre étude initiale suggère qu'il peut y avoir des différences quant à la portion des embryons résorbés entre les groupes maternels témoins et ceux déficients en folate, ainsi qu'entre les mères *Mthfr* 677 CC et *Mthfr* 677 TT. Cependant, en raison de la taille modeste de notre échantillon actuel, des données supplémentaires seront requises afin de permettre une étude statistique appropriée. L'analyse des métabolites a montré que les femelles non enceintes *Mthfr* 677 TT présentaient un niveau d'homocystéine élevé et une diminution de la méthionine, de la bétaïne et de la choline dans le plasma, avec une diminution des taux de protéine hépatique MTHFR, par rapport aux femelles *Mthfr* 677 CC.

Ces modèles murins sont importants car ils permettent d'étudier l'impact de variations alimentaires et génétiques sur le métabolisme du folate et de la choline. Pour 85-90% des femmes en âge de procréer, l'apport en choline est insuffisant dans l'alimentation et une supplémentation appropriée pourrait donc être critique, particulièrement pour les personnes présentant une déficience en MTHFR. La caractérisation de modèles pour étudier les déficiences en groupe méthyle peut contribuer à élucider le rôle du folate et de la choline chez les humains.

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List of Abbreviations

Abbreviation	
5,10-methyleneTHF	5,10-methylenetetrahydrofolate
5-methylTHF	5-methyltetrahydrofolate
ACh	acetylcholine
ACHY	S-adenosylhomocysteine hydrolase
AD	Alzheimer's disease
AI (behaviour)	anxiety index
AI (nutrition)	adequate intake
ANOVA	analysis of variance
BHMT	betaine-homocysteine methyltransferase
CBS	cystathionine β-synthase
CD	Control Diet
cDNA	complementary DNA
CDP	cytidine diphosphate
ChAT	choline acetyltransferase
CHD	congenital heart defect
ChDD	Choline-Deficient Diet
CHDH	choline dehydrogenase
CRL	crown rump length
DI	discrimination index
DMG	dimethylglycine
DNA	deoxyribonucleotide
DNMT	DNA methyltransferase
Dpc	days post coitum
E0.5	mouse embryonic age 0.5 days
E10.5	mouse embryonic age 10.5 days
E14.5	mouse embryonic age 14.5 days
EAR	estimated average requirement
FASD	Folic Acid-Supplemented Diet
FD	Folic Acid-Deficient Diet
GCPII	carboxypeptidase II
HCy	homocysteine
HHcy	hyperhomocysteinemia
Ig	immunoglobulin
IQ	intelligence quotient
IUGR	intrauterine growth restriction

MTHFR	methylenetetrahydrofolate reductase
MTR	methionine synthetase
MTRR	methionine synthetase reductase
NOR	novel object recognition
NTD	neural tube defect
OF	open field
PBS	phosphate-buffered saline
PC	phosphocholine
PE	phosphatidylethanolamine
PEMT	PE methyltransferase
PFA	paraformaldahyde
poly(A) tail	polyadenylic acid tail
PtdCho	phosphatidylcholine
RBC	red blood cell
RDA	recommended daily allowance
RNA	ribonucleic acid
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	standard error of the mean
SM	sphingomyelin
tHCy	total homocysteine
THF	tetrahydrofolate
TMAO	trimethylamine N-oxide
UTR	untranslated region
VLDL	very-low-density lipoprotein

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Format of Thesis

This thesis is written in the traditional format and is comprised of seven chapters. **Chapter I** contains a literature review of the topic, and the rationale, objectives, and hypotheses of the studies performed for this thesis are explained in **Chapter II**. **Chapter III** contains materials and methods for all studies. **Chapter IV** focuses on the reproductive studies performed using a low-choline diet and the *Mthfr*+/- murine model of mild MTHFR deficiency, as well as preliminary results of behavioural tests performed on low choline-fed *Mthfr*+/+ and *Mthfr*+/- females. **Chapter V** describes preliminary results of reproductive studies performed on the novel *Mthfr* 677 CC and *Mthfr* 677 TT mice and metabolite analyses of *Mthfr* 677 female mice. **Chapter VI** is a discussion of the data presented. **Chapter VII** concludes the thesis and discusses future directions.

Conventions

The names of genes are all italicised in this thesis. Human genes are uppercase (i.e., *MTHFR*) and murine genes are titlecased (i.e., *Mthfr*). Protein names are in normal font and are upper case for both human and murine forms (i.e., MTHFR). The *Mthfr* knockout mouse on a Balb/c background is represented with three possible genotypes and indicated as *Mthf*r+/+ (wild-type), *Mthfr*+/- (heterozygous knockout) or *Mthfr*-/- (homozygous knockout). "*MTHFR* 677C>T" indicates the human genetic *MTHFR* single nucleotide polymorphism variant. The novel mouse model representing this genetic variant, generated on a C57BL6/J background is indicated as "*Mthfr* 677" in this thesis and has two possible genotypes written as *Mthfr* 677 CC (wild-type) or *Mthfr* 677 TT (homozygous variant).

Contribution of Authors

The research and writing presented in this thesis (**all Chapters I-VII**) was performed and written by the candidate in assistance with Dr. Rima Rozen and members of her research group. The abstract for this thesis was translated into French by Dr. Daniel Leclerc. Dr. Rima Rozen provided editorial support for the chapters in this thesis. Figures representing folate and choline metabolic pathways in **Chapter I** were generated by the candidate and adapted from literature, as cited.

For **Chapters IV and V**, all murine colony maintenance tasks (routine weaning, genotyping) and reproductive experiments (tracking specialized diets, maternal and embryonic dissections, embryonic scoring, fixing and freezing of embryonic and placental tissues) were performed by the candidate, with the assistance of laboratory members (Dr. Karen Christensen, Miss. Yan Luan, and Dr. Marta Cosín-Tomàs) in exceptional cases.

For **Chapter IV**, maternal hepatic and cortical tissue from *Mthfr*+/+ and *Mthfr*+/- mice were collected by the candidate. Measurements of choline metabolites on these tissues were performed by Dr. Olga Malysheva from Dr. Marie Caudill's Laboratory at Cornell University in Ithaca, New York.

Morphological assessment of embryos for **Chapters IV and V** during dissection was performed twice: initially by the candidate and subsequently by Dr. Karen Christensen, who were both blinded to maternal genotype, embryonic genotype, and maternal dietary groups. Representative images of embryos used for this thesis were captured by Dr. Karen Christensen and edited by the candidate.

Behavioural experiments for **Chapter IV** were performed by the candidate, including placing mice on diets and measuring behavioural parameters. The mice used for behaviour tests were sacrificed by the candidate with assistance from Miss. Yan Luan.

Plasma and hepatic tissue for *Mthfr* 677 metabolite analysis for **Chapter V** was collected by the candidate and Dr. Karen Christensen. Measurements of folate metabolites in *Mthfr* 677 plasma were performed by members of Dr. Teodoro Bottiglieri's Laboratory at the Baylor Scott & White Health Research Institute in Dallas, Texas. Western blotting to assess hepatic MTHFR expression was performed and analysed by the candidate.

For both **Chapter IV and V**, all figures were generated by the candidate. Statistical analyses were performed by the candidate with assistance from Dr. Karen Christensen. R Scripts

used to assess embryonic delays, defects, and resorptions were originally written by Dr. Renata Bahous and Dr. Karen Christensen and were adapted by the candidate for these studies. Statistical analyses of embryonic crown rump length and somite measurements were done by Dr. Karen Christensen using IBM SPSS® Statistics. Interpretation of results was performed by the candidate in collaboration with Dr. Rima Rozen and Dr. Karen Christensen.

Chapter I: Literature Review

1.1 Folate

1.1.1 Folate Structure

Folate, also known as vitamin B9, is an essential water-soluble nutrient that was initially characterized for the treatment of megaloblastic anemia in the 1930s (1). The basic chemically stable structure of folate consists of a pteridine ring, p-aminobenzoic acid, and glutamate moieties (2). The term "folate" refers to various metabolic and oxidative states of this structure, as well as folic acid, the synthetic form of folate. Folates have a range of functions in the body and can act as coenzymes or cosubstrates for one-carbon metabolic reactions. The system of one-carbon metabolic pathways plays a crucial role for adequate nucleotide and protein synthesis, and methylation reactions (3).

1.1.2 Folate Intake and Absorption

Humans are unable to synthesize folate naturally; however small amounts of folate are generated by the intestinal microbiota which is absorbed by the intestinal wall for circulation. This phenomenon does not result in adequate levels for humans, and foods or supplements are the major source for folate (4). Leaf vegetables, such as spinach and asparagus, and animal products such as liver are high in folate. Folates that occur naturally in food have a polyglutamate tail, which requires conversion to a monoglutamate tail at the intestine using the enzyme carboxypeptidase II (GCPII) for absorption. In contrast, folic acid, found in synthetic supplements exists in a monoglutamate form and can be directly absorbed without enzymatic conversion (5).

1.1.3 Folate Metabolism

In the cell, folic acid is initially converted to 5,10-methylenetetrahydrofolate (5,10-methyleneTHF) through a series of reactions. 5,10-methyleneTHF can be used for purine synthesis or fed into the homocysteine remethylation pathway. The latter occurs when the cytosolic enzyme methylenetetrahydrofolate reductase (MTHFR) reduces 5,10-methyleneTHF to 5-methyltetrahydrofolate (5-methylTHF), the primary circulatory form of folate in humans. 5-methylTHF acts as a methyl group donor for methionine synthetase (MTR), which remethylates homocysteine to methionine. This reaction requires methionine synthase reductase (MTRR) and vitamin B12 for MTR function. Methionine is an essential amino acid used for protein synthesis and can be converted to S-adenosylmethionine (SAM), a ubiquitous methyl donor for DNA

methylation and the synthesis of neurotransmitters. SAM is used for the donation of methyl groups by methyltransferases, which forms S-adenosylhomocysteine (SAH). S-adenosylhomocysteine hydrolase (ACHY) can convert SAH back to homocysteine, propagating the homocysteine remethylation cycle (6) (**Figure 1**).

Choline, another essential nutrient, is used as an alternate for homocysteine remethylation in the folate-independent cycle. Choline dehydrogenase (CHDH) oxidises choline to betaine, which acts as a direct methyl donor for homocysteine in the reaction catalyzed by betaine-homocysteine methyltransferase (BHMT), generating dimethylglycine (DMG) and methionine. Choline plays important roles for the synthesis of cell membrane lipids and neurotransmitters, which will be discussed in subsequent sections. The folate and choline cycles further link as 3 molecules of SAM are used to convert phosphatidylethanolamine (PE) to phosphatidylcholine (PtdCho) by PE methyltransferase (PEMT). PtdCho is converted back to choline, which can be fed into the betaine-dependent cycle for homocysteine remethylation (7) (**Figure 2**). Folate-dependent homocysteine remethylation occurs in all tissues whereas the betaine-dependent cycle is primarily in liver (8).

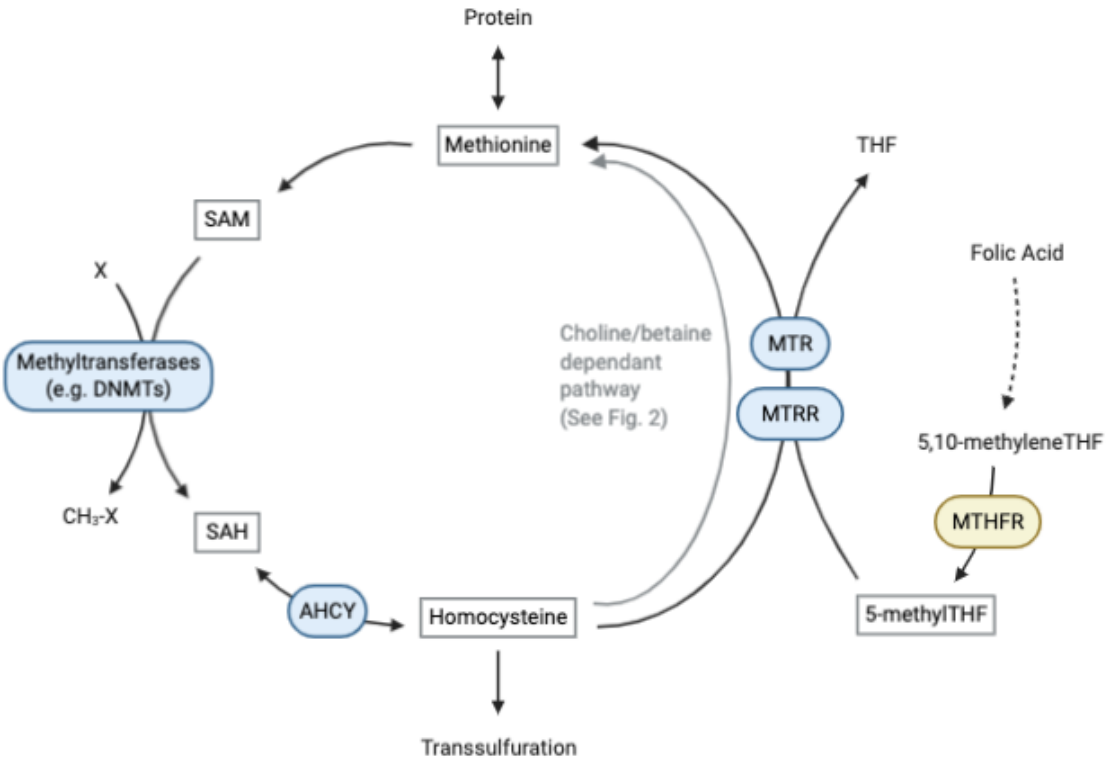

Figure 1. Folate in one-carbon metabolism and homocysteine remethylation. Enzymes are indicated in solid blue ovals, with MTHFR highlighted in yellow, and key metabolites are shown in open boxes. Legend: AHCY: S-adenosylhomocysteine hydrolase; DNMT: DNA methyltransferase; MTHFR: methylTHF reductase; MTR: methionine synthase; MTRR: MTR reductase; SAH: S-adenosylhomocysteine; SAM: S-adenosylmethionine; THF: tetrahydrofolate. Figure modified from (6).

1.1.4 Methylenetetrahydrofolate reductase (MTHFR)

The *MTHFR* gene encodes for metabolic enzyme methylenetetrahydrofolate reductase (MTHFR; EC 1.5.1.20). The prokaryotic enzyme, encoded by the gene *metF* in *Escherichia coli (E. coli)*, has functional roles in methylation and one-carbon metabolic pathways (9). Little was known about the eukaryotic gene and enzymatic structure and function until the past few decades. Early studies of MTHFR and its role in human disease revealed that the disorder homocystinuria, an inborn error of folate metabolism, was associated with severe MTHFR enzymatic deficiency (10). In the mid-90s, the *MTHFR* cDNA was isolated by the Rozen Laboratory and has been extensively studied to understand the genetic aberrations that result in MTHFR-related disease. Initially, pig liver MTHFR was purified and analyzed for the primary polypeptide sequence. A corresponding porcine RNA partial sequence, 90 nucleotides in length (11) was used to probe for the *MTHFR* cDNA in a human liver library and resulted in the isolation of a 1.3 kb product, exhibiting homology to both the porcine *MTHFR* and *metF* sequences. This partial product was hybridized with a separate human cDNA library to find the C-terminus, 3'UTR, and polyA tail. Subsequently, the Rozen Laboratory identified 2 isoforms of the *MTHFR* protein, a 77 kDa product, as well as a 70 kDa catalytically active isoform. An alternative splicing site at the N-terminus, which is conserved between humans and mice, results in a translational start site in the mRNA which codes for the 77 kDa product (12).

1.1.5 Deficiencies in MTHFR and Hyperhomocysteinemia

Homocysteine (HCy) is a non-proteinogenic sulfur amino acid, generated from methionine donating its methyl group. Normal homocysteine metabolic pathways ensure that its levels are not elevated in the body. On average, the plasma normally contains 10 μmol/L homocysteine, as higher levels can be detrimental (13). Homocysteine can be fed into the folate- or betaine- dependent remethylation pathways to methionine, as described above. Alternatively, the homocysteine transsulfuration pathway converts it to cysteine by the enzymes cystathionine β-synthase (CBS) and cystathionase (6,14). While remethylation of homocysteine occurs in most tissues, transsulfuration occurs in a few tissues, such as the liver. Disturbances in the detoxification of homocysteine, such as genetic mutations that result in folate deficiency, can result in elevated levels of homocysteine in the body. Hyperhomocysteinemia (HHcy) is a condition characterized by high levels of homocysteine in blood plasma, and is milder than homocystinuria, in which elevated levels are observed in blood and urine (15). High

homocysteine levels have been found to be a risk factor for conditions such as cardiovascular (16) and neurological diseases (17), and increased pregnancy complications (such as gestational hypertension or pre-term birth) (18). Homocysteine may disturb normal cell function in high levels, as it has been found to increase cellular oxidative stress and promote apoptosis (19). Furthermore, high homocysteine may disturb methylation reactions, possibly impacting the methylation status and epigenetic signature of genes, as well as protein function (20). Genetic aberrations or deficiencies in vitamins involved in folate pathways can result in homocysteine elevation. Genetic mutations in *MTHFR* and other enzymes in homocysteine metabolism (i.e. CBS, MTR) may result in the inborn error homocystinuria, a severe form of HHcy (21). Symptoms often first appear during childhood and can cause detrimental effects to the cardiovascular and neurological systems, as well as development (22). While multiple single nucleotide polymorphisms (SNPs) in *MTHFR* have been discovered over the decades, the 677C>T and 1298A>C variants are most commonly studied (23).

A point mutation at the nucleotide position 677 of *MTHFR* resulting in a nonsynonymous cytosine to thymine substitution (C677T) converts the normal alanine amino acid to a valine. The homozygous 677 TT signature was discovered to be a risk factor for mild HHcy and found to be present in 10-15% of many populations, such as North Americans whites, many European populations and east Asians (12,21). Some populations with Spanish/southern European roots (such as Hispanic Americans), were observed to have up to 25% prevalence (24). The Rozen Laboratory discovered that this mutation impacts the enzymatic stability of MTHFR and renders it thermolabile when treated with heat at 46°C. MTHFR extracted from lymphocytes from patients with coronary artery diseases was also observed to be thermolabile.. However, the genetic change, 677 C>T, was discovered after the initial thermolability finding. (25). Heat treatment of MTHFR from 677 TT individuals resulted in residual enzymatic activity of 30% of the control samples. The heterozygous 677 CT signature resulted in an intermediate decrease of activity. At body temperature (37°C), the mutated MTHFR in 677 TT individuals was also found to have reduced specific activity (~35% of controls) (12). Homozygous individuals were also found to have reduced plasma folate levels, as MTHFR produces 5-methylTHF, the major folate form. Furthermore, the reduction in 5-methylTHF resulted in elevated homocysteine due to inadequate remethylation of homocysteine (26). Inadequate folate consumption coupled with genetic deficiency of MTHFR also increases homocysteine levels. It is likely that increased

folate in diet or folate supplementation may cause stabilization of MTHFR enzymatic activity, allowing for the generation of 5-methylTHF (27). The clinical significance of folate supplementation for 677 TT individuals is promising and could help relieve the symptoms associated with HHcy.

The *MTHFR* 1298A>C variant is characterized by a missense SNP at position 1298, which results in glutamate to alanine amino acid conversion. In contrast to the more common 677C>T mutation, this variant does not result in a thermolabile enzyme; however enzymatic activity is reduced in human lymphocytic extracts. While homozygosity for the 1298A>C SNP does not seem to increase homocysteine levels, co-heterozygosity of both the 677C>T and 1298A>C variants may result in an increased risk for HHcy. (28,29).

1.1.6 MTHFR Deficiency and Pregnancy Complications

Folate deficiency can be clinically diagnosed through assessment of plasma and red blood cell (RBC) folate levels, which represent short- and long-term consumption, respectively. Less than 10 nmol/L of plasma folate or 340 nmol/L of RBC folate is considered deficient (30). However, pregnancy could have greater demands for folates due to its important roles in placental and embryonic development. Inadequate maternal folate during the perinatal and gestational periods of pregnancy have been found to be associated with increased risk of birth defects, such as neural tube defects (NTD) (31), congenital heart defects (CHD) (32), and low offspring birthweight (33). Furthermore, studies have found that there is a role in maternal folate supplementation to reduce the risk of NTD by 50-75% (34). The optimal RBC folate level in women of reproductive age is considered to be 906 nmol/L to reduce the risk of NTD in offspring (35). Since the 1990s, mandatory folate fortification of grains and cereal was adopted in Canada and United States, and now in over 50 countries worldwide, to help increase folate intake. Furthermore, maternal supplements for the perinatal and prenatal period contain folate to reduce the risk of NTD (30). However, for individuals that have genetic MTHFR deficiency, such as those with the *MTHFR* 677 TT signature, the current standards for dietary supplementation may not be enough. Alternatively, individuals with genetic MTHFR deficiency may also be sensitive to excessively high folate intake. Recent murine studies from the Rozen Laboratory showed that excessive (10-times higher than the recommendation for rodents) maternal folate supplementation negatively impacted offspring neurodevelopment, impaired memory, and reduced MTHFR expression in maternal and offspring livers, indicating pseudo-

MTHFR deficiency (36). Mice with high folate supplementation and MTHFR deficiency may be at greater risk because of a double hit with respect to MTHFR deficiency. The amount of folate required for MTHFR-deficient women during pregnancy has not been specifically determined. Studies of risk reduction by folate for NTD and other pregnancy complications have not looked at the various *MTHFR* genotypes. Defining the upper and lower limits to reduce pregnancy and developmental complications is important.

One of the major roles of folate is indirectly allowing for adequate methylation due to SAM production. Folate and MTHFR deficiency can result in reduced SAM levels, which may impact the maintenance of epigenetic markers during development (6). SAM is primarily used by DNA methyltransferases (DNMTs), a group of enzymes responsible for the transfer of methyl moieties onto DNA. DNMTs are sensitive to the levels of SAM and SAH (S-adenosylhomocysteine), and high levels of SAH result in a feedback inhibition of DNMT activity. Individuals with HHcy may have increased SAH hydrolase activity, which is the enzyme that produces SAH from homocysteine. Increased SAH, subsequently, could inhibit DNMT activity, promoting a hypomethylated environment (37). Reduced DNA methylation often results in increased gene expression, and folate deficiency is associated with global (genome-wide) hypomethylation (38). Furthermore, during early embryonic development, a genome-wide reduction of methylation occurs in the early zygote which is then followed by DNA remethylation (39). Inadequate SAM levels at this time could result in aberrations in methylation patterns and dysregulation of genes during embryogenesis.

1.1.6.1 The Mthfr Knockout Mouse

Exploring the impact of nutrition and diet in patients with MTHFR deficiency is important but can be complicated due to the limitations of clinical research using humans. Murine models, due to their genetic and reproductive similarities and fast gestation periods, are important to understanding genetic deficiencies. The structure of the *MTHFR* gene in humans is similar to the gene in mice, and the resulting MTHFR protein has high sequence homology (~90%) (40). The Rozen Laboratory initially generated a mouse model with a defective *Mthfr* gene. A targeted disruption, through the insertion of a *neo* cassette, of exon 3 was performed to inactivate the *Mthfr* gene. Homozygous (*Mthfr*-/-) mice did not have any detectable mRNA, and heterozygous (*Mthfr*+/-) mice had reduced expression relative to wild type (*Mthfr*+/+) mice. These mice also had reduced MTHFR specific activity, while homozygous mice had no

detectable enzyme activity. Phenotypic analyses of *Mthfr*-/- mice on a BALB/c background showed increased mortality, with the majority not surviving past weaning age (21 days). Homozygous mice that died young also had low body weights, abnormalities in motor and gait, as well as tremors. However, heterozygous mice and wildtype mice showed no differences in mortality and lived to healthy ages (41). *Mthfr*-/- pups also had a higher incidence of shorter and kinked tails, which mirror the phenotype observed in the curly tail (ct) mouse, a model for neural tube defects (42). In terms of neural development, homozygous mice also showed smaller cerebra at a young age. At old age, homozygous mice (that survived) and heterozygous mice both exhibited lipid deposition in the aorta, indicating atherogenic effects of MTHFR deficiency (41).

Hyperhomocysteinemia was observed in both *Mthfr*+/- and *Mthfr*-/- mice, as plasma levels of total homocysteine were increased in these mice when compared to wild-type. Furthermore, 5-methylTHF and SAM levels in these mice were decreased, and SAH was increased, indicating disturbances in the folate metabolic cycle and methylation potential. The homozygous *Mthfr*-/- mouse exhibits the variability of pathological phenotypes observed in humans with homocystinuria, or severe genetic folate deficiency. Homocystinuric patients have higher risk of neurological issues, developmental delay, and motor/gait issues, which is mirrored in the homozygous mice, indicating the importance of MTHFR expression for development. Heterozygous mice were phenotypically similar to humans with mild MTHFR deficiency, as seen in the *MTHFR* 677 TT genetic profile, due to the presence of hyperhomocysteinemia and decreased MTHFR activity (41).

Various reproductive studies have been performed on the *Mthfr*+/- mice to elucidate how genetic MTHFR deficiency impacts pregnancy outcomes. Dietary-gene studies have also shown the importance of folate levels during development. Pregnant *Mthfr*+/- females fed low folate have embryos with higher incidence of heart defects at gestational day 14.5, as well as higher rates of resorption (fetal death) and developmentally delayed embryos (43). Similarly, excessive folate in the diets of pregnant *Mthfr*+/- mice resulted in adverse effects as well, such as a higher rate of congenital heart defects in embryos, as well as increased embryonic loss and delay (44). Excessive dietary folate was also shown to result in similar reproductive abnormalities due to pseudo-MTHFR deficiency (45). MTHFR activity is partially modulated by phosphorylation, as the non-phosphorylated form is the more active isoform. In the liver of high folate-fed females,

phosphorylation of MTHFR is increased, rendering the enzyme less active. Hepatic expression of MTHFR also decreases due to excessive dietary folate, reducing the levels of 5-methylTHF and SAM. PtdCho levels are also reduced in these mice due to altered lipid metabolism, resulting in liver damage (46). High-folate fed *Mthfr*+/- mothers also have embryos with developmental delay, pups with altered choline metabolism in the brain (resulting in memory impairment), and pseudo-MTHFR deficiency (36). These murine studies reiterated the importance of defining a range and upper limit of folate intake for individuals with genetic MTHFR deficiency (677 TT profile).

Strain specific differences have been observed, as the *Mthfr* knockout model on a C57BL/6J background has less severe phenotypes than the BALB/c counterpart. C57BL6/J *Mthfr*-/- mice have better survival and a reduction in some pathological outcomes (47). However, both strains show similarities in the sense that *Mthfr*+/- littermates represent mild MTHFR deficiency, while the *Mthfr*-/- counterparts exhibit severe deficiency.

1.1.6.2 The Mthfr 677C>T Mouse

A mouse model with the exact SNP observed in humans (*MTHFR* 677C >T variant) was generated recently by collaborators from the Jackson Laboratory (Howell, Gareth/JAX). The stability of the mutated and thermolabile MTHFR protein in humans is restored by folate supplementation (12). This phenomenon has not been observed in the *Mthfr*+/- mouse model, which does not carry a thermolabile mutant enzyme, indicating the importance of a novel model that may be more accurate to studying human folate metabolism. Recently, the Rozen Laboratory has been performing enzyme activity and thermolability assays to study MTHFR kinetics in this new model. Furthermore, novel experiments to characterize the model were performed in this project to elucidate its importance for understanding reproduction outcomes related to MTHFR deficiency.

1.1.7 Folate and Neural Tube Defects (NTD)

The neural tube is the embryonic structure that eventually develops into the brain and spinal cord in vertebrates. The precursor to the neural tube is the neural plate, which is a sheet of epithelial cells that must undergo migration and differentiation to form the tube. In humans, the neural plate begins to close on gestational day 21 and is complete around day 42 (48). It is important to note that often many women do not know that they are pregnant when neurulation begins, as this can coincide with the first week after their expected menstruation (49). Inadequate

neural tube closure during development may result in neural tube defects (NTD), which can cause congenital deformities such as spina bifida or anencephaly. The frequency of NTD is 1-5 in 1000 live births. However, in countries or areas where inadequate maternal nutrition is of concern, rates of NTD may be higher (50). Since the neural tube closure period is so early in pregnancy, and often when the mother is not aware of pregnancy, adequate folate levels during the periconceptional (before gestation) period are important. Folate supplementation (4 mg/day) during the periconceptional period is thought to prevent up to 75% of NTD; however, as little as 0.4 mg/day supplementation has been demonstrated to provide protective effects against NTD (51). As mentioned above, these NTD studies did not examine *MTHFR* genotypes.

Neurulation is divided into two major stages. The primary stage, which occurs from days 21-28 of gestation, results in the formation of the brain and the upper spinal cord. Defective neural tube closure at this stage often results in an "open" neural tube (49). These can manifest as conditions such as anencephaly, a severe birth defect in which a child is born with absent portions of the brain, skull, and scalp, resulting in almost immediate death (52). Another condition, myelomeningocele, is a type of spina bifida which results in an incomplete development of the spine and is characterized by the neural tissue being contained in a meninges-covered sac (53). Secondary neurulation starts around gestational day 35 and ends at day 42, creating the lower part of the spinal cord. Defects occurring from errors in this stage result in "closed" NTDs, such as occult spina bifida (49). These NTD are not as severe as open NTD.

In mice, neurulation occurs similarly, beginning at gestational day 8.5 at the caudal region and completing at day 10.5 with closure at the upper sacral region (54). Brain development in the offspring of *Mthfr*+/- mice is altered when dietary folate or choline is low. These offspring have increased hippocampal apoptosis and decrease hippocampal volume, and short-term memory loss (55). The importance of folate and choline metabolites for the developing brain is not fully elucidated. However, these mice models may prove to be useful in studying their effects.

1.1.8 Folate, Intrauterine Grown Restriction (IUGR), and Low Birth Weight

Poor maternal nutrition, especially of concern in developing countries or areas of low socioeconomic status, is correlated with low birth weight of offspring. Intrauterine growth restriction (IUGR) occurs when the estimated weight of developing fetus is lower than the

expected weight for its gestational age. IUGR can result in babies born with a lower birth weight than normal. A low folate status and HHcy in mothers has been found to be associated with increased risk of IUGR (56). However, excessive folate intake, such as a recent study in a Dutch cohort, was also found to be linked to low fetal weight (57).

1.2 Choline

1.2.1 The Importance of Choline

Choline is an essential nutrient that can be generated de novo in humans, but often is needed through external sources due to its critical role in metabolite formation. The major pathway to generate choline is the phosphatidylethanolamine N-methyltransferase (PEMT) pathway in the liver (58). Choline has three major fates, which are oxidation to betaine (a methyl donor), acetylation to form acetylcholine (ACh), a major neurotransmitter and neurodevelopmental metabolite, or its phosphorylation to phosphatidylcholine (PtdCho), a cellular membrane component (59). Choline is linked to the folate one-carbon cycle through its oxidation to betaine, which acts as an alternate methyl donor to homocysteine when folate levels are low (6). Studies have shown that betaine expression increases during folate deficiency (60). The enzyme choline dehydrogenase (CHDH) is responsible for converting choline to betaine in hepatic and renal tissue (61). Betaine homocysteine S-methyltransferase (BHMT) then uses betaine to remethylate homocysteine back to methionine and also produces dimethylglycine (DMG). DMG can be demethylated to use as a methyl group donor for SAM-dependent DNMTs (**Figure 2**). These pathways indicate a role for choline in maintaining epigenetic methylation patterns. During MTHFR deficiency, the demand for choline-dependent methyl donation increases (6).

The importance of choline for cell membranes is shown by the role of its derivative PtdCho, which exists in all cellular lipid membranes. PtdCho is responsible for maintaining fluidity of plasma membranes (particularly synaptic cell membranes), as well as cellular proliferation. PtdCho is also used for the synthesis of very low-density lipoproteins (VLDLs) for the export of lipids in liver tissue. Low choline was found to result in leaky cell membranes, characterized by increased cell permeability (59,62). Furthermore, individuals with low choline intake may be at higher risk for fatty liver due to the lack of PtdCho available for lipid transport through lipoproteins (63). PtdCho can also donate its phosphocholine moiety to ceramide which forms sphingomyelin (SM) (59), one of the components of the myelin sheath, required for axonic

insulation and nerve signal transmission in nervous system cells (64). Choline plays other major roles in the nervous system, particularly through its metabolite ACh. In the central nervous system, cholinergic receptors have been found in the forebrain, cerebral cortex, and hippocampus, and maintain their cognitive functions (65). Alternatively, in the peripheral nervous system, ACh acts as a major neurotransmitter for the autonomic nervous system as well as a skeletal muscle cell activator (66).

1.2.2 Choline Intake and Metabolism

Choline can be naturally obtained through various animal and plant sources. The major form of choline in foods is PtdCho. Other choline metabolites such as SM, phosphocholine, glycerophosphocholine, and lysophosphatidylcholine, as well as free choline, can also be obtained in smaller quantities. In the body, these metabolites are interchangeable and constitute the total choline intake (67). Dietary betaine, however, is not a choline source as there is no conversion pathway of betaine to choline (68). Once choline is taken into the body, free choline, phosphocholine, glycerophosphocholine all enter through the bloodstream, as they are water-soluble. The lipid-soluble choline metabolites, such as PtdCho, SM, and lysophosphatidylcholine, are taken up by chylomicrons, small protein and lipid globules that transport fatty compounds from the intestine into the liver (60).

Choline metabolism occurs primarily in liver tissues; ACh production can occur in neurons and placental cells (69). In the liver, choline is initially fed into the cytidine diphosphate (CDP)-choline pathway, leading to production of PtdCho. The “spillover” pathway, which occurs when choline levels are high, is the oxidation of choline into betaine, which is an irreversible process. SAM, which is produced from methionine, can be fed back into the PEMT pathway, resulting in *de novo* choline synthesis. In this pathway, SAM donates methyl groups to phosphatidylethanolamine, producing PtdCho (70).

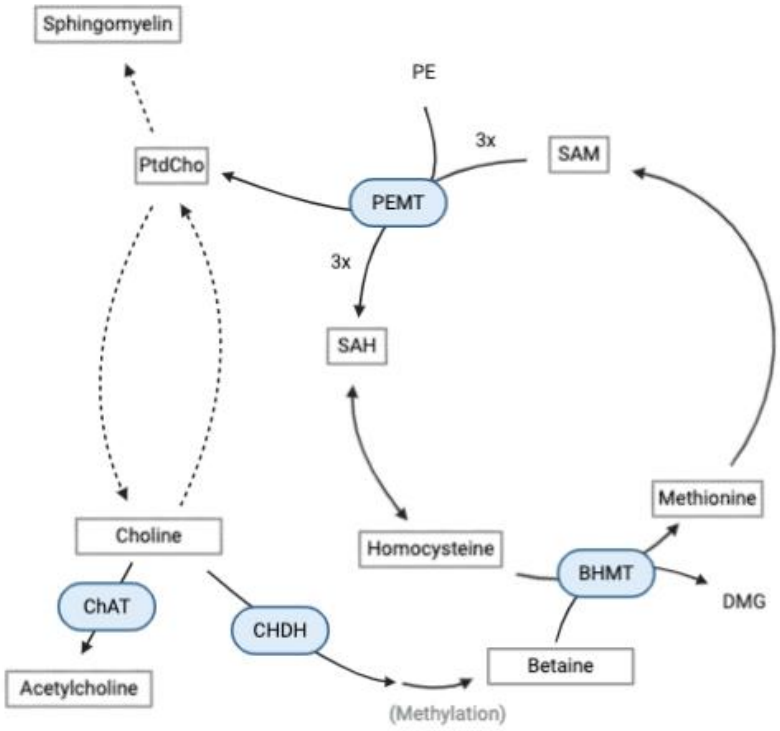

Figure 2. Choline metabolism and its link to folate through the homocysteine remethylation pathway. Enzymes are indicated in solid blue ovals, and key metabolites are shown in open boxes. Legend: BHMT: betaine-homocysteine methyltransferase; ChAT: choline acetyltransfease; CHDH: choline dehydrogenase; DMG: dimethylglycine; PE: phosphatidylethanolamine; PEMT: PE methyltransferase; PtdCho: phosphatidylcholine; SAH: S-adenosylhomocysteine; SAM: S-adenosylmethionine. Figure modified from (71).

The recommended amounts of choline intake vary depending on age, sex, and reproductive status (gestation or lactation). In the late 90s, the National Academies of Medicine put forth a dietary adequate intake (AI) level for healthy adults. This was determined by the amount of choline that was required to prevent an increase in hepatic expression of enzymes that result in liver damage. These values were determined to be 550 mg/day for adult men and 425 mg/day for adult women. During pregnancy, the demand for choline and its derivatives is likely higher, and the recommended AI is 450 mg/day during gestation and 550 mg/day for lactation (72). Animal products, such as beef, egg yolks, poultry, and pork can provide at least 60 mg choline per 100 g. Plant sources such as nuts, legumes, and leafy greens contain over 25 mg choline per 100 g (67). A limitation of AI values is that they are set when there is not sufficient data to determine an estimated average requirement (EAR). EAR is defined as the median intake value required to meet the nutritional demands of half of the healthy individuals in a population. It is used to calculate the recommended dietary allowance (RDA), which is the daily intake level to meet at least 97 percent of healthy individuals in a population. An AI, in contrast, is the recommended daily level in apparently healthy individuals that are assumed to have adequate nutritional levels (73). Issuing an AI for a particular nutrient instead of an EAR or RDA indicates that data are limited and there is need for further epidemiological research to elucidate the required intake for a specific nutrient. Research involving the importance of choline is relatively recent, and thus requires further study to understand its dietary value.

1.2.3 Choline and Pregnancy/Development

The demand for choline is seemingly increased during pregnancy due to its role in fetal development. Choline metabolites such as PtdCho and SM are needed in large quantities to keep up with the rates of cell division, nervous cell myelination, and growth required in a developing fetus (74). During development, ACh also acts on the developing brain as it is involved in pathways for brain cell proliferation and differentiation, neurodevelopment, and cell migration, among other roles. ACh allows for development of the hippocampus, which functions in learning and memory (75). Choline also plays important roles in placental and fetal epigenetic regulation due to its connection to the methylation pathways that result in SAM production (76). Maternal murine studies showed that low dietary choline in *Mthfr*+/- mice impacts neurodevelopmental outcomes in offspring (55). A genetic model for severe MTHFR deficiency, the *Mthfr*-/- mouse, was found to have increased expression of choline acetyltransferase, which converts choline to

ACh. This may be because these mice have reduced ACh in the hippocampus and increased enzymatic expression may be an attempt to compensate (77).

As choline is an important source for methyl groups, it is also required during development to ensure epigenetic methylation patterns in the placental and embryonic genomes. Altered choline intake could potentially impact gene expression patterns in the growing embryo (78). Aberrations in epigenetic programming during development may have lasting impacts on offspring growth and brain function. Maternal choline supplementation in murine models showed that there may be reduced risk of disease in offspring, and that choline modulates epigenetic programming during pregnancy (79). Maternal choline may also be important in regulating offspring stress response (modulation of cortisol-related pathways that may pose an increased risk of stress-related conditions, *e.g.* autoimmune disorders, or metabolic diseases, *e.g.* diabetes, in adulthood), as neonates born to mothers with adequate choline had lower levels of cord blood cortisol levels (80). Neonatal stress has lasting impacts later in life, such as increased risk of mental health disease such as depression and compromised immunological outcomes (81).

During development, adequate placental angiogenesis and differentiation is crucial to ensure that the growing embryo has access to maternal oxygen and nutrients for growth. Aberrations in placental vascularization may lead to increased risk of offspring IUGR and preeclampsia, a condition that results in poor developmental outcomes and risk of seizures to mothers (82). Increasing choline supplementation may help proper development of the placenta due to its influence on angiogenesis and placental cell function. A study using a mouse model of placental insufficiency, characterized by inadequate placental nutrient/oxygen transport, showed that maternal choline supplementation improved fetal developmental outcomes by improving placental morphology (83,84).

The influence of choline on neurodevelopment has been studied in rodent models. High gestational choline was founded to improve cognitive and memory outcomes of offspring during adulthood (85). Rodent studies also show that increased perinatal choline may be important to reduce risk of neurological conditions such as Alzheimer's disease (AD) (86), autism (87), and Down syndrome (88). Early-onset AD is associated with lower cholinergic neurons in the brain; maternal choline supplementation in a mouse model for AD improved the number of cholinergic neurons in the offspring brain (89). Maternal choline supplementation in mice that are a model

for Down syndrome resulted in offspring with better spatial memory and hippocampal development, as well as a permanent upregulation of PEMT and increased PtdCho in the brain (90). Porcine models have also shown that low maternal choline during pregnancy results in lower offspring brain weight and volume during early stages of life (91). In humans, prospective studies have been performed to understand the role of maternal choline on infant cognition. One study found that at week 16 of gestation, levels of choline and betaine in maternal plasma positively correlated with cognitive test results in 18-month old infants (92). Another study showed that 7-year old children born to mothers in the top quartile of choline intake (~400 mg/day) during gestation had better visual and color-location memory than mothers in the bottom quartile (~250 mg/day) (93). While choline may be important for some cognitive functions during development, it might not be necessary for all functions. Some maternal choline studies have found no association with the intellectual quotient (IQ) scores (94) or visual-motor ability of children (95). These findings indicate that the role of choline during gestation and early development is not well understood, and further provides rationale for why dietary choline studies during reproduction are still essential.

While the AI for pregnancy has been set at 450 mg/day, studies have found that only 10% of women in certain populations meet the daily AI (96). The recommendation of 450 mg/day is contested as well, as many studies that doubled choline intake during pregnancy (930 mg/day vs. 480 mg/day) improved neurological outcomes in offspring, placental DNA methylation, and neonatal stress (59). How much choline is actually required for better outcomes is not completely understood as of yet, and thus requires more human and mouse model reproductive studies. Regardless, it is known that many women do not receive enough choline daily. Furthermore, while folate is found in periconceptional and gestational supplements, choline is not. Improving the choline intake for women of reproductive age, as well as during pregnancy and lactation, may help promote healthy development.

Chapter II: Thesis Rationale, Objectives, and Hypotheses

2.1 Reproductive outcomes due to mild maternal MTHFR deficiency (*Mthfr*+/- mouse) and low dietary choline

While folate intake during pregnancy and the periconceptional period has been studied extensively and shown to improve gestational and offspring developmental outcomes, choline is still an understudied nutrient. Studies have shown that choline may be important during pregnancy. However, when the folate metabolic cycle is disturbed (such as in *MTHFR* 677 TT females), the demand for choline may be higher due to its use as a source for betaine and methyl donation (60). Choline's role in the developing brain is not completely elucidated as well. The Rozen Laboratory has previously shown that low maternal dietary choline and *Mthfr*+/- genetic status in mice results in memory impairment and poor neurodevelopment outcomes in pups (55). However, the importance of choline for embryonic neural development is not known. When maternal folate metabolism is deficient (*Mthfr*+/- mouse), the role of choline for neural tube closure (embryonic day E10.5) is unknown and thus should be studied. For this study, **Objective I** was to determine the impact of mild maternal MTHFR deficiency and low dietary choline on pregnancy outcomes and embryonic development. I **hypothesized** that pregnant *Mthfr*+/- females fed low choline diet will have increased risk of embryonic defects (such as NTD) and pregnancy complications, such as increased resorptions or implantation difficulties, due to their higher dependence on choline for HCy remethylation.

2.2 Reproductive outcomes due to low and supplemental maternal dietary folate in the novel *Mthfr* 677 TT mouse

Reproductive outcomes due to MTHFR deficiency and poor maternal nutrition are not completely understood as of yet. While the *Mthfr*+/- mouse model has been used extensively for reproductive studies and has been characterized as a model for human MTHFR deficiency (41), a model with the exact *MTHFR* 677C>T variation seen in humans has not been available until recently and therefore has never been studied for reproductive outcomes. For the first time, the *Mthfr* 677 TT mouse will be examined in reproductive studies. As it is known that human MTHFR with the 677 mutation is stabilized by folate (25,97), which is not possible in the *Mthfr*+/- model, understanding outcomes in the novel model is important to translate results to humans. In this study, for **Objective II**, the aim was to evaluate the effect of dietary folate and MTHFR deficiency on reproductive outcomes in the *Mthfr* 677 TT mouse model. I **hypothesized**

that low dietary folate and genetic deficiency of folate metabolism in *Mthfr* 677 TT pregnant females will increase risk of embryonic defects and pregnancy complications whereas supplemental folate will stabilize MTHFR and rescue this phenotype.

Chapter III: Materials and Methods

3.1 Animal Experimentation

Experimentation on mice was performed in accordance with the Canadian Council on Animal Care guidelines, and animal protocols were approved by the Animal Care Committee at The Research Institute of the McGill University Health Centre. All mice had *ad libitum* food and water and were held in facilities with 12-hour light/dark cycles. Animal euthanasia was performed using isofluorane general anesthesia followed by CO_2 asphyxiation as outlined by McGill Animal Care Standard Operating Procedure (SOP 301.03 – Rodent Euthanasia, McGill University).

Mthfr knockout mice, on a BALB/c background, and genotyping protocols used for this study had previously been generated and characterized (41). For colony maintenance, male *Mthfr*+/- mice were mated with female *Mthfr*+/+ mice to generate offspring of two genotypes (*Mthfr*+/+ and *Mthfr*+/-). These mice were fed commercial Global 18% Protein Rodent Diet 2018 (chow) (Teklad Diets, Madison, WI, USA). *Mthfr*+/+ and *Mthfr*+/- females used for experimentation were weaned at 3 weeks. At 4 weeks of age, they were put on a Control Diet (CD) (2 mg folic acid/kg diet; 2.5 g choline bitartrate/kg diet) (TD.01369, Envigo Teklad Diets, Madison, WI, USA) or a Choline-Deficient Diet (ChDD; 3-fold lower levels of choline than recommended) containing 2 mg folic acid/kg diet; 0.8 g choline bitartrate/kg diet) (TD.180221, Envigo Teklad Diets, Madison, WI, USA). Female mice used for embryonic studies were placed on diet for 4 weeks, at which point, mating was performed with male *Mthfr*+/+ mice. For behavioural studies, 4-week old *Mthfr*+/+ and *Mthfr*+/- females were placed on diet for 5 weeks, at which point behaviour tests were performed, and sacrificed after completion for collection of plasma, liver tissue, and brain cortices, which were frozen at -80°C.

Mice with the *Mthfr* 677C>T variant and accompanying genotyping protocols were generated by a collaborator (Howell G., JAX) on a C57BL/6J background. For colony maintenance, *Mthfr* 677 CT males and females were mated for progeny with three genotypes (*Mthfr* 677 CC, *Mthfr* 677 CT, and *Mthfr* 677 TT). Colony breeding mice were fed Global 18% Protein Rodent Diet 2018 (chow) (Teklad Diets, Madison, WI, USA). For reproductive experiments, *Mthfr* 677 CC and *Mthfr* 677 TT females were weaned at 3 weeks of age and put on three possible diets: Control Diet (CD) (2 mg folic acid/kg diet) (TD.01369, Envigo Teklad Diets, Madison, WI, USA), 2.5-fold greater folic acid than recommended, Folic Acid-

Supplemented Diet (2.5x FASD) (5 mg folic acid/kg diet) (TD.190221, Envigo Teklad Diets, Madison, WI, USA), or 2.5-fold lower folic acid than recommended, Folic Acid-Deficient Diet (FD) (0.8 mg folic acid/kg diet) (TD.190220, Envigo Teklad Diets, Madison, WI, USA). After 4 weeks on diet, mating was done with *Mthfr* 677 CC males.

All mice that were used for embryonic studies and mated with a male were observed for the presence of a vaginal plug, which indicated 0.5 days post coitum (dpc, E0.5). At 10.5 days post coitum, pregnant females were sacrificed, and maternal plasma, liver, and brain cortices were collected and frozen at -80°C. Uteruses were collected to dissect placentas and embryos. Placenta were either fixed whole in 4% paraformaldehyde (PFA) in 1X PBS or the maternal and fetal sides were separated to weigh and freeze the fetal placental tissue at -80°C. Embryonic yolk sac was collected to extract DNA for *Mthfr* genotyping, and embryos were scored for E10.5 developmental markers and defects. Some embryos were fixed in 4% PFA in 1X PBS while the rest were frozen at -80°C.

For all studies, full body weights of mice were collected at the start and end of diets and during sacrifice. Mice used for embryonic studies were additionally weighed at the 4-week time point and E0.5 (day of vaginal plug) for measurements of consumption during pregnancy. At sacrifice, weights of whole liver and brain were collected, as well as whole uterus weight for reproductive experiments. The number of corpora lutea on the left and right ovaries were counted as a measure of egg releases.

3.2 Behavior Testing

Behavioral studies were performed at one behavior experimentation room at the Vivarium of the Research Institute of the McGill University Health Centre. All mice were acclimated to the experimentation room for 30 minutes prior to the start of the experimentation. Behavioral and movement patterns were recorded and analyzed using the ANY-maze tracking software (Stoelting Co., Chicago, IL, USA).

3.2.1 Open Field Test

Mthfr+/+ and *Mthfr*+/- females used for behavior studies were analyzed for locomotor activity and anxiety in the open field (OF) test (98). An opaque Plexiglas OF box was disinfected, and a mouse was placed in the centre and recorded with a video camera for 5 minutes.

3.2.2 Novel Object Recognition Test

The novel object recognition (NOR) test can be used for analyses of murine short-term memory (99). A 10-minute habituation period to the OF box was done 24 hours prior to the NOR test. During the trial phase of the experimentation, a mouse was placed in the centre of the box containing two identical objects (object 1) placed at opposite positions and recorded for 10 minutes, then returned to the housing cages. After 60 minutes, the mouse was placed back into the OF box containing the familiar object (object 1) and a novel object (object 2) placed at opposite corners and recorded for 5 minutes. The amount of time spent exploring the objects was recorded in both phases. The discrimination index (DI) (100) is a measure of how much time was spent exploring the novel object compared to the familiar object and was calculated using the formula:

$$Discrimination\ Index = \frac{Time\ (Novel) - Time\ (Familiar)}{Time\ (Novel + Familiar)} \qquad Eq.\ 1$$

A positive discrimination index value indicates greater time with the novel object (as expected for a control mouse) and a negative value implies greater time spent with the familiar object.

3.3. Western Blotting

Western blotting was performed using crude hepatic protein extracts to assess MTHFR expression. 50 mg of frozen liver tissue was homogenized in radioimmunoprecipitation assay (RIPA) buffer with additional phosphatase (Halt, ThermoScientific) and protease inhibitors (Roche) using a stainless-steel bead for lysis (TissueLyser II, Qiagen). The Bradford Assay (Bio-Rad) was utilized to determine protein concentration using bovine serum albumin to generate a standard curve. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed to separate proteins based on polypeptide length on 8% stacking and separating gels. Gels were run for approximately 3 hours at 100V before transfer to a 0.45 μm nitrocellulose membrane (Amersham GE Life Sciences) in a wet transfer apparatus for 1 hour at 100V and blocking at room temperature in a 4% skim milk solution. Rabbit anti-MTHFR antibody (1:800, 70-77 kDa subunits), generated in house, was used for overnight incubation at 4°C. Secondary antibody incubation was done at room temperature for 1 hour using antibodies horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG (GE Life Sciences) followed by protein detection using electrochemiluminescence (ECL) Prime Western Blotting Detection Reagent

(GE Life Sciences) and imaging with the Amersham Imager 600 (GE Life Sciences). Densitometric analysis was performed using the Amersham Imager 600 Analysis Software (GE Life Sciences) using vinculin expression as a loading control.

3.4 Folate and Choline Metabolite Measurements

Measurements of choline metabolites in hepatic and cortical tissue from *Mthfr*+/+ and *Mthfr*+/- maternal CD/ChDD-fed pregnant females at E10.5 were obtained using liquid chromatography-mass spectrometry. The averages of three technical replicate measurements were measured for each sample. Choline, betaine, and methionine were measured in both hepatic and cortical tissue. In addition, measurements of glycerophosphocholine, phosphocholine (PC), phosphatidylcholine (PtdCho), sphingomyelin (SM), and lysophosphatidylcholine were obtained from hepatic tissue only, and acetylcholine (ACh) was measured in cortical tissue only.

Preliminary studies of folate cycle metabolite measurements in frozen plasma from chow-fed *Mthfr* 677 CC and *Mthfr* 677 TT 4-5 week old female mice was performed using liquid chromatography–electrospray ionization tandem mass spectrometry. The specific metabolites measured were total homocysteine (tHcy), 5-methyltetrahydrofolate (5-methylTHF), folic acid, S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), methionine, cystathionine, betaine, and choline. These measurements were performed by members of Dr. Teodoro Bottiglieri's Laboratory (Baylor Scott & White Health, USA).

3.5 Statistical Analyses

Maternal organ weights, corpora lutea, implantation rates, and were analysed by two-way analysis of variance (ANOVA) followed by Tukey's post-hoc multiple comparison test using GraphPad Prism v. 6. (GraphPad Software). Embryonic crown rump length, weights, and somite count measurements were analyzed using IBM SPSS® Statistics accounting for litter effects. Incidences of embryonic delay, defects, and resorptions were analysed using linear mixed models in R v. 4.0 (The R Foundation) accounting for litter effects. The unit for statistical analysis was the female mouse or litter and results were expressed as the mean ± the standard error of the mean (SEM). Analyses with p-values ≤ 0.05 were considered statistically significant.

Chapter IV: Results of low choline and mild MTHFR deficienc

y (*Mthfr*+/-) on reproductive and preliminary behaviour outcomes

4.1 Impact of low choline and mild MTHFR deficiency (*Mthfr*+/-) on maternal organ (brain and liver) weights

Embryos, maternal tissues, and plasma from pregnant mice from all four maternal experimental groups (2 diets, 2 genotypes) were collected. In total, 10-11 pregnant females per group were sacrificed for this study. Maternal tissues and organs were collected at the time of dissection (10.5 days post copulation). Body weights of pregnant *Mthfr*+/+ and *Mthfr*+/- females were measured. Whole maternal livers and brains were extracted for measuring. During gestation, murine hepatic weight often increases as a maternal physiological response to pregnancy (101). To account for variation due to size of individual mice, organ weights were normalized to body weight of the mouse. To avoid confounding due to differences in litter size, the weight of the uterus was subtracted from the total body weight before normalization. No differences were observed in the normalized maternal body weights. No diet, genotype or diet x genotype interactions were observed in the % liver weights (**Figure 3A**). In terms of % brain weights, ANOVA tests did not show any differences in diet, genotype or diet x genotype interactions across all four groups. However post hoc analysis showed that % brain weights of *Mthfr*+/- pregnant females fed low choline were significantly lower than *Mthfr*+/- females on control diet (**Figure 3B**, Tukey adjusted p value = 0.0255), indicating a diet effect on brain weights in pregnant mice with MTHFR deficiency.

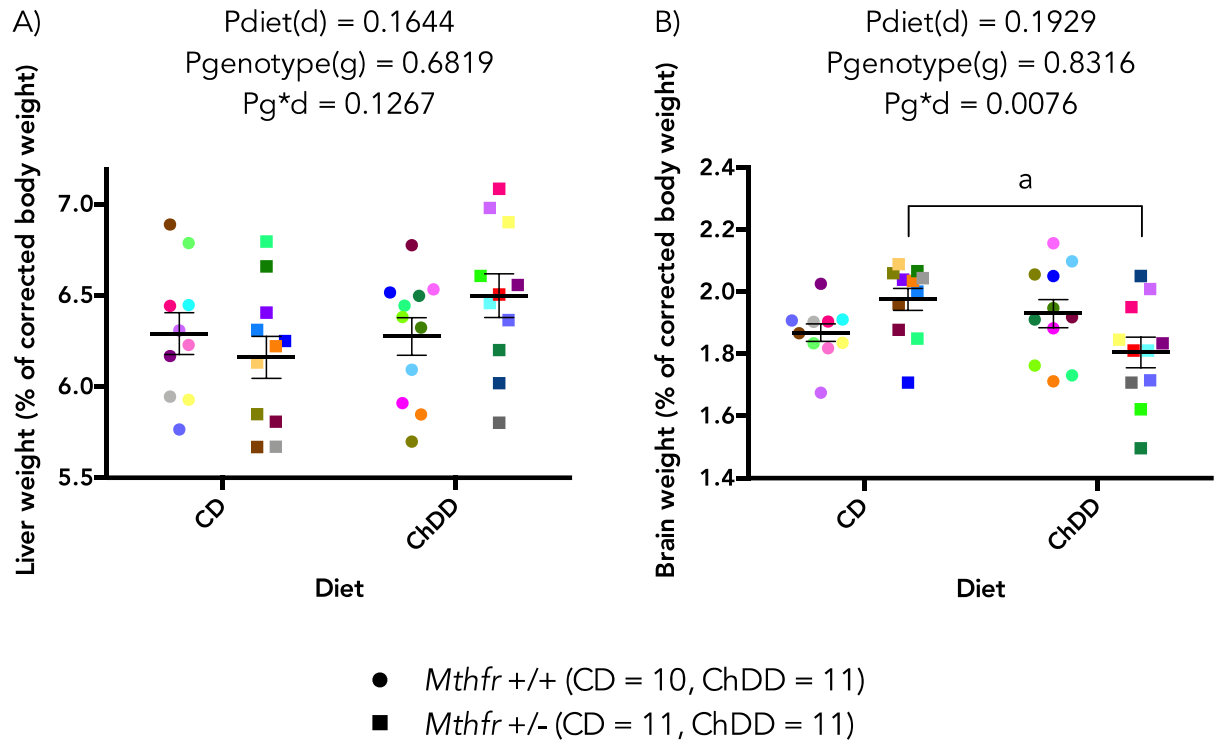

Figure 3. The weights of maternal (A) livers and (B) brains as a percentage of total corrected body weight. Mean ratios of (A) % liver weights: CD +/+ = 6.29, CD +/- = 6.16, ChDD +/+ = 6.26, ChDD +/- = 6.50 and (B) % brain weights: CD +/+ = 1.87, CD +/- = 1.98, ChDD +/+ = 1.93, ChDD +/- = 1.81 The corrected body weight is maternal body weight – weight of uterus. Results were analysed by 2-factor ANOVA with post hoc analysis and the Tukey method to account for multiple testing. Values shown are means and error bars represent standard error of the mean. Data points are colour coded to represent an individual mouse in each group. Colours in Figs. 3-8 correspond with each other. a = diet effect (Tukey adjusted p value = 0.0255) on % brain weight of *Mthfr*+/- pregnant females. Legend: CD: Control Diet; ChDD: Choline-Deficient Diet.

4.2 Modulation of hepatic and cortical choline metabolites due to low choline and mild maternal MTHFR deficiency (*Mthfr*+/-) at E10.5

Whole liver and brain cortex tissues were collected from maternal Mthfr+/+ and Mthfr+/- pregnant females at E10.5 from CD-fed and ChDD-fed groups (2 diets, 2 genotypes) using liquid chromatography-mass spectrometry. For choline metabolite measurements, hepatic tissue and right cortical brain tissue were analysed (n = 5/group). Hepatic measurements of methionine, betaine, choline, glycerophosphocholine, PC, PtdCho, sphingomyelin (SM), and lysophosphatidylcholine, DMG, and trimethylamine N-oxide (TMAO) were obtained. ChDD-fed pregnant females had significantly less hepatic betaine levels (**Figure 4B,** 2-factor ANOVA p value = 0.0112) than CD-fed females, particularly in the Mthfr+/- group (**Figure 4B**, Tukey adjusted p value = 0.0232). ChDD-fed pregnant females also had lower concentrations of hepatic DMG (**Figure 4I**, 2-factor ANOVA p value = 0.0100) and TMAO (**Figure 4J**, 2-factor ANOVA p value = 0.0217). No significant differences in hepatic concentrations of methionine (**Figure 4A**), choline (**Figure 4C**), glycerophosphocholine (**Figure 4D**), PC (**Figure 4E**), PtdCho (**Figure 4F**), SM (**Figure 4G**), and lysophosphatidylcholine (**Figure 4H**) were observed due to maternal diet, genotype, or diet x genotype interaction. Cortical measurements of methionine (**Figure 5A**), betaine (**Figure 5B**), choline (**Figure 5C**), and ACh (**Figure 5D**) concentrations were also obtained, however, no significant differences due to diet, genotype, or diet x genotype interaction were observed.

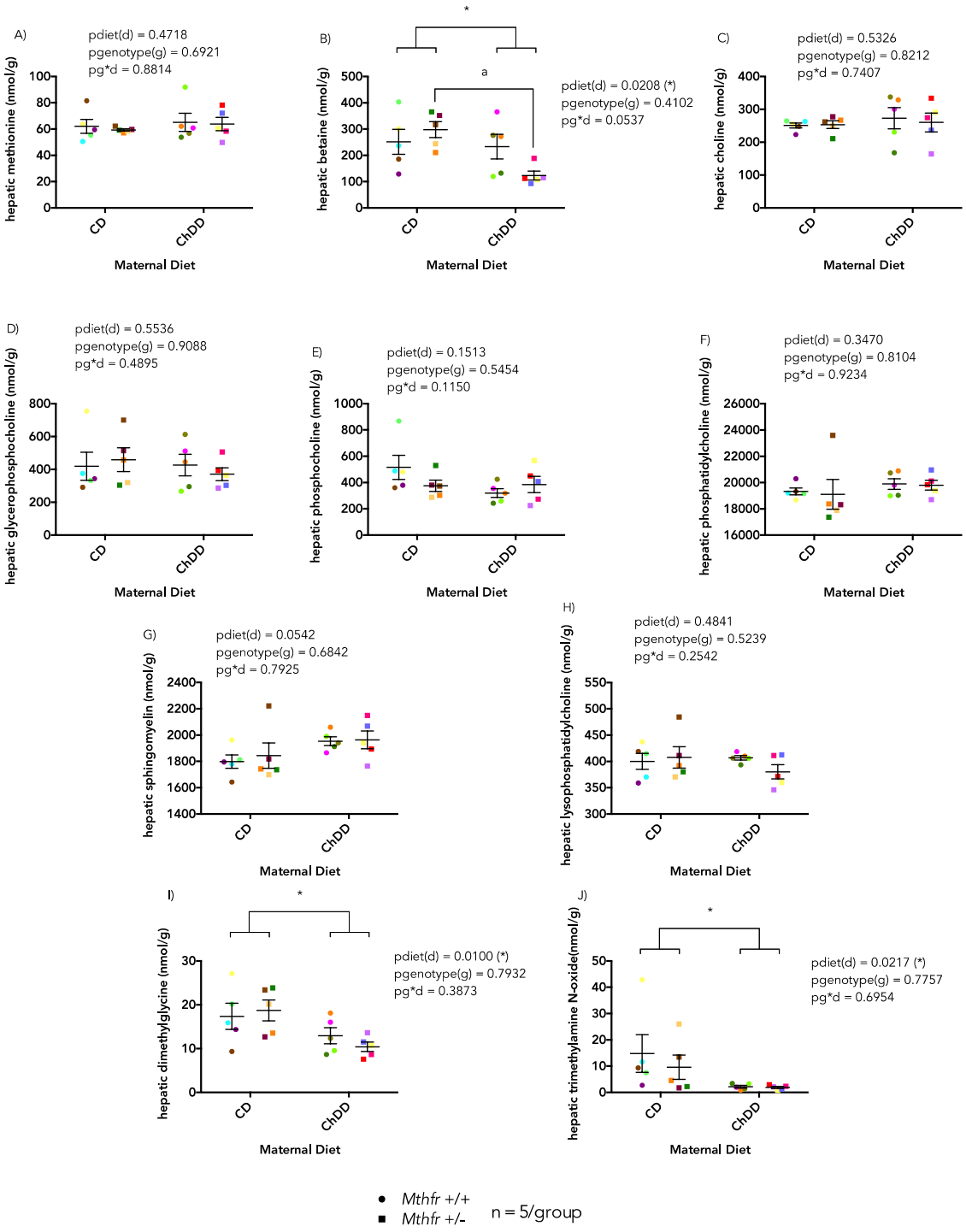

Figure 4. Choline metabolite levels in CD- and ChDD-fed *Mthfr*+/+ and *Mthfr*+/- pregnant female livers (n = 5/group) at 10.5 dpc. Concentrations of **(A)** methionine, **(B)** betaine, **(C)** choline, **(D)** glycerophosphocholine (GPC), **(E)** phosphocholine (PC), **(F)** phosphatidylcholine (PtdCho), **(G)** sphingomyelin (SM), **(H)** lysophosphatidylcholine, **(I)** dimethylglycine, and **(J)**

trimethylamine N-oxide were obtained. A significant difference in betaine concentration due to ChDD diet is shown. * indicates $p < 0.05$ (two-way ANOVA test) and “a” indicates reduced betaine particularly in the *Mthfr*+/- group (Tukey adjusted p value = 0.218). Data shown are mean values and error bars indicate standard error of the mean. Data points are color coded to represent individual mice. Colours in Figs. 3-8 correspond with each other. Results were analysed by 2-factor ANOVA with post hoc analysis and the Tukey method to account for multiple testing. Legend: CD: Control Diet; ChDD: Choline-Deficient Diet.

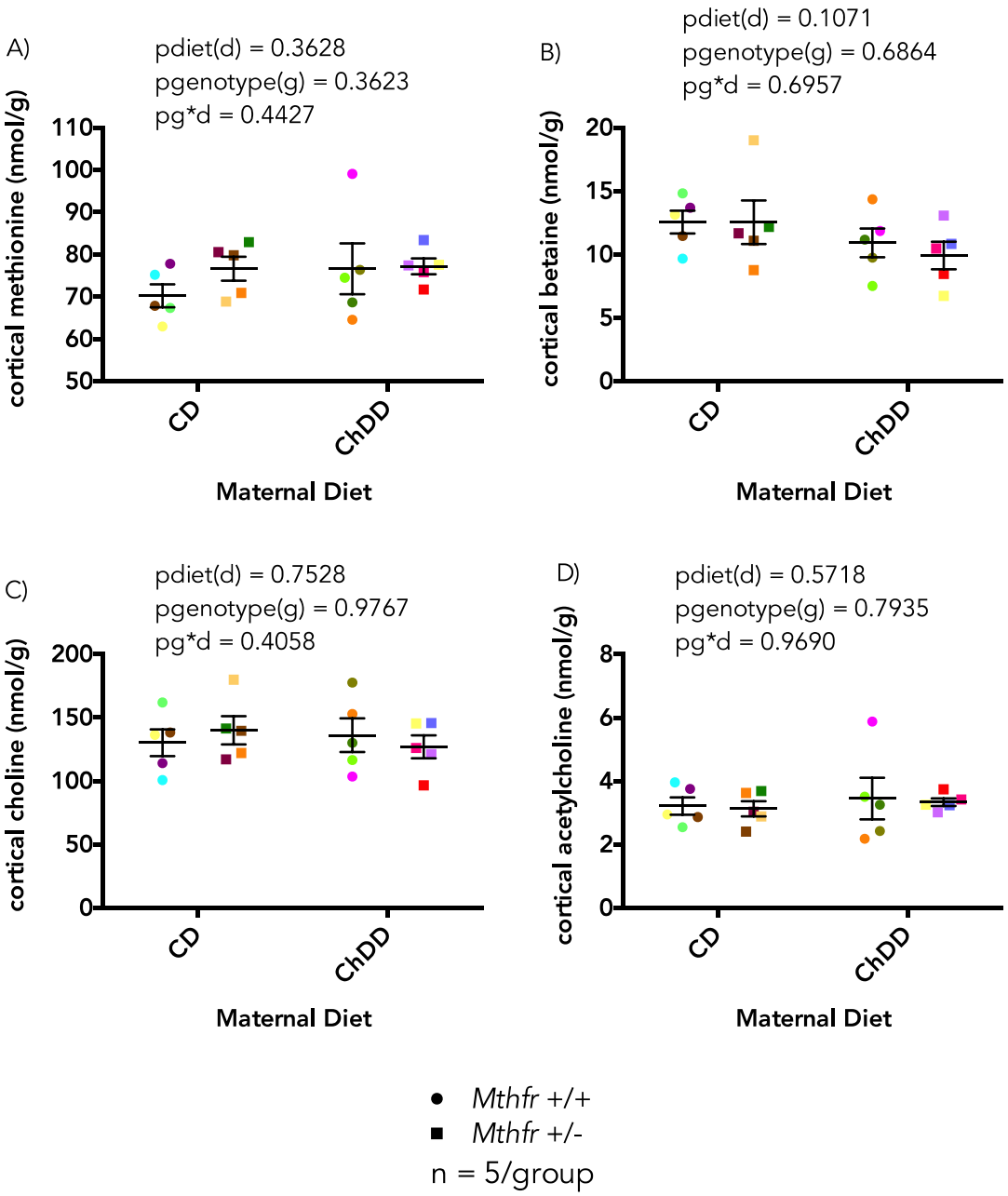

Figure 5. Cortical choline metabolite concentrations in CD- and ChDD-fed *Mthfr*+/+ and *Mthfr*+/- pregnant females (n = 5/group) at 10.5 dpc. Concentrations of **(A)** methionine, **(B)** betaine, **(C)** choline, **(D)** acetylcholine (ACh) are shown. Data shown are mean values and error bars indicate standard error of the mean. Results were analysed by 2-factor ANOVA with post hoc analysis and the Tukey method to account for multiple testing. Data points are color coded to represent individual mice. Colours in Figs. 3-8 correspond with each other. Legend: CD: Control Diet; ChDD: Choline-Deficient Diet.

4.3 Effect of low choline and mild MTHFR deficiency (*Mthfr*+/-) on pregnancy outcomes at E10.5

After ovulation, as the mature ova escapes the ovarian follicle, a remnant structure called the corpora luteum in the ovaries can be observed (102). Successful implantation of embryos can be assessed by a comparison of the number of embryos (normal/alive or resorbing) with the number of corpora lutea observed (indicating the number of ova released from the ovaries). Both the left and right ovaries were examined for the number of corpora lutea in *Mthfr*+/+ and *Mthfr*+/- mice on control or low choline diet. There were no differences observed in the total number of corpora lutea between maternal groups. Furthermore, no diet, genotype, or diet x genotype interaction effects were observed in the percentage of successful implantations (**Figure 6**). In mice, if fetal death occurs, the embryo and its corresponding placenta will begin to resorb, which is observable through the presence of necrosis. Complete resorptions are indicated by bloody spots without the presence of an embryo and/or discernable placental tissue. No diet, genotype, or diet x genotype interaction effects were observed in the percentage of resorbed embryos between the four maternal groups (**Figure 9B**). Comparison of *Mthfr*+/+ and *Mthfr*+/- embryos in *Mthfr*+/- mothers also showed no differences in the incidence of fetal resorptions based on maternal diet, embryonic genotype, or diet x embryonic genotype interactions (**Figure 10B**).

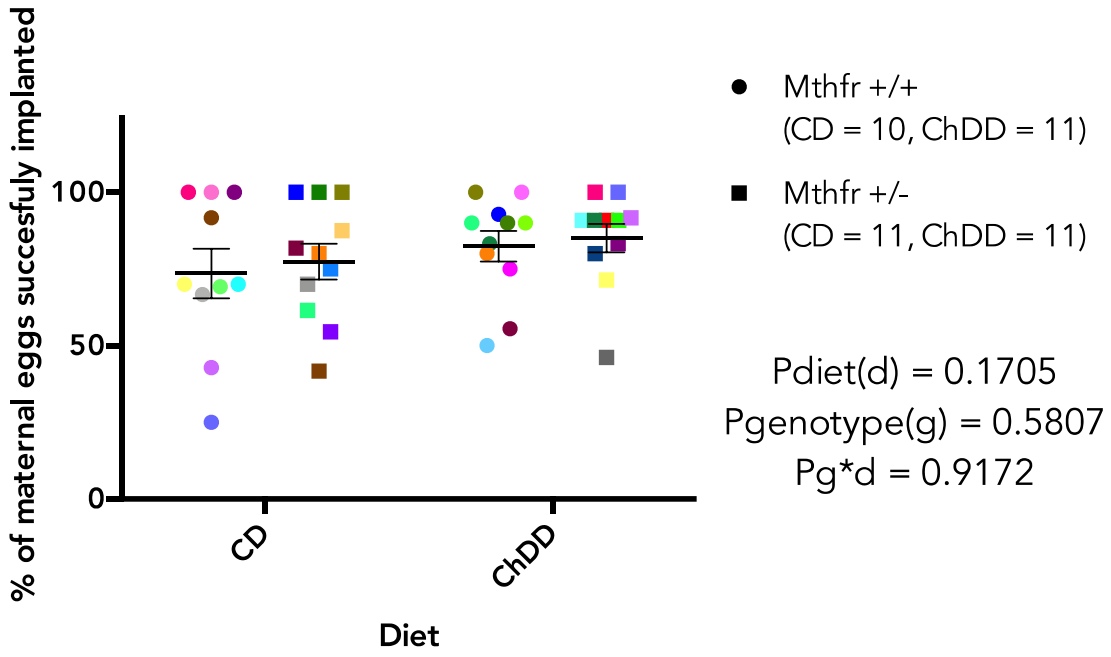

Figure 6. The ratio of successful implantation was calculated as total implantation sites over total number of corpora lutea (representative of how many ova were released during estrus). Mean ratios: CD +/+ = 73.5, CD +/- = 77.5, ChDD +/+ = 82.4, ChDD +/- = 85.1. Results analyzed by 2-factor ANOVA. Values shown are means and error bars are the standard error of the mean. Colours in Figs. 3-8 correspond with each other. Legend: CD: Control Diet; ChDD: Choline-Deficient Diet.

4.4 Effect of low choline and mild MTHFR deficiency (*Mthfr*+/-) on E10.5 embryonic development.

Embryos were collected from 10-11 litters from each maternal group (2 genotypes – *Mthfr*+/+ and *Mthfr*+/-, 2 diets – CD and ChDD). Embryos were scored for size markers such as crown rump length (CRL) and the number of somite pairs. Other markers, such as lens indentation, nasal placode invagination, formation of branchial aches, cerebral hemisphere formation, and otic vesicle formation (**Figure 11A**) were also used as relative markers to assess delay and defects in embryos from the same litter. No differences based on diet, maternal genotype, or diet x genotype interaction were observed on the CRL (**Figure 7A**) or number of somite pairs (**Figure 7B**) of *Mthfr*+/+ embryos from all four maternal groups. Delayed embryos were observed (**Figure 11B**). However, no significant differences were seen in the incidence of delayed embryos across all four maternal groups (**Figure 9A**). Embryonic defects were observed including incomplete cranial neural tube closure, resulting in anencephaly (**Figure 11C and D**), failure of embryonic turning, and incorrect tail orientation. No visible CHD, such as abnormal heart looping, was observed in any embryos examined. Significant differences in the incidence of defects based on diet, maternal genotype, or diet x genotype interaction were not observed (**Table 1**). Embryonic genotype effects between *Mthfr*+/+ and *Mthfr*+/- embryos from *Mthfr*+/- mothers on CD and ChDD were also analysed for developmental parameters. However, no differences based on diet, embryonic genotype or diet x embryonic genotype interaction were observed in embryonic CRL (**Figure 8A**) or number of somite pairs (**Figure 8B**). Similarly, no differences in embryonic delay (**Figure 10A**) or incidence of defects (**Table 2**) were observed based on diet, embryonic genotype, or diet x embryonic genotype interaction.

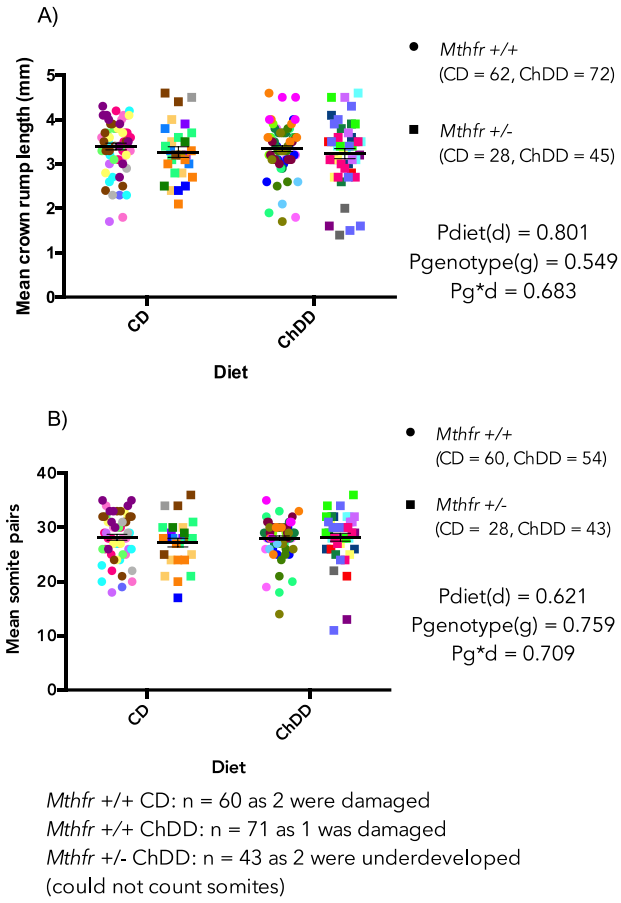

Figure 7. Maternal diet and *Mthfr* genotype effects on **(A)** mean crown rump length (mm) (CRL) and **(B)** mean somite pair counts of E10.5 *Mthfr*+/+ embryos. **(A)** CRL was measured for all *Mthfr*+/+ embryos that were not resorbed. Mean values: CD +/+ = 3.40, CD +/- = 3.27, ChDD +/+ = 3.34, ChDD +/- = 3.23 **(B)** Somite pairs were measured for all *Mthfr*+/+ embryos that were not resorbed and somites were distinguishable. Mean values: CD +/+ = 28.2, CD +/- = 27.2, ChDD +/+ = 28.1, ChDD +/- = 28.2. Note in the bottom of figure B explains discrepancies in the total number of embryos between the two parameters measured. **(Both A and B)** "*Mthfr*" genotype in the legend refers to maternal genotype and numbers in the legend refer to the total number of *Mthfr*+/+ embryos from which measurements could be obtained. Data points are color coded per litter (i.e. embryos from the same mother) in each maternal group. Colours in Figs. 3-8 correspond with each other. Results were analyzed for significance between maternal diet (d), maternal genotype (g), or genotype*diet interaction (g*d) using linear mixed model accounting for litter effects. Values shown are means and error bars represent the standard error of the mean. Legend: CD: Control Diet; ChDD: Choline-Deficient Diet.

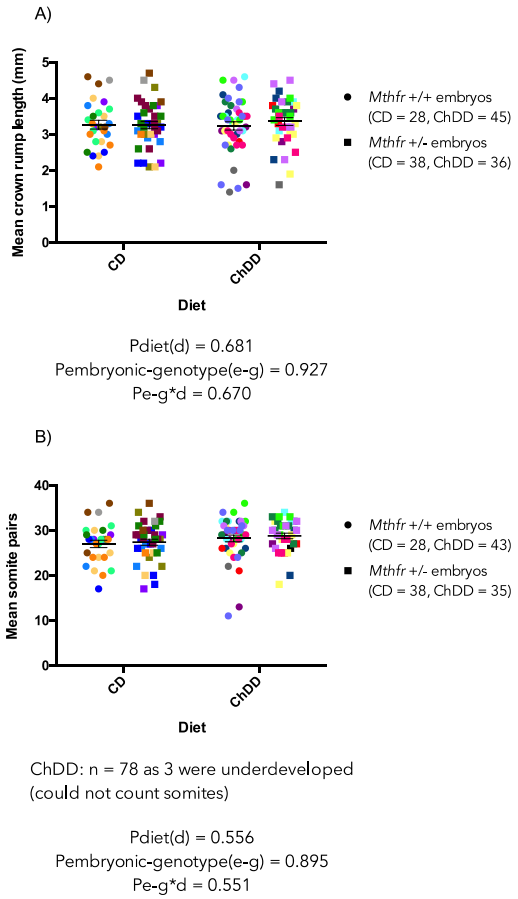

Figure 8. Comparison of **(A)** mean crown rump length (mm) (CRL) and (**B)** mean somite pair counts of E10.5 *Mthfr*+/+ and *Mthfr*+/- embryos from *Mthfr*+/- mothers fed CD and ChDD. **(A)** CRL was measured for all embryos that were not resorbed. Mean values: CD +/+ = 3.27, CD +/- = 3.26, ChDD +/+ = 3.23, ChDD +/- = 3.35 **(B)** Somite pairs were measured for all embryos that were not resorbed and somites were distinguishable. Mean values: CD +/+ = 27.0, CD +/- = 27.4, ChDD +/+ = 28.2, ChDD +/- = 28.8. Note in the bottom of figure B explains discrepancies in the total number of embryos between both parameters measured. **(Both A and B)** "CD" and "ChDD" refers to maternal diet and numbers in the legend refer to the total number of *Mthfr* embryos from which measurements could be obtained. Data points are color coded per litter (i.e. embryos from the same mother) in each maternal group. Colours in Figs. 3-8 correspond with each other. Results were analyzed for significance between maternal diet (d), embryonic genotype (e-g), or genotype*diet interaction (e-g*d) using linear mixed model accounting for litter effects. Values shown are means and error bars represent the standard error of the mean. Legend: CD: Control Diet; ChDD: Choline-Deficient Diet.

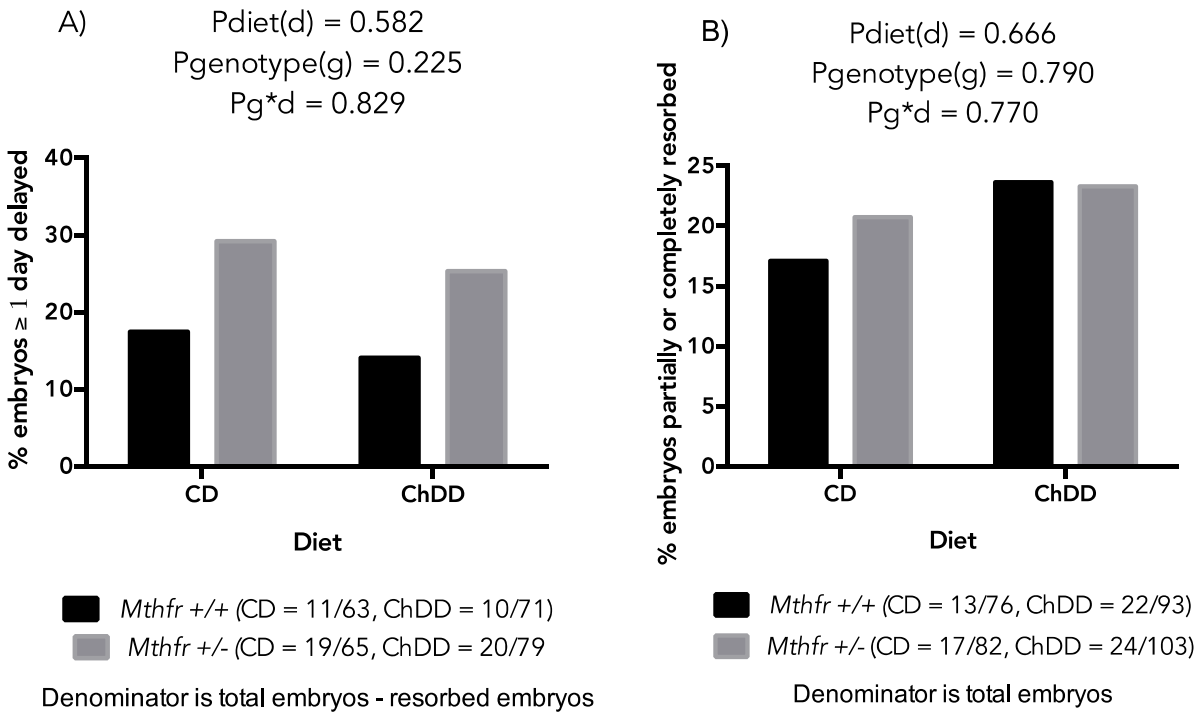

Figure 9. Maternal diet and *Mthfr* genotype effects on the percentage of **(A)** embryonic delay and **(B)** embryonic resorptions. **(A)** A delayed embryo is ≥ 1 day behind development in relation to the oldest embryo in the litter, based on morphological scoring. The ratio of delay is calculated as the total number of delayed embryos over the total number of characterized embryos. **(B)** Complete resorptions were bloody/necrotic implants, and partially resorbed embryos had placentae corresponding with either remnants of an embryo or no embryo. The ratio of resorption is calculated as the total number of resorbed embryos over the total number of embryos. "*Mthfr*" in the legend refers to maternal genotype. Results were analyzed for significance between maternal diet (d), maternal genotype (g), or genotype*diet interaction (g*d) by binary logistic regression accounting for litter effects. Legend: CD: Control Diet; ChDD: Choline-Deficient Diet.

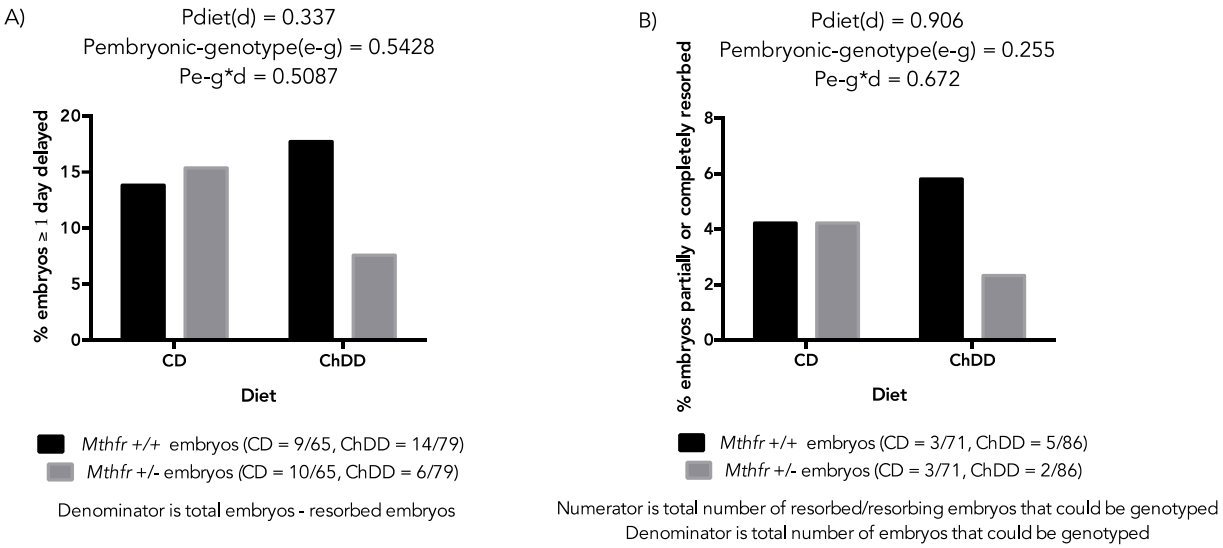

Figure 10. Maternal diet and embryonic *Mthfr* genotype effects on the percentage of **(A)** embryonic delay and **(B)** embryonic resorptions in *Mthfr*+/- pregnant females. **(A)** A delayed embryo is ≥ 1 day behind development in relation to the oldest embryo in the litter, based on morphological scoring. The ratio of delay is calculated as the total number of delayed embryos over the total number of characterized embryos. **(B)** Complete resorptions were bloody/necrotic implants, and partially resorbed embryos had placentae corresponding with either remnants of an embryo or no embryo. The ratio of resorption is calculated as the total number of resorbed embryos over the total number of embryos. "*Mthfr*" in the legend refers to maternal genotype. Results were analyzed for significance between maternal diet (d), embryonic genotype (e-g), or genotype*diet interaction (e-g*d) by binary logistic regression accounting for litter effects. Legend: CD: Control Diet; ChDD: Choline-Deficient Diet.

Table 1. Summary of embryonic defects observed in CD or ChDD-fed pregnant female *Mthfr* mice. An embryonic defect is abnormal heart looping, failure of embryo to turn, incomplete neural tube/head closure and/or incorrect tail direction. A normal embryo is one without a developmental defect and includes delayed embryos. The ratio of defective embryos is calculated as the number of embryos with defect(s) over the total number of embryos. Results were analyzed using binary logistic regression accounting for litter effects. p maternal diet = 0.507, p maternal genotype = 0.600, p diet*genotype = 0.562. Legend: CD: Control Diet; ChDD: Choline-Deficient Diet.

Maternal Diet	Maternal Genotype	Embryos with Defects	Total Embryos	Defective (%)
CD	*Mthfr*+/+	2	63	3.2
	Mthfr+/-	1	65	1.5
ChDD	*Mthfr*+/+	2	71	2.8
	Mthfr+/-	3	79	3.8

Table 2. Summary of embryonic defects and genotypes observed in CD or ChDD-fed pregnant female *Mthfr*+/- mice. An embryonic defect is abnormal heart looping, failure of embryo to turn, incomplete neural tube/head closure and/or incorrect tail direction. A normal embryo is one without a developmental defect and includes delayed embryos. The ratio of defective embryos is calculated as the number of embryos with defect(s) over the total number of embryos. Results were analyzed using binary logistic regression accounting for litter effects. p maternal diet = 0.628, p embryonic genotype = 0.644, p diet*e-genotype = 0.791. Legend: CD: Control Diet; ChDD: Choline-Deficient Diet.

Maternal Diet	Embryonic Genotype	Embryos with Defects	Total Embryos	Defective (%)
CD	*Mthfr*+/+	0	28	0
	Mthfr+/-	1	37	2.7
ChDD	*Mthfr*+/+	1	45	2.2
	Mthfr+/-	2	34	5.9

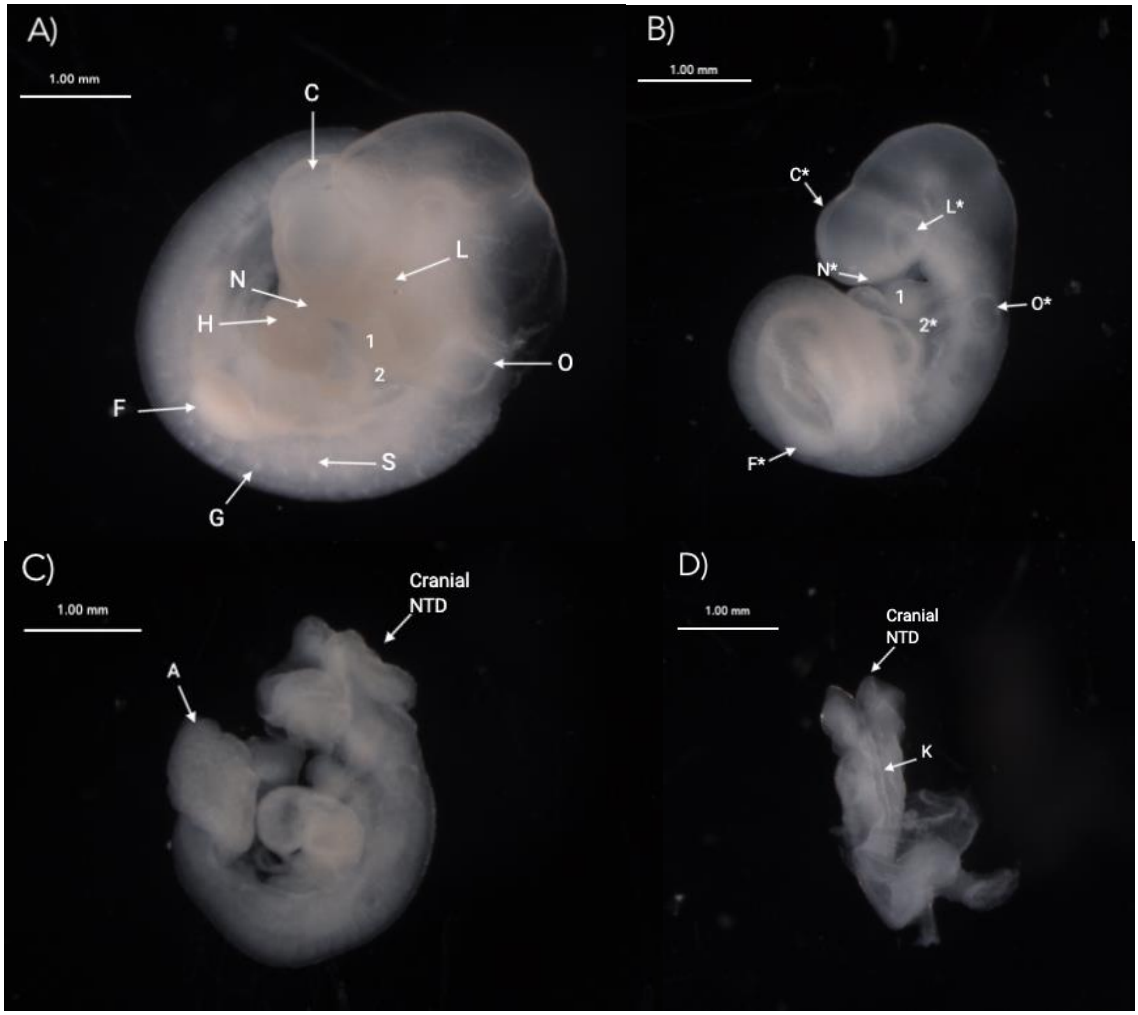

Figure 11. Representative images of E10.5 embryos from *Mthfr*+/+ and Mthfr+/- mothers fed CD (Control Diet) and ChDD (Choline-Deficient Diet). **(A)** Normal E10.5 embryo with tail orientation to the right of the body. Legend: 1: 1st branchial arch; 2: 2nd branchial arch; C: cerebral hemispheres; F: forelimb bud; G: cervical ganglion; H: heart; L: lens (eye) invagination; N: nasal placode invagination; O: otic vesicle (pointed); S: somite. **(B)** Developmentally delayed embryo dissected at 10.5 dpc with tail orientation to the left of the body. Legend: 1: 1st branchial arch; 2*: smaller 2nd branchial arch; C*: smaller cerebral hemispheres; F*: smaller forelimb bud; L*: lens without clear invagination; N*: flat nasal placode; O*: otic vesicle (round). **(C)** Embryo 1 with open craniofacial neural tube and anencephaly. Legend: A: allantois. **(D)** Posterior view of embryo 2 with open craniofacial neural tube and anencephaly. Legend: K: kinked/wavy neural tube closure.

4.5 Preliminary behavioral outcomes due to low dietary choline and MTHFR deficiency (*Mthfr*+/-) in non-pregnant mice of reproductive age

9-week old *Mthfr*+/+ and *Mthfr*+/- non-pregnant and virgin females were used for behaviour tests to examine if low choline and MTHFR deficiency could impact behavioural, motor, and memory outcomes. 3-4 individual females were tested in each of the four groups. Due to low sample size and low power for statistical tests such as ANOVA, analyses could not be performed. In the OF test, outcomes such as the total distance travelled, mean speed of travel, total grooming and rearing times, total time spent active, and the anxiety index could be measured for 3-4 females per group (**Figure 12**). Mean values of these behavioural parameters are summarized in **Table 3**. For the NOR test, which is dependent on the amount of time spent with the novel or familiar object (**Eq. 1**), only 1-3 individual females per group were measured (**Figure 13**), as some females that were measured in the OF test did not exhibit object exploration during the NOR test. The NOR test was also underpowered, and statistical tests such as ANOVA to compare differences in the discrimination index were not possible.

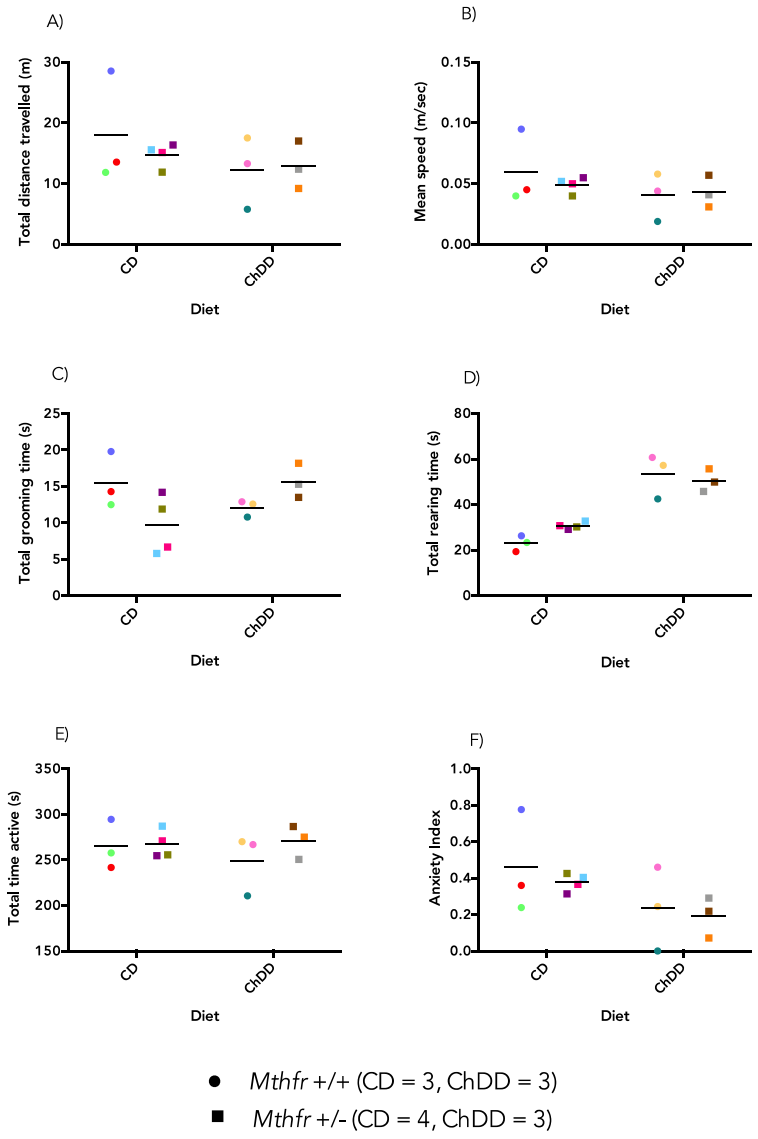

Figure 12. Results of open field test on reproductive-age non-pregnant and virgin female *Mthfr*+/+ and *Mthfr*+/- mice on diet (CD/ChDD) for 5 weeks. Figure legend indicates *Mthfr* genotype status of females, with sample sizes included. **(A)** Total distance traveled over 5 minutes in metres. **(B)** Mean speed (metres/second) of travel. **(C)** Total time (s) spent grooming and **(D)** rearing **(E)** Total time (s) spent active (grooming, rearing, and travelling). **(F)** Anxiety index, as measured by the total distance travelled in the 4 inner/center zones divided by total distance travelled. Data shown are individual values and lines indicate mean. Data points are color coded to show results of individual mice. Specific mean values are shown in Table 3. Due to low power, analysis by 2-way ANOVA was not possible. Legend: CD: Control Diet; ChDD: Choline-Deficient Diet.

Table 3. Mean values of behavioural parameters in open field test of CD/ChDD-fed *Mthfr*+/+ and *Mthfr*+/- mice of reproductive age. Parameters are visualized in Figure 12. Due to low power, analysis by 2-way ANOVA was not possible. Legend: CD: Control Diet; ChDD: Choline-Deficient Diet.

Diet	Genotype	Mean distance travelled (m)	Mean speed (m/sec)	Mean grooming time (s)	Mean rearing time (s)	Mean total active time (s)	Mean anxiety index
CD	*Mthfr*+/+ (n = 3)	17.9	0.060	15.5	23.0	264.7	0.46
	Mthfr+/- (n = 4)	14.8	0.049	9.65	30.7	267.1	0.37
ChDD	*Mthfr*+/+ (n = 3)	12.2	0.040	12.1	53.5	249.2	0.24
	Mthfr+/- (n = 3)	12.9	0.043	15.7	50.1	270.8	0.19

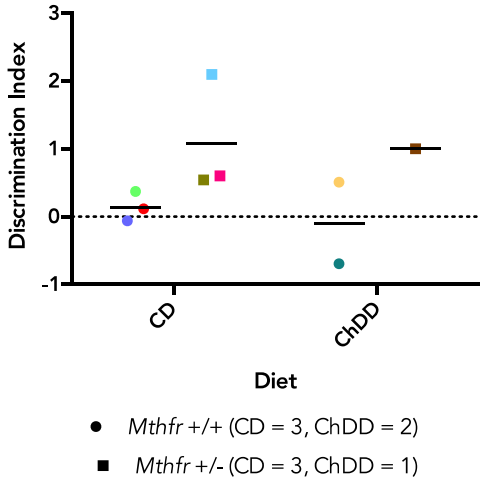

Figure 13. Discrimination Index (DI) (See Eq. 1), of *Mthfr*+/+ and *Mthfr*+/- non-pregnant and virgin females of reproductive age on diet (CD/ChDD) for 5 weeks. Some mice did not explore either objects during the NOR testing phase and were excluded from the analyses as DI could not be calculated. Figure legend indicates *Mthfr* genotype status of females, with sample sizes included. Data points are color coded to corresponding values in Figure 10. Mean DI for *Mthfr*+/+ females: CD: 0.141, ChDD: -0.094. Mean DI for *Mthfr*+/- females: CD: 1.080, ChDD: 1. Legend: CD: Control Diet; ChDD: Choline-Deficient Diet.

Chapter V: Preliminary results of reproductive outcomes in the novel *Mthfr* 677 TT model for MTHFR deficiency due to low and supplemental dietary folate

5.1 Impact of low and supplemental folate on maternal organ weights (liver and brain) and pregnancy outcomes in *Mthfr* 677 TT mice

For the reproductive study, 2-3 litters were collected in each of the 6 maternal experimental groups (2 genotypes, 3 diets). At 10.5 dpc, maternal plasma, liver, and brain tissue were collected. Total body, uterus, liver, and brain weights were measured. To account for changes in liver size due to pregnancy, a corrected body weight was calculated as the total body weight minus the weight of the uterus. % organ weights were measured as a fraction of total body weight. Due to low sampling size, statistical analysis such as ANOVA tests were not performed. Individual variation of % organ weights between CD-FD and CD-FASD groups were visualized for both % liver (**Figure 14A and B**) and % brain. (**Figure 14C and D**). Ovaries of pregnant females were observed for the presence of corpora lutea, the physical remnants of ova releasing after ovulation. The total number of corpora lutea were counted for all individual females, as well as the number of implantations. The percentage of successful implantation was calculated as the total number of implantations/the total number of corpora lutea (representing the number of ova released). Again, due to low sampling and power, statistical analyses could not be performed. Individual variation of % successful implantation can be visualized between CD-FASD (**Figure 15A**) and CD-FD (**Figure 15B**) maternal groups. All litters were examined for fetal death, which is seen as necrotic and or/bloody resorption sites. In 5 out of the 6 maternal groups, resorptions were observed (**Table 4**). Binary logistic regression analyses were planned; however, due to low power and sampling, detailed statistical tests could not be performed. A χ^2 test was performed to evaluate the numbers of resorbed embryos between CD-FD groups (diet and genotype) and resulted in a p-value of 0.0001. Other parameters could not be evaluated through a χ^2 test due to non-integer values.

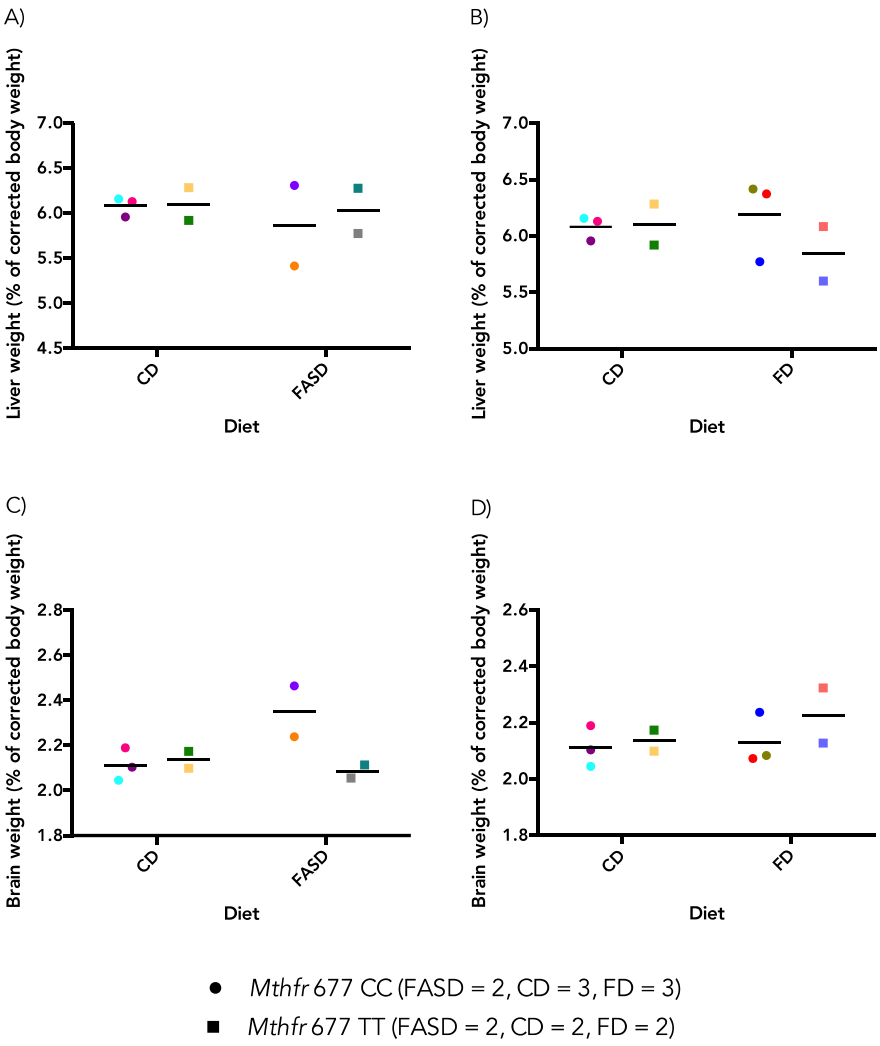

Figure 14. The weights of maternal **(A/B)** livers and **(C/D)** brains as a percentage of total corrected body weight. Comparisons between CD and **(A/C)** FASD and **(B/D)** FD are shown. The corrected body weight is maternal body weight – weight of uterus. Values shown are individual data points with lines representing means. Due to low power, results were not analyzed by 2-factor/3-factor ANOVA. Data points are color coded to represent individual mice and correspond to colors between Figs. 14-17. Legend: CD: Control Diet; FASD: Folic Acid Supplemented Diet; FD: Folic Acid Deficient Diet.

Mean % liver weights for *Mthfr* 677 CC females: FASD: 5.86, CD: 6.08, FD: 6.19

Mean % liver weights for *Mthfr* 677 TT females: FASD: 6.03, CD: 6.10, FD: 5.84

Mean % brain weights for *Mthfr* 677 CC females: FASD: 2.35, CD: 2.11, FD: 2.13

Mean % brain weights for *Mthfr* 677 TT females: FASD: 2.08, CD: 2.14, FD: 2.23

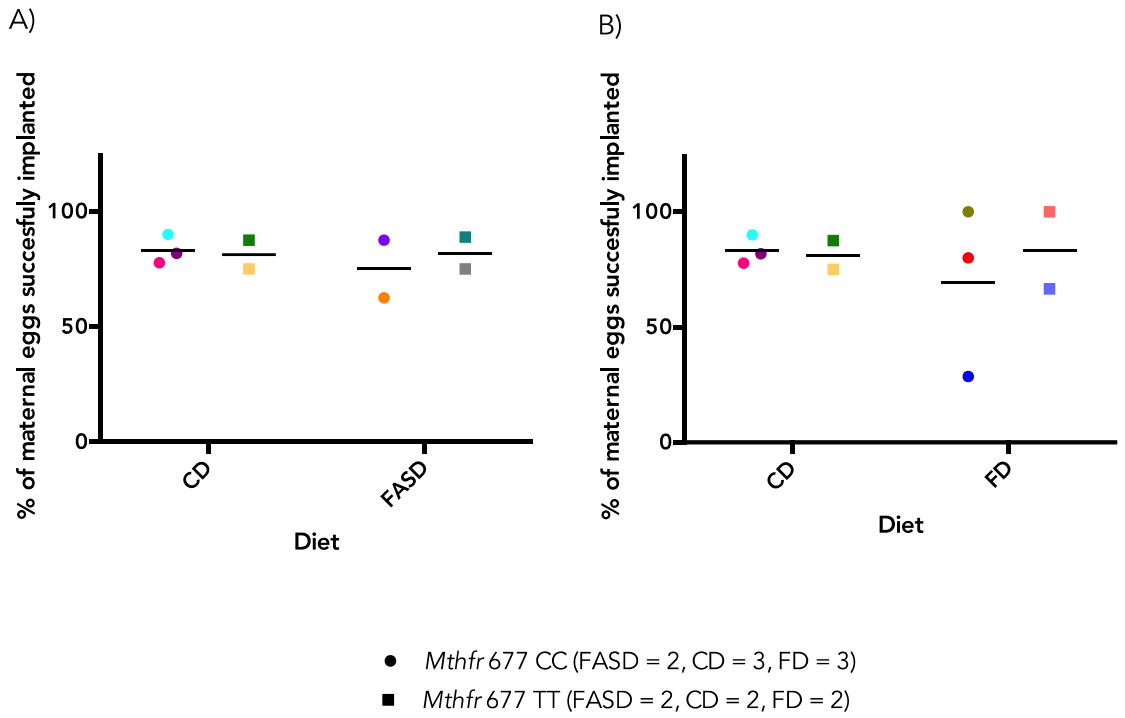

Figure 15. The ratio of successful implantation was calculated as total implantation sites/total number of corpora lutea (representative of how many ova were released during estrus). Comparisons shown between CD and **(A)** FASD and **(B)** FD. Data shown are individual values with lines indicating the mean. Due to low power, results were not analyzed by 2-factor/3-factor ANOVA. Data points are color coded to represent individual mice and correspond to colors between Figs. 14-17. Legend: CD: Control Diet; FASD: Folic Acid Supplemented Diet; FD: Folic Acid Deficient Diet.

Mean % implantation for *Mthfr* 677 CC females: FASD: 75.0, CD: 83.2, FD: 69.5

Mean % implantation for *Mthfr* 677 TT females: FASD: 81.9, CD: 81.3, FD: 83.3

5.2 Effect of low and supplemental dietary folate and *Mthfr* 677 TT status on embryonic development at E10.5

E10.5 embryos were collected from all 6 maternal groups, with 2-3 litters per group. Embryos were scored for morphological markers, such as CRL and somite pair counts to assess growth. Furthermore, all embryos were examined for normal E10.5 markers, visualized in **Figure 18A and B**. These markers include lens indentation and lens plate formation, cerebral hemisphere development, nasal placode invagination, otic vesicle formation, heart development, branchial arches formation, and forelimb/hindlimb bud swelling. Mean CRL was analyzed through linear mixed models comparing CD-FASD (**Figure 16A**) and CD-FD (**Figure 16B**) maternal groups. However, no significance was observed based on maternal diet, maternal genotype, or diet x genotype interaction. Similarly, comparisons of mean somite pair count in CD-FASD (**Figure 17A**) and CD-FD (**Figure 17B**) groups did not result in differences based on diet, maternal genotype, or interaction. Embryos that were a least one day behind development in relation to the oldest embryo in the litter were marked as developmentally delayed. Delayed embryos were observed in CD-*Mthfr* 677 TT (9.1%), FD-*Mthfr* 677 CC (12.5%), and FD-*Mthfr* 677 TT (13.3%) maternal groups (**Table 4**). Due to low sampling and power, however, detailed statistical analyses using binary logistic regression analyses was not possible. A representative image of a delayed embryo collected at 10.5 dpc was taken (**Figure 18C**), which had smaller cerebral hemispheres, a smaller 2nd branchial arch, flat nasal placodes, and poorly developed otic vesicles when compared to normal E10.5 embryos. One defect was observed, which came from a CD-fed *Mthfr* 677 TT mother (**Table 4**). This was a craniofacial NTD, as visualized by the open space in the head (**Figure 18D**). Similar to delay, low sampling and power made it difficult to perform detailed statistical analysis on the effect of diet and genotype on the incidence of defects.

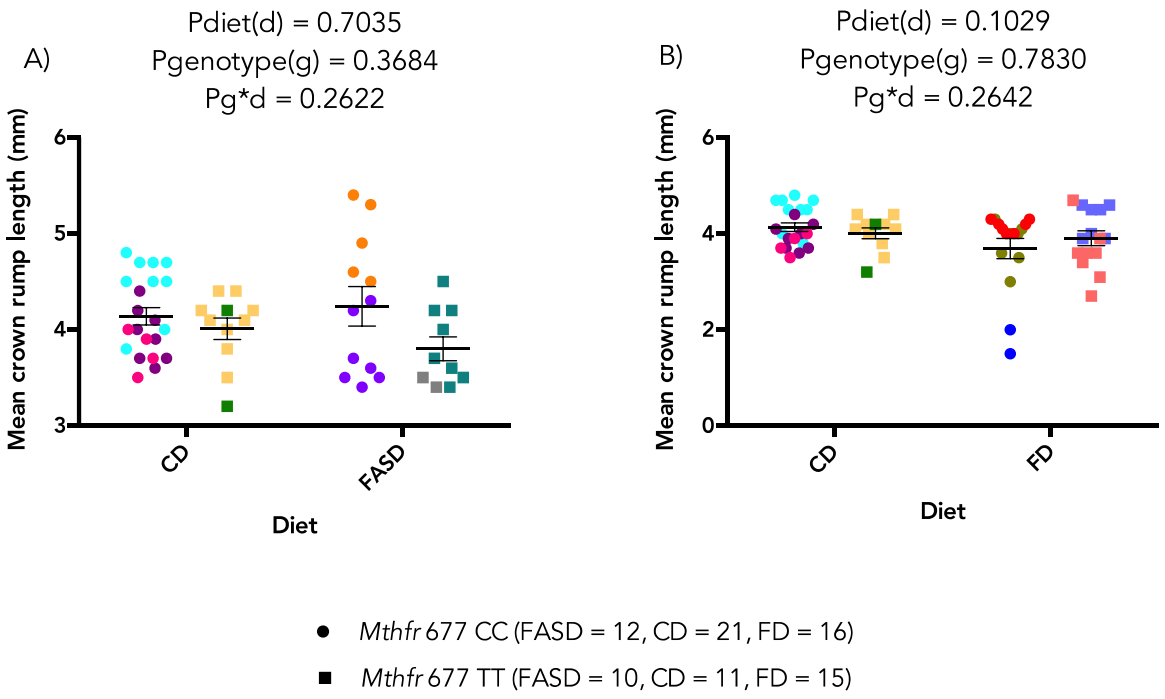

Figure 16. Maternal diet and *Mthfr* 677 genotype effects on mean crown rump length (mm) of E10.5 embryos. Comparisons between CD and **(A)** FASD and **(B)** FD are shown. CRL was measured for all embryos that were not resorbed. "*Mthfr*" genotype in the legend refers to maternal genotype and numbers in the legend refer to the total number of embryos from which CRL could be obtained. Results were analyzed for significance between maternal diet (d), maternal genotype (g), or genotype*diet interaction (g*d) using linear mixed model accounting for litter effects. Values shown are means and error bars represent the standard error of the mean. Data points are color coded to represent individual litters in each group and correspond to colors between Figs. 14-17. Legend: CD: Control Diet; FASD: Folic Acid Supplemented Diet; FD: Folic Acid Deficient Diet.

Mean embryonic CRL *Mthfr* 677 CC females: FASD: 4.24, CD: 4.14, FD: 3.70

Mean embryonic CRL for *Mthfr* 677 TT females: FASD: 3.8, CD: 4.01, FD: 3.91

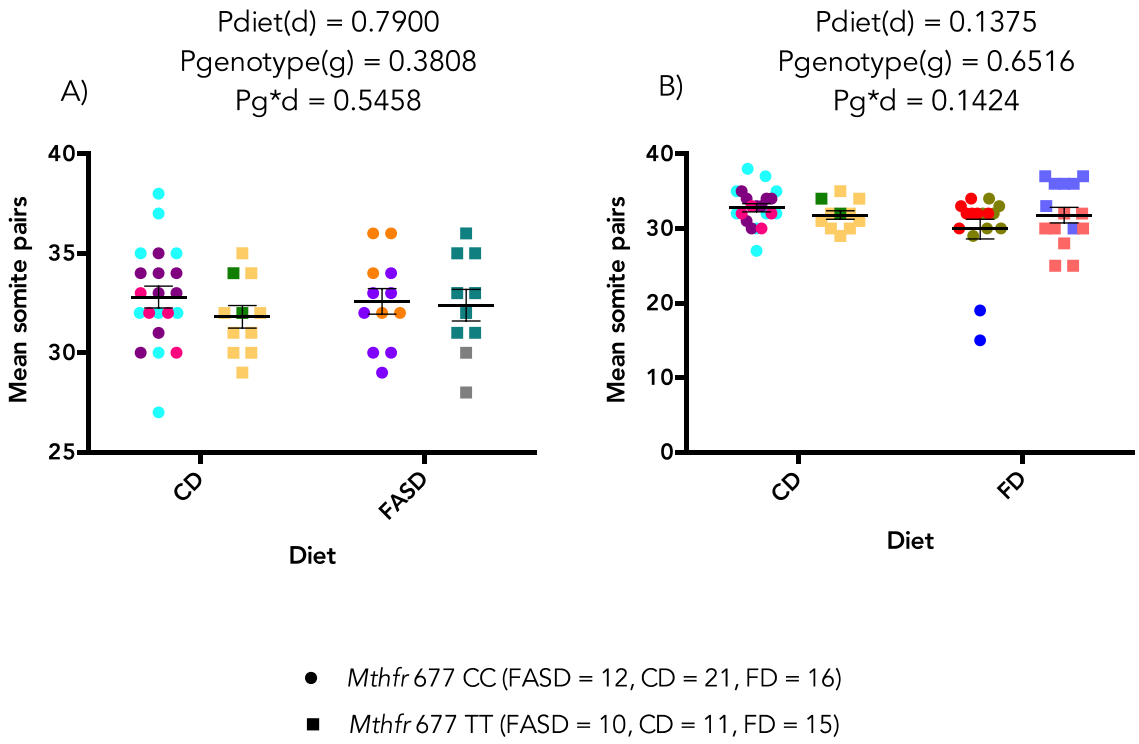

Figure 17. Effect of maternal diet and *Mthfr* 677 genotype on mean somite pairs of E10.5 embryos. Comparisons between CD and **(A)** FASD and **(B)** FD are shown. Somite pairs were measured for all embryos that were not resorbed and somites were distinguishable. "*Mthfr*" genotype in the legend refers to maternal genotype and numbers in the legend refer to the total number of embryos. Results were analyzed for significance between maternal diet (d), maternal genotype (g), or genotype*diet interaction (g*d) using linear mixed model accounting for litter effects. Values shown are means and error bars represent the standard error of the mean. Data points are color coded to represent individual litters in each group and correspond to colors between Figs. 14-17. Legend: CD: Control Diet; FASD: Folic Acid Supplemented Diet; FD: Folic Acid Deficient Diet.

Mean embryonic somite pairs for *Mthfr* 677 CC females: FASD: 32.6, CD: 32.8, FD: 29.9

Mean embryonic somite pairs for *Mthfr* 677 TT females: FASD: 32.4, CD: 31.8, FD: 31.8

Table 4. Summary of embryonic defects, delays, and resorptions observed in FASD, CD, or FD-fed pregnant female *Mthfr* 677 mice. An embryonic defect is abnormal heart looping, failure of embryo to turn, incomplete neural tube/head closure and/or incorrect tail direction. A normal embryo is one without a developmental defect and includes delayed embryos. One defect (open cranial neural tube) was observed, indicated by an asterisk. The ratio of defective embryos is calculated as the number of embryos with defect(s) over the total number of normal embryos. A delayed embryo is ≥ 1 day behind development in relation to the oldest embryo in the litter, based on morphological scoring. The ratio of delay is calculated as the total number of delayed embryos over the total number of characterized embryos. Complete resorptions were bloody/necrotic implants, and partially resorbed embryos had placentae corresponding with either remnants of an embryo or no embryo. The ratio of resorption is calculated as the total number of resorbed embryos over the total number of embryos. Chi-squared analysis of contingency table comparing % resorptions between FD and CD (diet & genotype) resulted in a significant p-value (0.0001). Other parameters were not analysed. Analyses on any parameters could not be performed between FASD-CD (due to non-integer values). Legend: CD: Control Diet; FASD: Folic Acid Supplemented Diet; FD: Folic Acid Deficient Diet.

Maternal Diet	Maternal Genotype	Defective embryos/normal embryos	Delayed embryos/normal embryos	Resorptions/total embryos
FASD	*Mthfr* 677 CC (n = 2)	0/12	0/12	0/12
	Mthfr 677 TT (n = 2)	0/10	0/10	1/11 (9.1%)
CD	*Mthfr* 677 CC (n = 3)	0/21	0/21	2/23 (8.7%)
	Mthfr 677 TT (n = 2)	1/11 (9.1%)	1/11 (9.1%)	5/16 (31.3%)
FD	*Mthfr* 677 CC (n = 3)	0/16	2/16 (12.5%)	3/19 (15.8%)
	Mthfr 677 TT (n = 2)	0/15	2/15 (13.3%)	1/16 (6.3%)

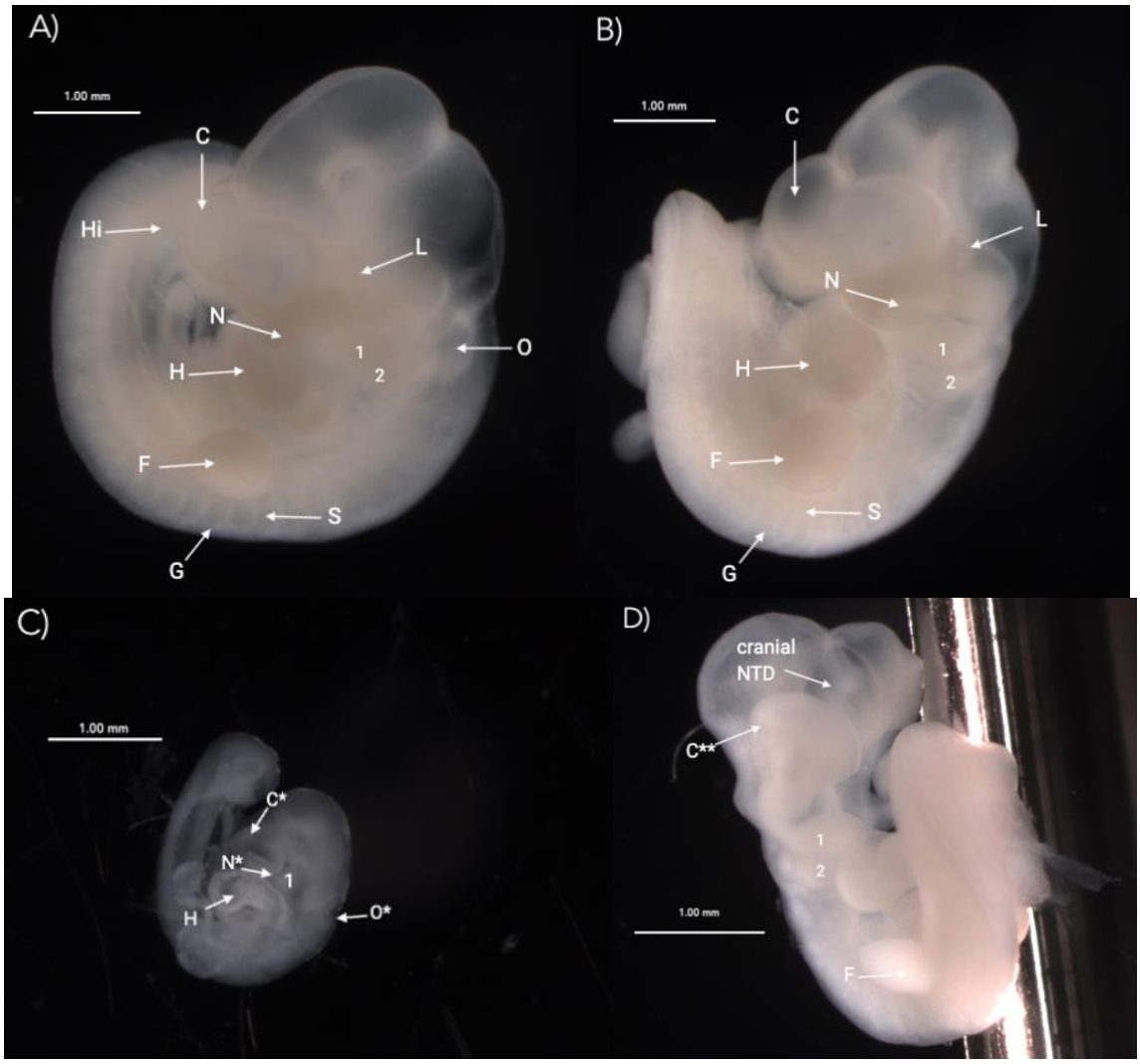

Figure 18. Representative images of E10.5 embryos from *Mthfr* 677 CC and *Mthfr* 677 TT mothers fed CD (Control Diet), FD (Folic Acid-Deficient Diet) and FASD (Folic Acid Supplemented Diet). **(A)** Side view and **(B)** front view of normal E10.5 embryos with tail orientation to the right of the body. Legend: 1: 1st branchial arch; 2: 2nd branchial arch; C: cerebral hemispheres; F: forelimb bud; G: cervical ganglion; H: heart; Hi: hindlimb bud; L: lens (eye) invagination; N: nasal placode invagination; O: otic vesicle (pointed); S: somite. **(C)** Developmentally delayed embryo dissected at 10.5 dpc. Legend: 1: 1st branchial arch; C*: smaller cerebral hemispheres; H: heart; N*: flat nasal placode; O*: otic vesicle (round). **(D)** Frontal view of embryo with open craniofacial neural tube and defects in facial development. Legend: Legend: 1: 1st branchial arch; 2: 2nd branchial arch; C**: defective cerebral hemisphere formation; F: forelimb bud.

5.3 Preliminary analyses of one-carbon metabolites in reproductive age *Mthfr* 677 TT female mice

Reproductive age (4-5 weeks old) *Mthfr* 677 CC and *Mthfr* 677 TT females (n = 4/group) on rodent chow were sacrificed for collection of blood/plasma for metabolite analyses using liquid chromatography–electrospray ionization tandem mass spectrometry. Plasma were analysed for the concentration of tHCy, 5-methylTHF, SAM, SAH, SAM:SAH ratio, methionine, cystathionine, betaine, and choline. *Mthfr* 677 TT females had significantly increased plasma tHCy (**Figure 19A**, Student's t-test p value = 0.0222) compared to *Mthfr* 677 CC females. Concentrations of plasma methionine (**Figure 19F**, Student's t-test p value = 0.0139), betaine (**Figure 19H**, Student's t-test p value = 0.0060), and choline (**Figure 19I**, Student's t-test p value = 0.0257) were significantly decreased in *Mthf*r 677 TT females compared to *Mthfr* 677 CC females. No significant differences were seen in the concentrations of plasma 5-methylTHF (**Figure 19B**), SAM (**Figure 19C**), SAH (**Figure 19D**), cystathionine (**Figure 19G**), or the ratio of SAM:SAH (**Figure 19E**) between *Mthfr* 677 CC and *Mthfr* 677 TT females. Hepatic tissue was also collected at the time of dissection in these females and was used to assess protein levels of MTHFR through western blotting. Results showed that the expression of total MTHFR in *Mthfr* 677 TT (n = 4) hepatic tissue was decreased (**Figure 20A and B**) when compared to *Mthfr* 677 CC (n = 3) females. One sample in the *Mthfr* 677 CC group was excluded due to experimental artifacts.

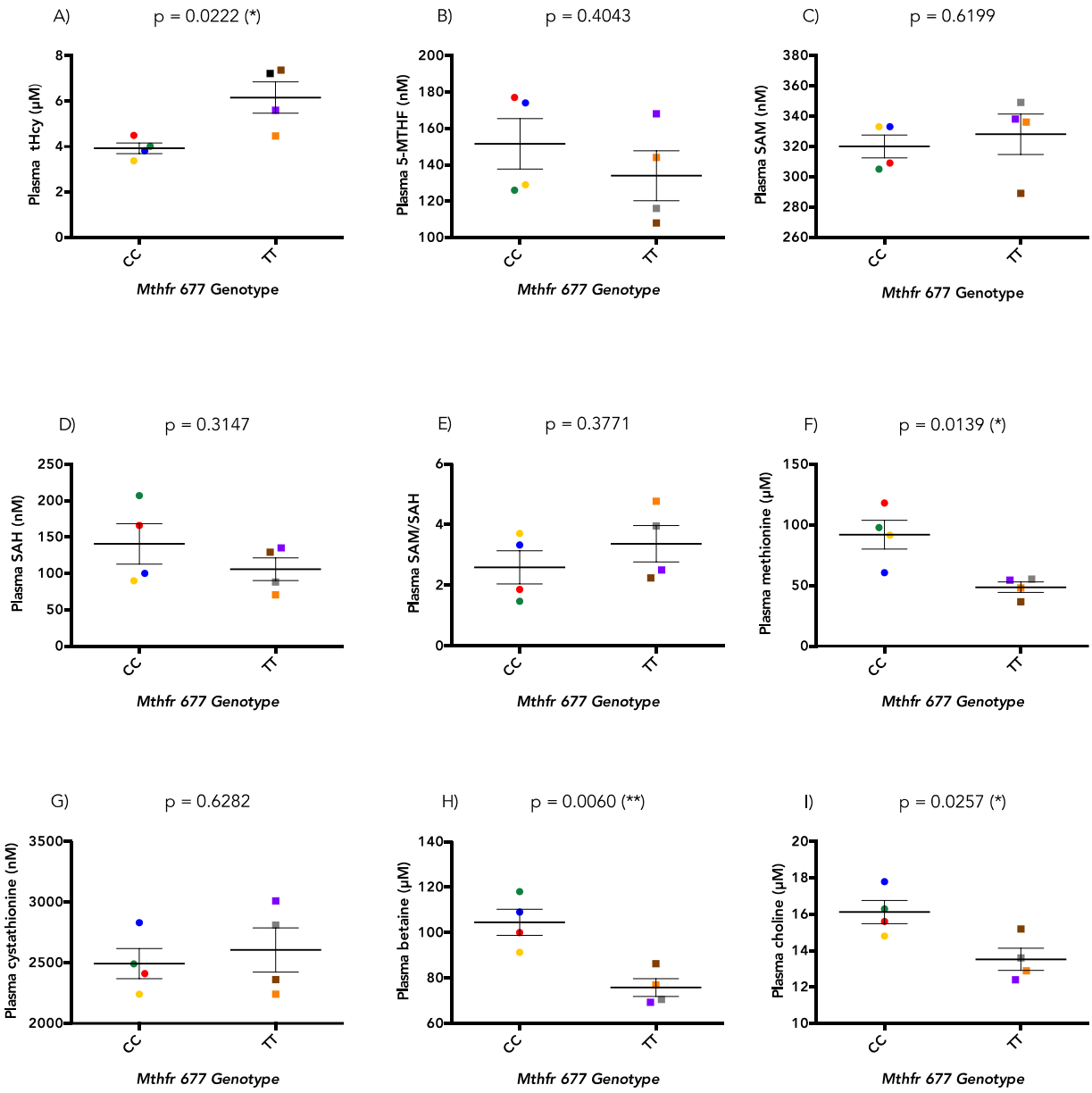

Figure 19. Plasma folate metabolite levels in *Mthfr* 677 CC and *Mthfr* 677 TT females (n = 4/group) of reproductive age (4-5 weeks) fed rodent chow. Levels of **(A)** total homocysteine, **(B)** 5-methyltetrahydrofolate, **(C)** S-adenosylmethionine (SAM), **(D)** S-adenosylhomocysteine (SAH), **(E)** Ratio of SAM:SAH, **(F)** methionine, **(G)** cystathionine, **(H)** betaine and **(I)** choline are shown. Significant differences are indicated in the levels of homocysteine, methionine, betaine, and choline. * indicates $p < 0.05$, ** indicates $p < 0.01$. Results were analyzed by Student's t-test. Data shown are mean values and error bars indicate standard error of the mean. Data points are color coded to represent individual mice correspond to colors in Figure 20.

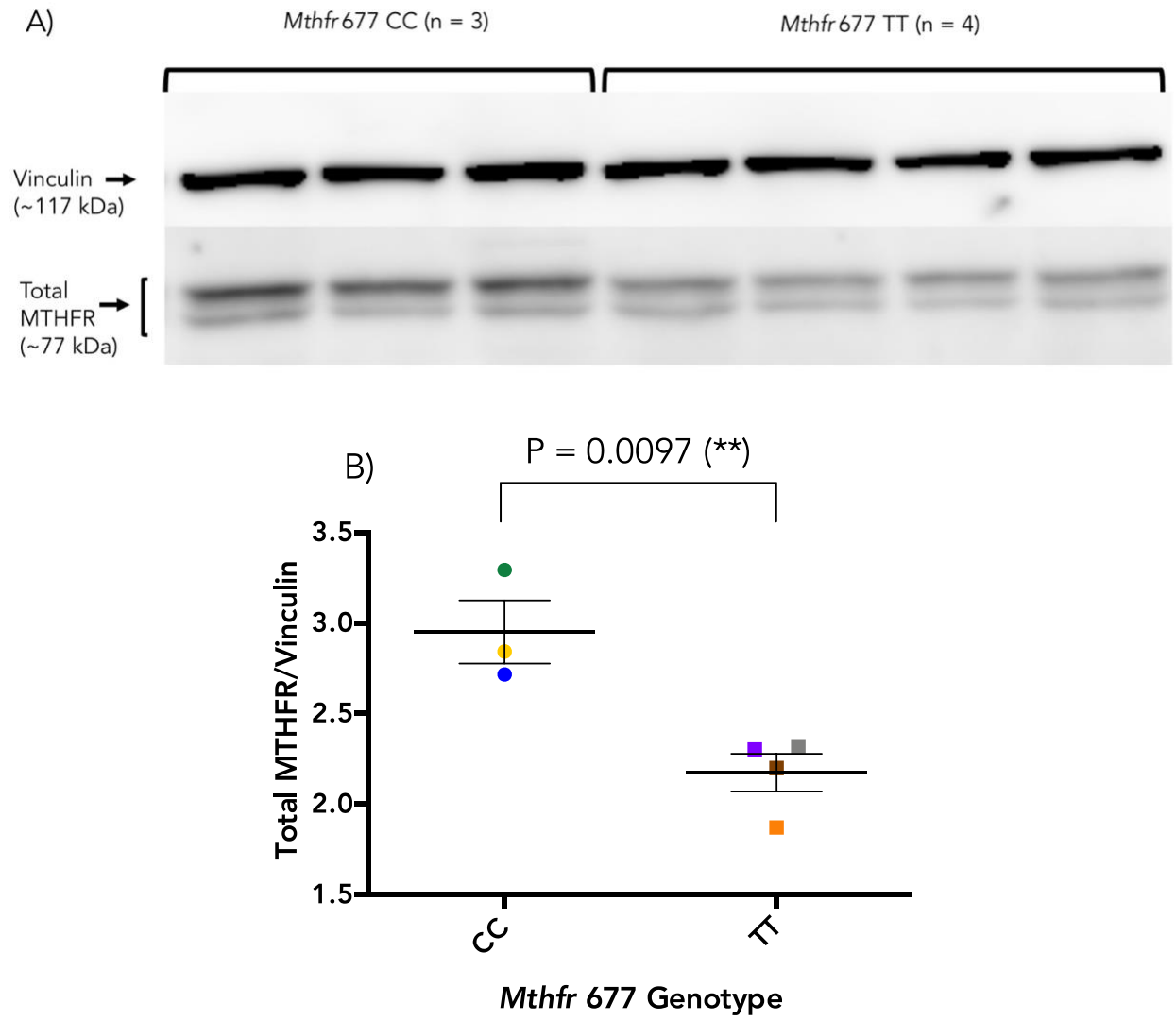

Figure 20. MTHFR protein expression in hepatic tissue from reproductive age (4-5 weeks old) *Mthfr* 677 CC and *Mthfr* 677 TT females fed rodent chow. **(A)** Western blot indicating protein bands of vinculin (loading control) and total MTHFR. **(B)** Expression level of total MTHFR/vinculin in hepatic tissue extracts. Bullet points indicate individual samples. Significant reduction in expression in *Mthfr* 677 TT extracts was observed. Results were analyzed by Student's t-test. ** indicates $p < 0.01$. Data shown are means and error bars indicate standard error of the mean. Data points are color coded to represent individual mice correspond to colors in Figure 19.

Chapter VI: Discussion and Future Directions

6.1 Low dietary choline and MTHFR deficiency (*Mthfr*+/-) on reproductive outcomes, maternal choline metabolite levels, and brain development during pregnancy at E10.5

Folate deficiency, either through inadequate consumption of folates or through genetic aberrations such as the *MTHFR* 677C>T variant, increases the risk of negative pregnancy outcomes or embryonic defects such as NTD. Maternal supplementation with folate has been found to rescue this adverse effect by up to 75% (34). Folic acid is metabolized to 5-methylTHF, the major circulatory form of folate, and a methyl donor for the remethylation of homocysteine to methionine (**Figure 1**). When folate metabolism is disturbed, choline is used as an alternate methyl donor for homocysteine remethylation through its derivative betaine (**Figure 2**). This pathway is upregulated when folate metabolism is altered. A murine model for MTHFR deficiency was developed by a targeted knockout of the *Mthfr* gene and has been used for reproductive studies by the Rozen Laboratory (41). Pregnant *Mthfr*+/- mice have an increased risk of embryonic CHD and delay when dietary folate is low at E14.5 (43). The impact of choline when MTHFR is altered on reproductive and embryonic outcomes such as the risk of NTD is largely unknown. The objective of this study was to investigate the effects of low dietary choline (3-times deficient than the requirement for rodents) and *Mthfr*+/- status on pregnancy outcomes and embryonic development. It was hypothesized that low choline will increase the risk of pregnancy complications and embryonic defects due to an increased demand for choline when folate metabolism is altered. Maternal hepatic TMAO levels were measured, and were decreased as a result of ChDD (**Figure 4J**), which was expected as TMAO is generated via gut microbial metabolism when dietary choline is present (103). Most of the maternal hepatic choline metabolites measured were not affected by either diet, *Mthfr* genotype, or diet-genotype interaction (**Figure 4**), including methionine, 5-methylTHF, and choline. However, hepatic betaine and DMG levels were decreased as a result of low dietary choline, particularly when genetic *Mthfr* deficiency is present (**Figure 4B and I**), indicating an increased demand for betaine in these mice. 10-11 litters per group were collected and we did not observe an increased risk of pregnancy complications as there were no differences in the rate of implantation (**Figure 6**) or the incidence of embryonic resorptions (**Figure 9B**) at E10.5 due to diet, genotype, or diet-genotype interaction. In a human assisted reproductive technology study, the *MTHFR* 677 TT

genetic profile was found to negatively impact oocyte retrieval, even when patients were supplemented with the RDA of folate (104). However, no differences in the number of ova released were observed in this study, possibly due to differences in hormonal regulation that modulate ovulation between humans and mice. The fertility outcomes of *Mthfr*+/- female mice when dietary folate or choline is low are not well known. Overall, the risk of poor embryonic development was not altered due to low dietary choline, maternal genotype, embryonic genotype, or diet-genotype interaction, as embryos from all four groups had no differences in size, or the incidence of delay and defects (**Figures 5-8**). It is possible that 3-times lower choline in the diet was not deficient enough to result in negative outcomes, and mice may not be sensitive to low choline at this level. While there was no increased risk of NTD observed in this study, previous work in the Rozen Laboratory has found that low maternal choline and maternal *Mthfr*+/- status results in poor memory outcomes in pups (55). While neurulation (at stage E10.5) may not be impacted, neurogenesis during later stages of embryo development may be altered but this is unknown. An unexpected outcome was that low choline resulted in lower % brain weights of pregnant *Mthfr*+/- mice at the time of sacrifice (**Figure 3B**). The demand for choline is increased during pregnancy in humans, as it is required for embryonic brain development (71). In this study, pregnant mice were approximately 9 weeks old at sacrifice, which is considered adolescence as rapid neurodevelopment occurs until 3 months of age in mice (105). No differences were observed in cortical measurements of choline metabolites (**Figure 5**). The differences in brain weight between pregnant mice were of total brain weight, not specifically the cortex. It is possible that the demand for choline metabolites in the cortex was not impacted. However, it may still be possible that maternal choline metabolism and placental transfer of choline is increased in pregnant *Mthfr*+/- mice to ensure proper embryonic development, which could be assessed in the future. This may result in lower transport of choline derivatives to the mother's brain for neurogenesis and could be poorly impacting maternal brain development. Another useful parameter to assess may be the methylation status in ChDD-fed *Mthfr*+/- mice, by analyzing SAM and SAH levels in the liver and brain, to understand how methylation patterns and gene expression may be impacted. This was planned for this study, however, due to research restrictions during the COVID-19 pandemic, these data could not be collected before the completion of this thesis.

6.2 Behavioural outcomes due to low dietary choline and *Mthfr*+/- status

Choline supplementation has been found to improve behavioural outcomes in rodents (85-90), and high choline intake status during gestation in humans correlates with positive neurological outcomes in offspring (84). Choline derivatives are important for neurogenesis and brain development during gestation and early life. In this study, preliminary behavioural outcomes due to low choline and *Mthfr*+/- status in reproductive-age female mice were assessed (**Figure 12-Figure 13**). However, due to low sampling (2-3 mice/group), detailed statistical analysis was not possible. Measuring these parameters in more mice would be useful to understand behavioral outcomes due to low choline. A detailed analysis of brain development during adolescence could be performed by collection of brain tissue for metabolic and histological analyses on *Mthfr*+/- female mice on ChDD. Whether poor behavioural outcomes due to *Mthfr*+/- status and low choline during pregnancy also occur is not known, and a detailed behavioural study during pregnancy may yield interesting results.

6.3 Reproductive outcomes due to *Mthfr* 677 TT status and low or supplemental folate in maternal diet

In humans, the *MTHFR* 677C>T variant is the result of a SNP that causes an alanine to valine substitution and an MTHFR enzyme with increased thermolability and lower specific activity which is stabilized with folate supplementation (12). While the *Mthfr* heterozygous knockout mouse (*Mthfr*+/-) has HHcy and is a useful reproductive model for dietary studies of MTHFR deficiency, it does not have a thermolabile MTHFR enzyme (41). The novel *Mthfr* 677 TT mouse was developed by collaborators and used for the first time for a preliminary reproductive study. The objective of this study was to investigate the effects of *Mthfr* 677 TT status and low or supplemental dietary folate on the risk of embryonic development and pregnancy outcomes. Few litters (2-3 per group) were collected, and detailed statistical analysis was not possible due to low power. Further sampling of up to 9-12 litters per group may be necessary to fully understand reproductive outcomes for this model. No preliminary results were observed in the risk of implantation defects (**Figure 15**). However, there may be differences in the rate of resorptions between CD and FD-fed maternal groups (**Table 4**). It is possible that low folate (even as low as 2.5-times lower than the control) and/or *Mthfr* 677 TT status may result in greater embryonic resorption. However, this needs to be further studied to be fully elucidated. While no statistical tests could be performed to compare the risk of embryonic defects, one

embryo with an NTD was observed from a CD-fed *Mthfr* 677 TT mouse (**Figure 18D**). Currently, enzymatic activity studies are being performed in *Mthfr* 677 TT mice tissue to characterize its folate metabolic status. Future studies on hepatic tissue from pregnant *Mthfr* 677 TT mice on low and supplemental folate would be useful to understand if pregnancy further alters MTHFR activity or stability in these mice, and if folate supplementation rescues these phenotypes.

6.4 Modulation of folate and choline metabolites in *Mthfr* 677 TT females.

The *MTHFR* 677 TT status in humans is associated with increased homocysteine (as seen in mild HHcy) and altered folate and choline metabolism due to an increased demand for choline for homocysteine remethylation (21). Furthermore, human lymphocytes from *MTHFR* 677 TT individuals were found to have thermolabile MTHFR enzymes with reduced specific activity (25). For the first time, folate metabolite status in hepatic tissue was analysed in *Mthfr* 677 TT female mice in this study. 4 female mice of reproductive age per group (*Mthfr* 677 CC and *Mthfr* 667 TT) were used for hepatic tissue collection. *Mthfr* 677 TT females had increased plasma tHCy levels, and lower levels of plasma methionine, betaine, and choline (**Figure 19**). This is consistent with human individuals with *MTHFR* 677 TT genetic status (21), as well as *Mthfr*+/- mice, who have mild HHcy and an increased demand for the choline-betaine pathway for homocysteine remethylation (41). It would be interesting to know if low dietary choline during pregnancy could alter reproductive outcomes and the risk of NTD in the *Mthfr* 677 TT mouse. Also, western blotting showed that hepatic expression of MTHFR is reduced in *Mthfr* 677 TT female mice compared to wild-type (**Figure 20**). This is a novel result as western blot expression of MTHFR protein in human tissues from *MTHFR* 677 TT individuals has not been studied. No differences were seen in the levels of plasma cystathionine, SAM, SAH, the SAM:SAH ratio, or 5-methylTHF (**Figure 19**). Cystathionine is produced from the transsulfuration of homocysteine, an alternate pathway to decrease homocysteine levels (14). However, cystathionine-β-synthase, the enzyme that catalyzes this reaction is primarily expressed in liver, not blood (15). Analyzing concentration of folate metabolites in hepatic tissue may be useful to understand if any tissue-specific modulation is occurring. Low folate or MTHFR deficiency (*Mthfr*+/- mouse) results in altered methylation potential due to decreased SAM, increased SAH, and a lower SAM:SAH ratio (36,41). This was not observed in the females used in this study, as this could be due to low sample size (only 4 per group were analyzed). Another parameter to assess could be age and sex

to understand if methylation status is impacted with age or in males. 5-methyTHF, the primarily circulatory form of folate, is also reduced due to the *MTHFR* 677 TT variant; however, this was not observed. These females were on rodent chow, with 24-hour access to *ad libitum* food, which contains 4 mg folate/kg diet, the standard for rodents. It is possible dietary folate may be lessening the impact of genetic MTHFR deficiency. Furthermore, *MTHFR* 677 TT individuals have an enzyme with lessened activity compared to *MTHFR* 677 CC individuals, but it is not completely inactive. It may be possible that *Mthfr* 677 TT mice also have a basal level of activity, or that the dietary folate in rodent chow may be rescuing its activity to wild-type levels, thus explaining why 5-methylTHF and methylation potential were not altered.

Chapter VII: Conclusion and Future Directions

In this thesis, two studies of alterations in one-carbon metabolism and their impacts on reproductive outcomes were outlined. For the first study using ChDD and *Mthfr*+/- mice, it was described that low dietary choline and maternal *Mthfr*+/- status resulted in lower brain weights during pregnancy, as well as decreased hepatic betaine and DMG. However, it did not negatively impact pregnancy outcomes nor the risk of embryonic defects. The choline-deficient diet may not have been deleterious enough to impact reproduction, or these females may have a compensatory mechanism to increase placental transport of choline metabolites to the developing embryo. However, this is unknown, and could be assessed through further analysis. There were no differences in many choline metabolite in the liver or cortex of the low choline fed mice, indicating a compensatory method to maintain levels of choline metabolism. Further analysis of methylation potential (through SAM/SAH measurements) may be useful and are planned. Choline is a largely understudied nutrient, in particular with regards to its role during pregnancy and embryonic neurulation. Many pregnant women do not receive enough choline through diet, and may require supplementation, in particular if they have MTHFR deficiency (such as the *MTHFR* 677C>T SNP) to lessen the risk of reproductive complications. In the second study, a novel model for MTHFR deficiency, the *Mthfr* 677 TT mouse, was used to analyze the impact of low and supplemental dietary folate. The data collected for this project are preliminary and must be further investigated with more sampling to fully elucidate reproductive outcomes. However, for the first time, it was found that *Mthfr* 677 TT female mice have altered homocysteine remethylation and choline-betaine levels compared to the *Mthfr* 677 CC mice. Furthermore,

hepatic expression of MTHFR is reduced in female *Mthfr* 677 TT mice. A low sample size of 4 was sufficient to observe differences in metabolite levels in non-pregnant mice on rodent chow and indicating this model seems to be promising for future studies on dietary folate/choline and genetic MTHFR deficiency. However, reproductive studies often require further sampling (at least 8-10 litters), particularly if the outcome of interest is already rare (*i.e.* an NTD), to discern if the event occurs due to random chance or as an outcome of treatment such as changes in dietary nutrients. Ultimately, the goal of murine studies is to help humans impacted by deleterious genetic variants, such as the *MTHFR* 677 TT signature, and to understand how nutrient supplementation can be beneficial to those affected by the adverse effects of MTHFR deficiency.

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Appendix

Centre universitaire de santé McGill
Institut de recherche

McGill University Health Centre
Research Institute

This is to certify

Maryam Tahir

Has completed the following workshop courses at the Research Institute of the McGill University Health Center (RI-MUHC):

MOUSE MODULE 1
On
September 6, 2018

Techniques successfully completed:
Handling/Restraint
Isoflurane/CO2 euthanasia
Cardiac Puncture (blood collection) under anesthesia
Cervical Dislocation under anesthesia
Decapitation under anesthesia
Pneumothorax

Carla Iacovelli
RI-MUHC Training Coordinator

Centre universitaire de santé McGill
Institut de recherche

McGill University Health Centre
Research Institute

This is to certify

Maryam Tahir

Has completed the following workshop courses at the Research Institute of the McGill University Health Center (RI-MUHC):

MOUSE MODULE 2
On
October 4, 2018

Techniques successfully completed:
Subcutaneous injection
Intraperitoneal injection
Saphenous vein blood collection

Carla Iacovelli
RI-MUHC Training Coordinator

Table of Contents

35- Personnel List

List the names of all individuals who will be in contact with animals in this study and their employment classification (co-investigator, technician, research assistant, undergraduate / graduate student, teaching assistants and fellow). Do not add a person if he/she will not be starting to work soon.

Note that the **Principal Investigator's name** is automatically included BUT his/her name needs to be clicked in order to answer the questions and select the species and Activities performed by the PI.

If this is a renewal, an amendment or a copy from an existing protocol, you must **click each of the names to ensure all questions for this person are answered and Activities assigned.**

Training is mandatory for all personnel listed here. Refer to Training Requirements for details.

For new people added to the list, it may take a few days to receive access to Darwin. If access is urgently needed, please request it via the Web form."

Everyone is covered under an **Occupational Health Program (OHP)**. For all students as well as McGill staff and faculty, see the Environmental Health and Safety Web site to apply. For staff in hospitals with a Health Office, please contact them; if no Health Office, you may apply to McGill's OHP. To find out more, visit the OHP section of the Theory course.

If you have removed procedures, drugs, euthanasia methods, hazards and surgeries, please uncheck the box for the corresponding Activity for each person who had been assigned to perform or use it.

NOTE: Sometimes the red dot does not change to green for this section (intermittent issue), continue with the next sections and submit the protocol or amendment as usual; it will not prevent you from submitting it to the FACC. If you are unable, please contact Darwin Support, darwin@mcgill.ca.

Need help?

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Rozen, Rima	934-1934X23839	rima.rozen@mcgill.ca	[redacted]	Yes	
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Tahir, Maryam	5149341934 23281	maryam.tahir@mail.mcgill.ca	[redacted]	Yes	Yes

Note that if a person is removed from all the protocols under this Principal Investigator (PI), then Darwin login access will be revoked entirely.

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