Enhancing Folic Acid Dietary Intervention Research in Mouse Models

By

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Abstract

Folates are a group of enzyme co-factors responsible for carrying out cellular one-carbon metabolic reactions. This one-carbon metabolic pathway plays a critical role in the de novo production of purines, thymidylates and the precursors required for various methylation reactions. Adequate folate status is vital for growth, development, and maintenance of health in both humans and animals. However, like many essential nutrients, there are concerns over potential adverse health effects associated with both inadequate and excess folate consumption. Specifically, folic acid (FA), the synthetic form of folate used in fortified foods and supplements, has been associated with both beneficial and potentially adverse effects. Hence, characterization of safe and adequate FA intakes while avoiding the risk of an adverse health effect is important for the nutritional risk assessment of FA. Animal-derived data play an important role in the elucidation of the specific mechanisms of action linked to FA intake. However, poor reporting of study details, and the inconsistent use of diets and animal models, hinders knowledge translation from animals to humans. Here I report the outcomes of two studies. The first was a scoping review of the literature to determine the reporting quality of studies examining the effect of dietary FA interventions in mice. The findings of our scoping review showed that 14% of studies did not report ≥1 generic reporting item(s) (i.e., sex, strain and age) and 41% did not report ≥1 nutrition-specific reporting item(s) (i.e., base diet composition, intervention doses, duration, and exposure verification). This incomplete reporting of findings notably limits their generalizability, reproducibility and interpretation. The second study was designed to facilitate the knowledge translation of animal-derived data to human nutrition by establishing biomarkers of folate intake, status and function in mice. This FA dose-response study allowed me to identify a biomarker of folate deficiency, namely a homocysteine concentration ≥ 3.88 umol/L as a functional marker of deficiency. I also

propose that an unmetabolized FA concentration ≥ 7.71 nmol/L represents a marker of excess FA intake. The observations made in these two studies will inform future study designs for assessing the effects of FA on health outcomes in mouse models.

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Table of Contents

List of Abbreviations

10-formyl THF	10-formyltetrahydrofolate
5-methyl THF	5-methyltetrahydrofolate
5, 10-methylene THF	5, 10-methylenetetrahydrofolate
5,10-methenyl THF	5,10-methenyltetrahydrofolate
AI	Adequate Intake
BMD	Benchmark Dose
CRC	Colorectal Cancer
DHF	Dihydrofolate reductase
DRIs	Dietary Reference Intakes
EAR	Estimated Average Requirement
FA	Folic Acid
FTHFS	Formyltetrahydrofolate Synthetase
Hcy	Homocysteine
MTHFC	Methylenetetrahydrofolate Cyclohydrolase
MTHFD	Methylenetetrahydrofolate Dehydrogenase
MTHFD1	Methylenetetrahydrofolate Dehydrogenase 1
MTHFR	Methylene Tetrahydrofolate Reductase
MTR	Methionine Synthase
NTDs	Neural Tube Defects
PCFT	Proton-coupled folate transporter
RBC	Red Blood Cell
RDA	Recommended Dietary Allowances
RFC	Reduced folate carriers
SAH (or AdoHcy)	S-Adenosyl-Homocysteine
SAM (or AdoMet)	S-Adenosyl-Methionine
THF	Tetrahydrofolate
TMP	Thymidine Monophosphate
TS	Thymidylate Synthase
UL	Tolerable Upper Intake Level
UMP	Uridine monophosphate

List of Figures

List of Tables

CHAPTER 1: INTRODUCTION

1.1. Overview of Folate

Folates are a group of essential water-soluble vitamins, also known as vitamin B_9, obtained through the diet. The term folate refers to a family of polyglutamate enzyme co-factors responsible for one-carbon metabolic reactions in the nucleus, mitochondria, and cytoplasm (1). Sources of naturally-occurring folates are green leafy vegetables (e.g. spinach), legumes (e.g. cabbage, broccoli), citrus fruits (e.g. oranges and grapefruit juices), and other fruits such as bananas, apples, avocadoes and tomatoes (2,3). It is also naturally occurring in yeast, cheese, poultry (e.g. egg yolks) and meat products such as liver, kidneys or tongue (2). These naturally occurring folates consist of a two-ring structure with one to six glutamate molecules peptide bonded to a carboxyl group p-aminobenzoylglutamic acid (3,4). Additionally, folates can also be obtained in its synthetic form "folic acid" (FA) in enriched foods or supplements. Synthetic FA (**Figure 1a**), also known as pteroylglutamic acid, is more bioavailable and stable than naturally occurring folates. As a result, it is used in food supplements and fortification initiatives across the world. FA enriched foods in Canada include white wheat flour, enriched ready-to-eat cereals, and enriched pastas and cornmeal (5). FA is the most oxidized form of folate with a single glutamate molecule peptide bonded to a carboxyl group glutamine hence, accounting for its stability and increased ability to be absorbed by cells; it does not occur in nature (3).

1.1.1. Absorption and transport of folate

Once obtained from the diets, monoglutamate folates are absorbed into the upper intestinal tract by a proton-coupled folate transporter (PCFT). The low pH dependent PCFT transporters

have a very low affinity for the polyglutamate forms of folate. Consequently, folates need to be deconjugated into a monoglutamate form through the hydrolysis action of glutamate carboxypeptidase II in the intestine (4). Mediated by the Na^+/H^+ proton gradient, folate monoglutamates are absorbed across the apical brush-border membrane of the proximal jejunum by PCFTs and transported to the liver via the hepatic portal system. Unlike naturally occurring folates, intestinal hydrolysis is not required for monoglutamate FA for transport into intestinal cells. Upon arrival in the liver, folates are further metabolized in preparation for liver storage, transport to peripheral tissues via the systemic circulation, and reabsorption into circulation through the kidneys (4). Folates enter the systematic system from the liver via the hepatic vein and are transported into peripheral tissues through the actions of reduced folate carriers (RFC) (4). The most abundant form of folate in the bloodstream is 5-methyltetrahydrofolate (5-methyl THF). Unlike PCFT, RFC transporters have a very low affinity for the FA. Therefore, FA metabolism to reduced bioactive folates occurs in the liver before transport to systemic circulation. However, in cases where FA intakes are supraphysiological, unmetabolized FA can enter systemic circulation.

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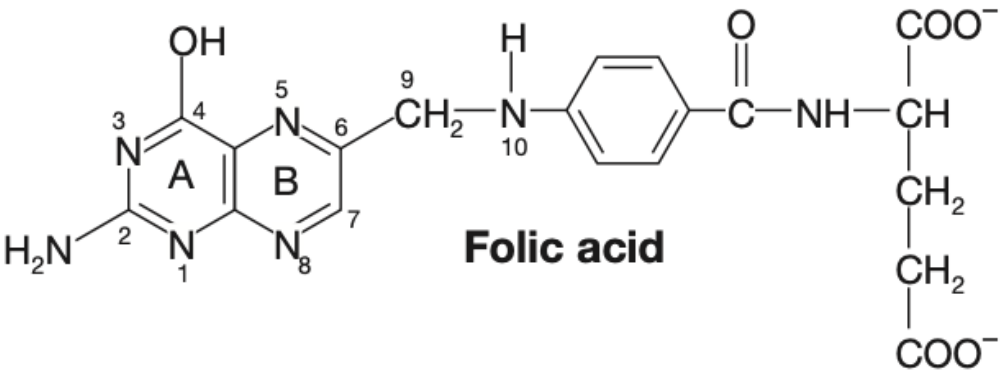

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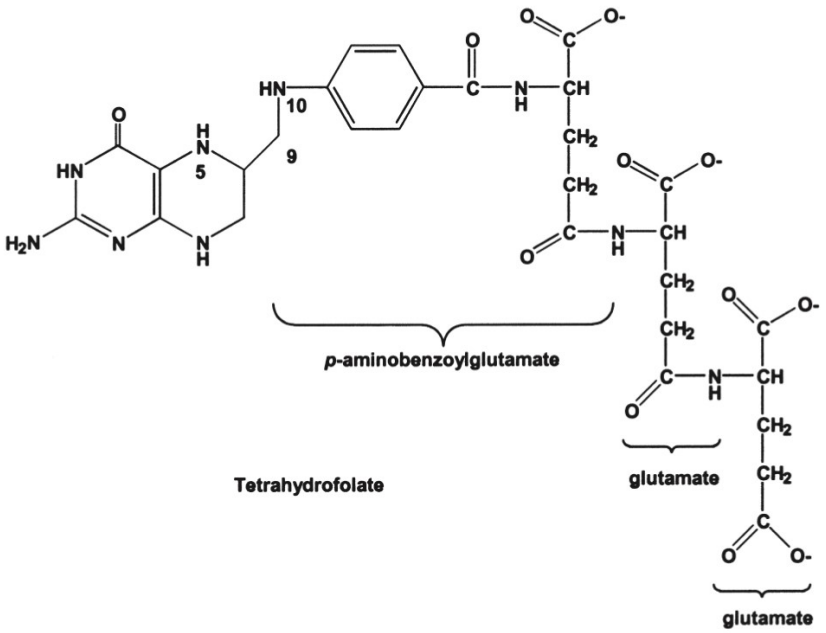

Figure 1: Folate Structure. **A)** Synthetic Folic Acid Structure. **B)** Tetrahydrofolate (THF) structure with 3 glutamate moieties attached. Different folate forms consist of the attachment of one-carbon groups at different positions of the THF structure. This could consist of a methyl group (CH3) at position N-5 (5- methyltetrahydrofolate), a methylene group (CH2) linking N-5 and N-10 (5,10- methylenetetrahydrofolate), a methenyl group (CH) linking N-5 and N-10 (5,10- methenyltetrahydrofolate), or a formyl group (CHO) at position N-10 (10-formyletetrahydrolate).

1.1.2. Folate metabolism

Folic acid is not bioactive and must therefore be reduced to enter 1 carbon metabolism. It is irreversibly reduced to dihydrofolate (DHF) by the activity of the NADPH-dependent enzyme dihydrofolate reductase (DHFR) and subsequently to tetrahydrofolate (THF), the form in which it enters the cellular folate pool. Within cells, folylpolyglutamate synthase enzymes restore linkage of polyglutamate chains onto folate monoglutamate (5-methyl THF and DHF) molecules, resulting in reduced THF polyglutamate (**Figure 1b**). Monoglutamate folates have low affinity for the folate-binding enzymes, whereas polyglutamates have high affinity, thereby restricting them within the cell (6). The polyglutamated form can have up to 7 glutamate residues attached (7).

THF polyglutamate, the bioactive form of folate acts to carry and transfer one-carbon units in the mitochondrial, nuclear or cytoplasmic compartments. The mitochondrion contains about 40% of cellular folates while the nucleus contains about 10% (8). The mitochondrion uses one-carbon metabolism to form formate, which in turn is exported to the cytoplasm where it is further utilized as the source of one-carbon units in cytoplasmic metabolic reactions. Formate is synthesized from formaldehyde, a by-product of serine, glycine, dimethylglycine and sarcosine catabolism in the mitochondria (8). In the cytoplasm, formate is involved in the interconversion of THF, 10-formyl THF 5,10-methenyl THF, and 5,10-methylene THF mechanisms governed by the trifunctional enzymatic activity of the NADPH-dependent enzyme methylenetetrahydrofolate dehydrogenase 1 (MTHFD1) (9). First, formate is incorporated at the C2 and C6 carbons of the purine ring of THF to 10-formyl THF via the enzymatic activity of formyltetrahydrofolate synthetase (FTHFS; first enzymatic activity of MTHFD1) (1,9). The cofactor 10-formly THF is used for *de novo* purine biosynthesis (**Figure 2**) in the cytoplasm (8). Next, methenyltetrahydrofolate cyclohydrolase (MTHFC; second enzymatic activity of MTHD1) reduces 10-formyl THF to 5,10-methenyl THF,

which is subsequently further reduced to 5,10-methylene THF by methylenetetrahydrofolate dehydrogenase (MTHFD; third enzymatic activity of MTHFD1) (9). The latter is the enzyme cofactor required for *de novo* thymidylate biosynthesis in the nucleus (Figure 2). The thymidylate synthase enzyme (TS) catalyzes the methylation reduction of deoxyuridylate (dUMP) to form thymidylate (dTMP) (8). During the production of thymidylate, DHF is also generated and reduced back to THF by the DHFR to re-initiate the cycle (8).

5,10-methylene THF can alternatively be reduced to 5-methyl THF by the NADPH-dependent enzyme methylene tetrahydrofolate reductase (MTHFR) (Figure 2). 5-methyl THF functions as the methyl donor in methionine synthesis. Homocysteine (Hcy) is methylated to methionine through a vitamin B_{12}-dependent reaction catalyzed by methionine synthase (MTR) (8). Biosynthesis of methionine is essential for the production of S-adenosyl-methionine (SAM or AdoMet), the universal cellular methyl donor for various methylation reactions, including that of DNA, RNA, neurotransmitter, phospholipids, proteins (e.g. histones) and other small molecule (10). S-adenosylhomocysteine (SAH or AdoHcy) produced upon the donation of a methyl group from AdoMet is converted back to homocysteine through the action of S-adenosylhomocysteine hydrolase.

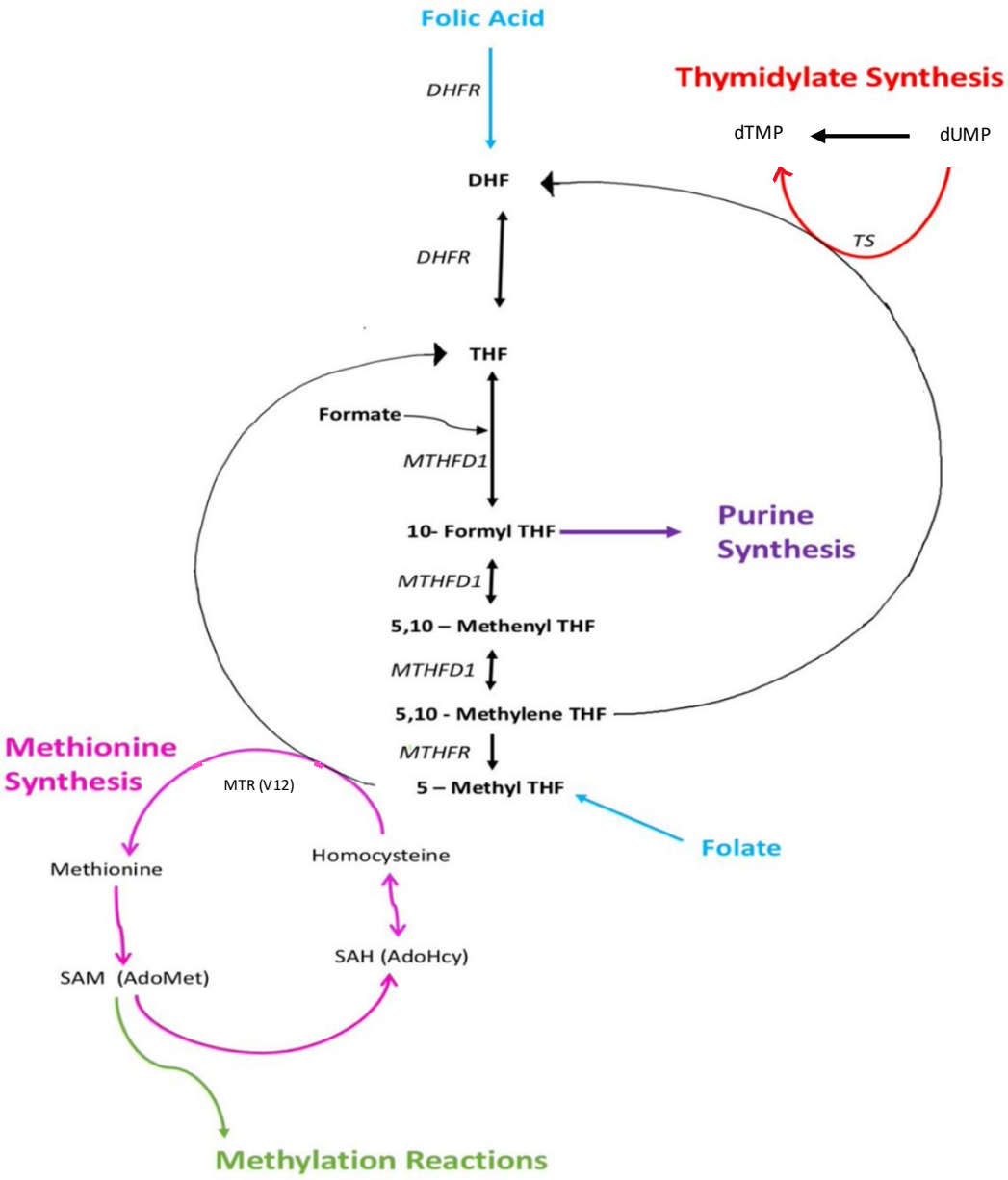

Figure 2: One-Carbon Metabolism of Folate. DHF: dihydrofolate reductase, MTR: methionine synthase, MTHFD1: methylenetetrahydrofolate dehydrogenase 1, MTHFR: methylenetetrahydrofolate reductase, SAH: S-adenosylhomocysteine (AdoHcy), SAM: S-adenosylmethionine (AdoMet), THF: tetrahydrofolate, TS: thymidylate synthase. Figure modified from Crider KS, 2012 and Stover PJ, 2009 (8,10).

1.2. Folate and Human Pathology

Folate metabolism plays an essential role in the normal development, growth, and maintenance of optimal health (11). Any disruption in the one-carbon metabolic reactions may lead to various adverse health outcomes. Neural tube defects and megaloblastic anemia are two well-characterized health outcomes known to be causally related to folate intake and status. Other health risks, such as congenital anomalies, cancers, cardiovascular diseases and cognitive decline have also been associated with folate intake (8).

1.2.1. Folate and Neural tube defects

Globally, neural tube defects (NTDs) affect an estimate of over 300,000 fetuses or infants every year (12). The neural tube is the embryonic precursor for the brain and spinal cord making up the central nervous system (13). NTDs arise when the neural tube fails to close during the first 18 – 28 days after conception, a period with increased demand for nucleotides and methylation reactions for the rapidly developing embryo. The most common NTDs are spina bifida and anencephaly. Anencephaly is fatal and affects over 4000 pregnancies in the US annually (14). It is a result of the incomplete closure of the anterior end of the neural tube compromising the development of the fetus – often leading to fetal or perinatal death. Spina bifida is characterized by the incomplete closure of the posterior end of the neural tube. Although there is an increased chance of a viable pregnancy, the child is prone to various disabilities ranging from loss of bowel and bladder control to paralysis due to the impaired development of the spinal cord and lower backbones (15,16).

The UK Medical Research Council (MRC) randomized double-blinded clinical trial published in 1991 was one of the first important studies that demonstrated a strong correlation between

dietary FA and incidence of NTD-affected pregnancies (17). The study demonstrated that a daily intake of 4000 μg of FA by high-risk women (women who have had a previous NTD-affected pregnancy) lowered recurrence by 72%. A 1992 study conducted by Czeizal and Dudás in low-risk women treated with 800 μg of FA demonstrated similar results (18). Lastly, another trial using a daily dose of 400 μg of FA in a population-based community trial in China by Berry et al. also demonstrated similar results (19). Berry et al. and Czeizal et al.'s study became the basis for recommendations by governing bodies including the WHO and Health Canada that low-risk women consume 400-800 μg (20).

Mandatory folic acid fortification was implemented in Canada and the United States in 1998 to increase folate intake, raise the population's baseline folate status, and inevitably decrease the prevalence of NTDs (3). Mandatory fortification included the addition of FA to white wheat flour with 150 μg FA added to every 100 g of flour. In Canada, other foods such as pasta and cornmeal can also be enriched with FA but must be labelled "enriched" on the package (21) . A 2007 study conducted by De Wals et al. demonstrated that the prevalence of NTDs in Canada reduced to 0.86 per 1000 birth from 1.58 per 1000 births after the fortification program was implemented (22). This translates to a 46% reduction (95% confidence interval, 40 to 51) in prevalence (22). Following suit, 87 other countries around the world implemented various models of fortification programs (3,23).

1.2.2. Folate and Megaloblastic Anemia

Megaloblastic anemia is a group of anemias characterized by impaired DNA synthesis due to vitamin B_{12} and/or folate deficiency during red blood cell (RBC) production (24,25). Both vitamin B_{12} and folate are cofactors in methionine synthesis. Deficiency in either one of these cofactors

results in increased homocysteine concentrations, as well as trapped 5-methyl THF within cells in a phenomenon known as 'methyl trap' (25,26). The methyl trap arises from reduced activity of the MTR enzyme when vitamin B_{12} is unavailable as a co-factor and/or 5-methyl THF is unavailable to transfer a methyl group to homocysteine to form methionine (Figure 2). Consequently, 5-methyl THF is not converted back to THF - which is eventually depleted and folate-mediated one carbon metabolism is inhibited. As a result, nucleotide synthesis and therefore DNA synthesis via the folate pathway is impaired. This is especially damaging for progenitor cells. During the G_1 phase of the cell cycle, progenitor cells continue to grow as RNA and protein production is relatively unaltered by the folate pathway. However, impaired DNA synthesis halts the cell cycle at the S phase and subsequent cell division step is obstructed. In RBCs, this results in enlarged erythroblasts with asynchronous maturation of the nuclear and cytoplasm (25). Physiologically and clinically, these abnormally enlarged erythroblasts are identified as megaloblastic (26). Megaloblastic anemia caused by impaired DNA synthesis and/or methylation reactions can lead to serious neurological damages if the underlining causes (i.e., folate and/or vitamin B_{12} deficiency) is left untreated.

1.2.3. Other health risks associated with folate deficiency

Low folate status and/or high plasma homocysteine concentrations have been associated with various diseases including cardiovascular diseases (CVD) and strokes (27). Various meta-analyses and observational studies have demonstrated associations between low folate status and increased risk of ischemic heart diseases and strokes by 32 and 59% respectively (28). Similarly, numerous studies have also shown an association between elevated plasma homocysteine and rapid cognitive decline, and an increased rate of dementia and Alzheimer's disease (27). Alzheimer's disease is

characterized by the accumulation of cellular amyloid- in the brain (29). Other case reports have also shown an association between folate deficiency and infertility but the causal mechanisms have yet to be determined (30). Further *in vivo* studies are required to demonstrate biological plausibility and explore mechanisms that govern these relationships.

1.2.4. Folate and Cancers

The Canadian Cancer Society states that cancer is the leading cause of death in Canada, with an estimate of 220,440 new diagnoses in 2020 alone (31). The role of folate intakes on cancer risks is divided in the literature. On one hand, concerns have been raised over the hypothesis that FA supplementation allow the progression of existing tumor cells by supplying the necessary nucleotides required for their rapid proliferation while inactivating tumor-suppressor genes via methylation (10,27). On the other hand, other meta-analysis studies such as those carried out by Pierth et. al, revealed that high folate intakes can provide a protective effect for some cancer (e.g. pancreatic cancer), have little to no effect for some (e.g. lung cancer) while potentially increasing the risk of others (e.g. Head and Neck squamous cell carcinoma and esophageal cancer) (23). The exact mechanism of these associations has yet to be outlined.

Colorectal cancer (CRC) is one of the most rigorously studied cancers in relation to folate intakes. In Canada, it is the third leading cause of cancer death, representing an estimate of 12.9% and 10.9% of all new cancer diagnosis in men and women respectively (32). While some studies have demonstrated that folate deficiency increases the risk of CRC, other studies have shown that increased FA intakes provide a protective effect against CRC. One of the proposed mechanisms brought forth is the increased DNA mutation frequency and impaired methylation mechanisms associated with folate deficiency in this highly proliferative tissue (33). Four case-controlled

studies conducted by Kim et al. showed that individuals who had a high total folate intake (≥ 900 ug/day) were 35% less likely to develop CRC than individuals who had a low total folate intake (≤ 200 ug/day) (34). Further studies have also shown that gene variations in the MTHFR and SHMT enzymes in the folate pathways have been associated with a protective effect against CRC (33).

1.3 Nutritional risk assessments

Proper and adequate nutrition is vital for growth, development and maintenance of health in both humans and animals. Nutrition research examines the relationship between nutrient intake and health outcomes including disease of deficiency and chronic disease. Data derived from animal and cell culture models inform the mechanisms of actions of nutrients and other food substances (non-nutritional components of food) and supports their casual relationships to health outcomes. For both essential nutrients and food substances obtained through foods and supplements, an exhaustive nutritional risk analysis is required to inform optimal intake.

Nutritional risk analysis encompasses both science-based nutritional risk assessment and policy-based risk management components working together to provide an informed and communicable intake guideline for vitamins and minerals (**Figure 3a**). As defined by the British Dietetic Association "nutritional risk assessment is defined as the systemic and structured process of collecting and interpreting information on foods and food supplements in order to make decisions about the nature and cause of nutrition-related health issues that affect an individual" (35,36). Namely, nutritional risk assessments set out to identify any adverse health effects associated with both inadequate and/or excess intakes of a given nutrient or other food substance based on available scientific research derived from humans, animals and *in vitro* studies (36).

Evidence supporting the relationships between nutritional intakes and health outcomes is used to inform nutritional risk assessments (**Figure** 3**b**). One example of a risk assessment-based framework for determining nutritional risk is that used for establishing the Dietary Reference Intakes (DRIs) for essential nutrients. The DRIs are based on the assumption of a U-shaped curve arising from the fact that there are adverse health comes associated with both inadequate and excess nutrient intakes, a feature that is unique to essential nutrients. The DRIs, developed by the National Academies of Sciences, Engineering and Medicine (NASEM), presents the risks associated with inadequate nutrient intake as well as the potential health risks associated with excessive intakes (37). The DRIs apply to the general healthy population and include the following values: Recommended Dietary Allowances (RDA), Estimated Average Requirement (EAR), Adequate Intake (AI) amounts as well as the Tolerable Upper Intake Levels (UL). Ultimately, the DRIs are used to establish public health nutrition policies in Canada and the United States and have been adopted by various other countries.

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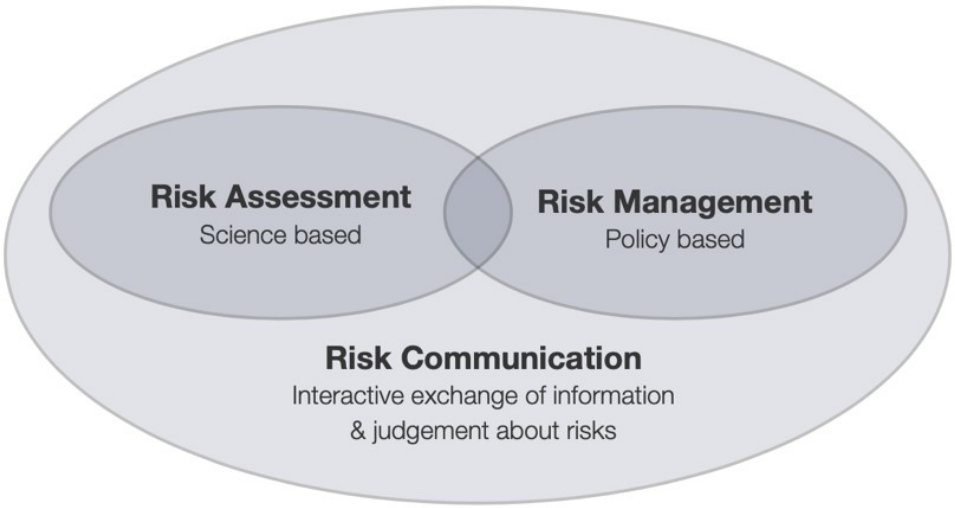

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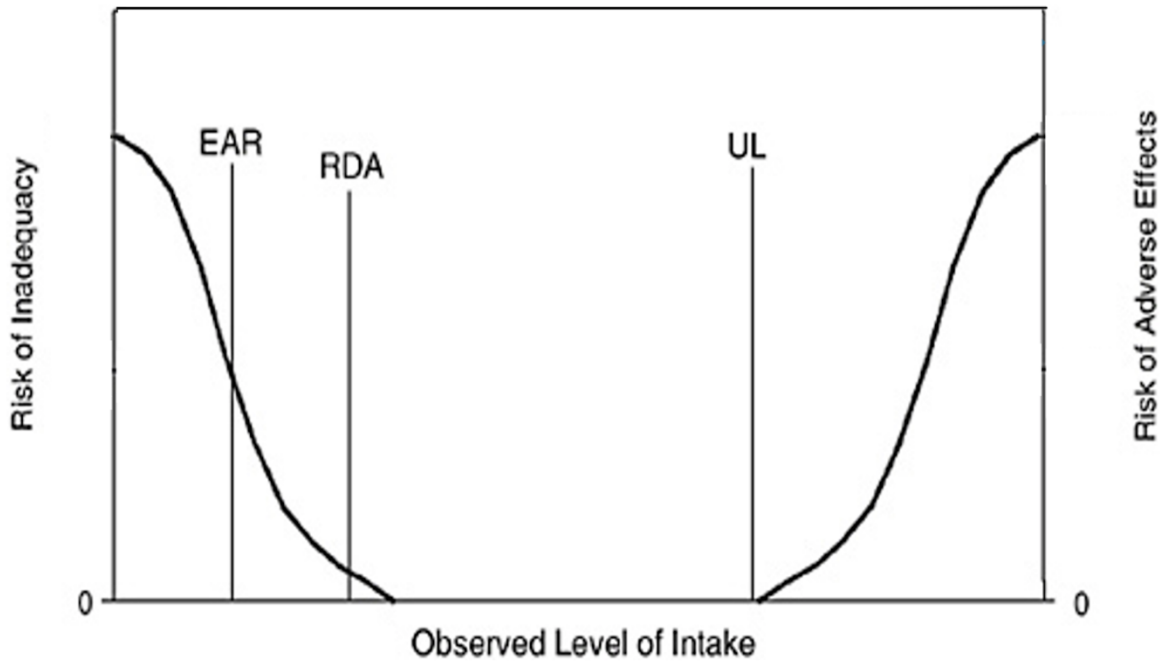

Figure 3: Nutrition Risk Analysis. A) The three key components of nutritional risk analysis as outlined by the 2006 FAO/WHO report (38). **B**) U-shaped nutritional risk assessment curve displaying both the requirement and potential adverse associated with nutritional intake. Each curve represents the adverse health effects associated with low (left) and high (right) intakes. Abbreviations: EAR, Estimated Average Requirement; RDA, Recommended Dietary Allowance; UL, Tolerable Upper Intake Level (35,39).

1.3.1 Folate nutritional risk assessment

The EAR and RDA for folate are primarily based on the minimum required folate intake required to ensure a red blood cell (RBC) folate status that prevents the development of megaloblastic anemia (40–42). RBC folate represents long-term folate status and is the preferred biomarker for assessing folate status (41). Secondary biomarkers such as serum and plasma folate can be used to determine recent folate intake. Hcy concentration can be used as a functional indicator of folate status since it is dependent on the rate of remethylation to form methionine, which requires the donation of a methyl group from 5-methyl THF. Note that Hcy is also influenced by the concentrations of other B vitamins such as vitamin B_{12}. At the population level, the WHO recommends a minimum RBC folate concentration of 226.5 nmol/L (100 ng/mL) to prevent the development of megaloblastic anaemia and a concentration of 906 nmol/L (400 ng/mL) for maximal protection against NTDs (41).

The folate DRIs are based on the intake of Dietary Folate Equivalent (DFE) values whereby 1 unit of natural folate is equivalent to 1.7 units of its synthetic form FA. This is because natural folate is ~ 35% less bioavailable than its synthetic counterpart (42,43). The RDA for infants and children (0-8 years) 80-200 ug DFEs/day depending on age, 300-400 ug DFEs/day for male and female teenagers (9-18 years) and 400 ug DFEs/day for male and non-pregnant females adults (> 19 years) (42). The RDA is increased to 600 ug DFEs/day for women during pregnancy and 500 ug during lactation (11,42). The UL is set for FA only, not natural folate, at 1000 ug/day in adults over 19 years (11,42). The UL represents a usual intake of FA that is not associated with increased risk for an adverse outcome (**Figure 3**) – this is the intake above which there is increasing risk for masking of vitamin B_{12} deficiency masking.

To date, no adverse effect has been causally associated with FA over-supplementation. However, the potential risk associated with the accumulation of unmetabolized FA in circulation and tissues is one of the hallmark concerns about over-supplementation. For example, a study carried out by Troen et. al. demonstrated an association between increased unmetabolized FA levels and a 23% decrease in natural killer (NK) cell activity among obese women (44). Some potential mechanisms proposed include the hypothesis that high FA intakes can oversaturate the DHFR enzyme resulting in increased levels of unmetabolized FA in plasma and tissues - especially in intestine tissues where FA is absorbed (4,45,46). Other studies have proposed that high DHF concentrations, due to high FA intakes, can function as non-competitive inhibitor of thymidylate synthase and a competitive inhibitor of MTHFR (47–49). On the other hand, it has also been hypothesized that any excess FA is excreted rather than reabsorbed in the kidneys (27). Ethically, these hypotheses cannot be directly tested in humans. However, a study design in animal models such as rodents could allow us to examine these potential mechanisms and determine the dose upon which elevated unmetabolized FA start to appear in plasma or tissues and whether they are related to adverse outcomes.

With the total FA intake increased due to food fortification and prenatal supplementation in addition to natural folates in our diets, there have been concerns over adverse health effects associated with over supplementation. The NASEM defined a Tolerable Upper Intake Level (UL) of 1000 μg FA per day. The UL was based on potential for FA to cause masking of vitamin B_{12} deficiency when the FA UL is exceeded (13). Two recent Canadian pregnancy cohort studies found that folate status was very high and revealed evidence of unmetabolized FA in circulation as a result of the combined exposure of FA from fortified foods and prenatal supplements (50). Such findings reinforce the call to further accurately define safe folate consumption ranges.

1.3.2 Mechanisms for Folate pathologies: Genomic Integrity and Epigenetics

One of the hallmark concerns of FA supplementation is vitamin B_{12} deficiency masking. Vitamin B_{12} deficiency is commonly misdiagnosed when present alongside folate deficiency (6). To treat folate deficiency, increased FA intakes are encouraged. As the FA enters the cell, it is metabolized to DHF and subsequently THF – replenishing the cell's THF supply. This allows the folate pathway to by-pass the methyl trap, resume nucleotide synthesis and reduce the prevalence of megaloblastic anemia. The replenished supply of the bioactive THF permits cells to complete the cell cycle and divide successfully. Nonetheless, megaloblastic anemia also functions as an early indicator of vitamin B_{12} deficiency. Without the anemia, vitamin B_{12} deficiency can go undetected and can lead to irreversible progression of neurological degeneration and cognitive decline (6). These two sides of folate supplementation and deficiency points out the conundrum in determining the safe and adequate range for folate intakes.

Folate-mediated one-carbon metabolic reactions play an essential role in the biosynthesis of purines, thymidylate and methionine. As seen with megaloblastic anemia, folate and/or vitamin B_{12} deficiency can cause a phenomenon called the methyl trap which prevents the regeneration of THF. Eventually, the depletion of THF within the cells results in the impaired synthesis of the purine nucleotides from 10-formyl THF and thymidylate from 5,10-methylene THF downstream. The reduced capacity to produce three of the four nucleotides required for DNA synthesis has serious consequences for rapidly proliferating cells. Previous studies have shown that impaired *de novo* purine synthesis resulted in impaired DNA repair, reduced capacity for DNA replication with associated mid-cell cycle arrest and increased occurrence of DNA mutagenesis due to an imbalanced nucleotide pools (51,52).

Additionally, intracellular dUMP levels (and eventually dUTP after serial phosphorylation) increase as the TS-dependent conversion to dTMP is compromised. These elevated uracil levels notably result in an increased rate of uracil misincorporation (24). When DNA repair mechanisms recognizes the error, Uracil DNA glycosylase enzymatically removes dUTP (UDG) from the DNA strand via the base excision repair pathway (53,54). However, DNA polymerase's attempt to patch and fill the gap with dTTP is hindered by intracellular shortages resulting in an increased chance of uracil re-incorporation (24,53). This results in a futile loop of attempting to correct misincorporation errors (53). Consequently, this leads to double-strand DNA breaks that leave chromosomes fragmented, unstable and prone to further mutations.

Furthermore, given that methionine can be converted to AdoMet, the universal methyl donor, folates also play a role in cellular methylation potential. The methionine synthesis pathway is influenced by the availability of 5-methyl THF to donate a methyl group to Hcy via the activity of the vitamin B_{12}-dependent MTR enzyme. DNA methylation patterns play a critical epigenetic role and influence gene expression. In addition to regulating the accessibility of transcription factors to the genome, methyl group on cytosine functions as anchors to DNA binding proteins and the recruitment of transcriptional silencing complexes (10). CpG methylation patterns are also critical in preserving nucleosome packaging as they dictate how tightly chromosomes pack around histones. This in turn functions as a gene regulation mechanism (55). Hypermethylation reaction have also been raised as a concern of FA supplementation. It has been hypothesised to silence promotor activity, thereby inactivating tumor-suppressor genes, regulatory and DNA repair genes or other functions that prevent carcinogenesis (27).

Even though there are multiple potential mechanisms by which folate intake could impact health outcomes, most remain hypothetical. Human observational studies are limited as they

cannot identify causal mechanism making mechanistic studies a necessity – this is where performing animal research is crucial. *In vivo* animal studies allow us to test these hypotheses and mechanisms in animal feeding studies. We know that folate is causally related to NTDs, but we do not know yet the exact mechanism. Each day we inch closer to defining these mechanisms using animal models. Nonetheless, although proposed safe FA intakes in humans is well established, their equivalents have yet to be defined in the commonly used animal models in folate research. Mouse models are essential for folate research to identify plausible casual pathways linking folate dietary intakes and health outcomes. However, it is also essential to accurately interpret animal-derived data to the relevant human context, something that can only be achieved when studies are designed to reflect comparable human intakes and findings are transparently and wholly reported.

1.4 Animals in research

While we know that there are causal relationships between folate and NTDs we do not know yet what the mechanisms are, and we cannot ethically examine these issues in humans. This is where animal research becomes an important part of assessing risk. They allow us to investigate and validate the mechanisms and causal associations. The functional and physiological similarities between humans, and some animal models, such as mice, have allowed researchers to study the progression of diseases, pathologies, responses to drugs and treatments, identify mechanisms and their interactions in a complex biological system (56). *In vivo* studies allows researchers to build onto data derived from *in vitro* studies by providing additional information on the effects, interactions and biochemical mechanisms involved in multisystem organisms (56).

In vivo studies are essential for research and continue to inform our knowledge. Scientific breakthroughs such as Type 1 diabetes treatments in 1921, vaccine development, as well as our

basic understanding of healthy biology and disease pathology can be traced back to animal-derived data (57). The laboratory mouse (*Mus musculus*) is one of the most prominently used animal models in the biomedical field. Biologically and physiologically similar to humans, their small size, relatively easy maintenance, high fertility rate, early maturity and large litter sizes render them as the animal model of choice for preclinical studies (57,58). Nonetheless, the use of animal models for research is also debated. Opponents argue that despite similarities to humans, there are distinct differences in animal models that reduces the validity of information obtained from these studies. Alongside animal welfare and protection concerns, opponents also argue that no single mouse model is capable of fully mimicking natural population variation or disease pathology in humans (57). This is particularly true for the readily available inbred mouse strains favored because their homogenous genetic background permits the production of results with high reproducibility and statistical power (57). Translation of this information to the human context can be challenging and often unsuccessful when compared to the heterogeneous nature of diseases in genetically diverse human populations (57,58). Furthermore, other concerns, such as size difference and its correlation to metabolic rates, as well as evolutionary trait differences, dispute the relevance of animal-model informed data as it relates to human research.

These valid concerns coupled with the undisputed importance of animal-based research created the foundation for the development of guidelines for animal use in research. These guidelines establish a balance between encouraging optimum knowledge translation from animal studies while minimizing the potential for unneeded research or harm (57). While conducting animal research, institutional Animal Ethics Committees adhere to the fundamental 3Rs – Refinement, Reduction and Replacement. Adherence to these ethical practices ensured that the minimum number of animals are used to produce reproducible data with precision and statistical significance.

Potential harm is minimized and implicit justification is provided for the use of a particular animal model and the intervention under study (59). Furthermore, animal reporting guidelines, such as the Animal Research: Reporting of In Vivo Experiments (ARRIVE) checklist, were developed partially to address concerns about transparency and reproducibility of findings, and to aid knowledge translation to human research (60). While animal research is important, it is only as good as the study designs and reporting of the research to ensure it can be translated to human health. Together, the ethical use of animal models and proper reporting of animal study design, conduct and outcomes play a critical role in informing future research, from basic biological principles to health care and policies. Essentially, guidelines help to ensure the best use of animals and maximize the ability to use the data derived from their research.

1.5 Objectives

1) I will perform a scoping review of recent mouse-based folate research as a case study to assess reporting of generic and nutrition-specific items in basic nutrition research.
2) I will identify the range of folic acid intakes in mice used in control, deficient and supplemented diets used in the folate research field.
3) I will identify strain and sex specific responses to folate exposure in mice.
4) I will determine the relationship between folate intakes using "traditional" exposure (plasma and tissue folate) and metabolic/health outcome (Hcy, macrocytic anemia) indicators in mice to better relate folate exposures in rodents to those in humans.
5) I will validate genome stability markers in blood cells (RBC micronuclei) as a potential functional outcome indicator of folate status in mice.

1.6 Hypotheses

1) I hypothesize that an evidence scan of the folate research field will identify a pattern of poor reporting quality of animal-based studies and the lack of a standardized definition of control, deficient and supplemented folate diets.
2) I hypothesize that the current 'control' diet (2mg/kg) is higher than required to minimize the functional and blood cell phenotypes in mice.
3) I hypothesize that RBC micronuclei will act as a functional indicator of folate deficiency.

CHAPTER 2: POOR REPORTING QUALITY IN BASIC NUTRITION RESEARCH: A CASE STUDY BASED ON A SCOPING REVIEW OF RECENT FOLATE RESEARCH IN MOUSE MODELS (2009-2019)

2.1 Abstract

Transparent reporting of nutrition research enables rigour, reproducibility and relevance to human nutrition. We performed a scoping review of recent articles reporting on dietary folate interventions in mice as a case study to determine the reporting frequency of generic study design items (i.e., sex, strain and age) and nutrition-specific items (i.e., base diet composition, intervention doses, duration, and exposure verification) in basic nutrition research. We identified 646 original research articles in the EMBASE, Ovid Medline, FSTA, Global Health and IPA databases published between January 2009 and May 2019 in which a dietary folic acid (FA) intervention was used in mice. Of these, 246 original peer-reviewed articles including 152 studies in non-pregnant and 96 in pregnant mice were identified. Most studies reported sex (100%), strain (98%) and age (85%). The majority of studies used C57BL/6 (51%) or BALB/c (13%) mice aged 3-9 weeks. Non-pregnancy studies were more likely to use only male mice (57%). Dietary FA interventions varied considerably and overlapped: deficiency (0-2 mg/kg), control (0-16 mg/kg) and supplemented (0.1-60 mg/kg). Only 60% of studies used an open-formula base diet with a declared FA content and folic acid exposure was verified by folate status biomarkers in only 59% of studies. The duration of interventions ranged from 0-60 weeks for non-pregnancy studies. The duration of interventions (0-21 weeks) and the period of intervention (before and/or during pregnancy and/or lactation) in pregnancy studies were variable. Overall, 14% of studies did not report ≥1 generic reporting item(s) and 41% did not report ≥1 nutrition-specific reporting item(s). The variability

and frequent lack of reporting of important generic and nutrition-specific study design details in nutrition studies limits their generalizability, reproducibility and interpretation. The use of reporting checklists for animal research would enhance reporting quality of key study design and conduct factors in animal-based nutrition research.

2.2 Introduction

Nutrition research is essential for establishing nutrient requirements and dietary guidance to ensure the proper growth, development and maintenance of health for both humans and animals. The ability to perform accurate and reliable nutritional risk characterization is dependent on the quality and quantity of animal-based research. Animal studies provide an opportunity to directly observe biochemical and physiological interactions and responses to food substances that otherwise cannot be observed in humans (57). As such, basic nutrition research, especially in animal model systems, informs causal relations between specific food substances and health outcomes and allows for the elucidation of specific mechanisms of action. Nonetheless, despite the large quantity of published animal studies, study quality and reporting are crucial for ensuring that observations are accurate and reproducible.

Findings from pre-clinical studies often provide the plausibility needed to support the design of clinical trials. However, knowledge translation from animal studies to human research is dependent on transparent reporting. Poor and incomplete reporting of scientific findings raises concern for all stakeholders, including researchers, journals, funding agencies, academic institutions and medical and public health professionals (61). Findings from animal studies are used extensively to inform nutrition risk assessments, however, when these findings are poorly reported their utility and validity is limited (62). A systematic review of animal-based studies

published between 1999-2005 demonstrated common major omissions of basic animal characteristics (e.g. strain, sex, age, etc.) and their environmental conditions (63). These study design characteristics do not only influence the results obtained but are required to reproduce and appraise the findings (60,64). For instance, underreporting of animal strains can mask existing biases towards the use of specific inbred strains and limits the ability to generalize findings to other strains and more broadly to other species and human populations (65,66).

Reporting guidelines such as 'Animal Research: Reporting of In Vivo Experiments' (ARRIVE) were developed to provide a checklist of the minimum details required in an animal-based scientific publication with the aim to mitigate generic issues related to study design and analysis. Issues addressed include the reporting of both primary and secondary outcomes, key methodology (i.e., analytical method used; source and validation of reagents; quality control measures) and results (i.e., unit of analysis; measure of precision)(67)(68). Animal characteristics (e.g. species, strain, sex and developmental stages), their housing and husbandry conditions (including welfare-related assessments), and relevant baseline data are highlighted because they can impact experimental outcomes (68). The guidelines addresses study design issues such as sample size determination to allow replicability while minimizing selection bias, and the statistical methods used to analyze experimental outcomes (68). Errors in study design can result in systematic biases and distorted intake-response relations of reported findings, emphasizing the need for clear and transparent reporting of study design details. Generic reporting tools do not focus on nutrition-specific issues that should be reported in basic nutrition research studies in addition to generic study design details. Nutrition-specific reporting issues include the composition of base diets, duration of dietary intervention, dietary intervention dose, and verification of

intervention/exposure by assessing the diet directly or by assessing nutritional intake/status indicators.

Our objective was to perform a scoping review of recent basic nutrition research to determine the frequency of reporting of a selection of fundamental generic study methodology items, such as sex, strain and age of mice studied, as well as the frequency of reporting of nutrition-specific items, such as base diet, nutrient intervention dose, exposure verification and intervention duration. As a case study to assess reporting of generic and nutrition-specific items in basic nutrition research, we focused the scoping review on recent folate research in mice (last 10 years), a field with recent exponential growth in publications. We assumed that reporting in folate research would generally reflect reporting in other nutrition research areas.

2.3 Methods

2.3.1 Search Strategy

A literature search to retrieve articles that included studies of the effect of dietary folic acid in mouse models was conducted by the Health Canada Library. All scoping review methodology protocols are based on Preferred Reporting Items for Systematic Reviews and Meta-Analyses Extension for Scoping Reviews (PRISMA-ScR) guidelines (69).

Electronic databases EMBASE, Medline, Food Science and Technology Abstracts (FSTA), Global Health and International Pharmaceutical Abstracts (IPA) were searched in the Ovid interface. All database searches were restricted to 2009-present (May 2019) and English language published articles. The keywords and their variants used to identify relevant articles in each database included: folic acid, folic acid deficiency, folate metabolism, folic acid blood level, folate, diet, diet supplementation, dietary supplement, mouse model, mice or mouse. The specific syntax expressions used to identify studies with folic acid were (folic acid/ or folic acid deficiency/

or folate metabolism/ or folic acid blood level/) OR ((folic acid or folate) adj5 (deficien* or enriched or exposure or fortifi* or intake* or metaboli* or restrict* or supplement*)).tw,kw; those used to identify articles with dietary interventions were (diet supplementation/ or dietary supplement/ or diet/) OR ((diet or diets or dietary or nutrition*).tw,kw.). And finally, the expressions used to identify articles with mouse models were (mouse model/) OR ((((mice or mouse) adj3 model*) or mouse?model*).tw,kw.) OR (exp mouse/ or (mice or mouse).tw,kw.) OR (animal experiment/ or animal model/ or controlled study/ or (control or experimental or studies or study).tw.). The search was run up to May 3, 2019. The full search strategies are presented in **Supplementary Tables 1-4**. The search results were uploaded into the web-based bibliographic manager RefWorks 2.0 (ProQuest LLC, Bethesda, MD) database. Duplicate articles identified between databases were removed in RefWorks (**Figure 4**).

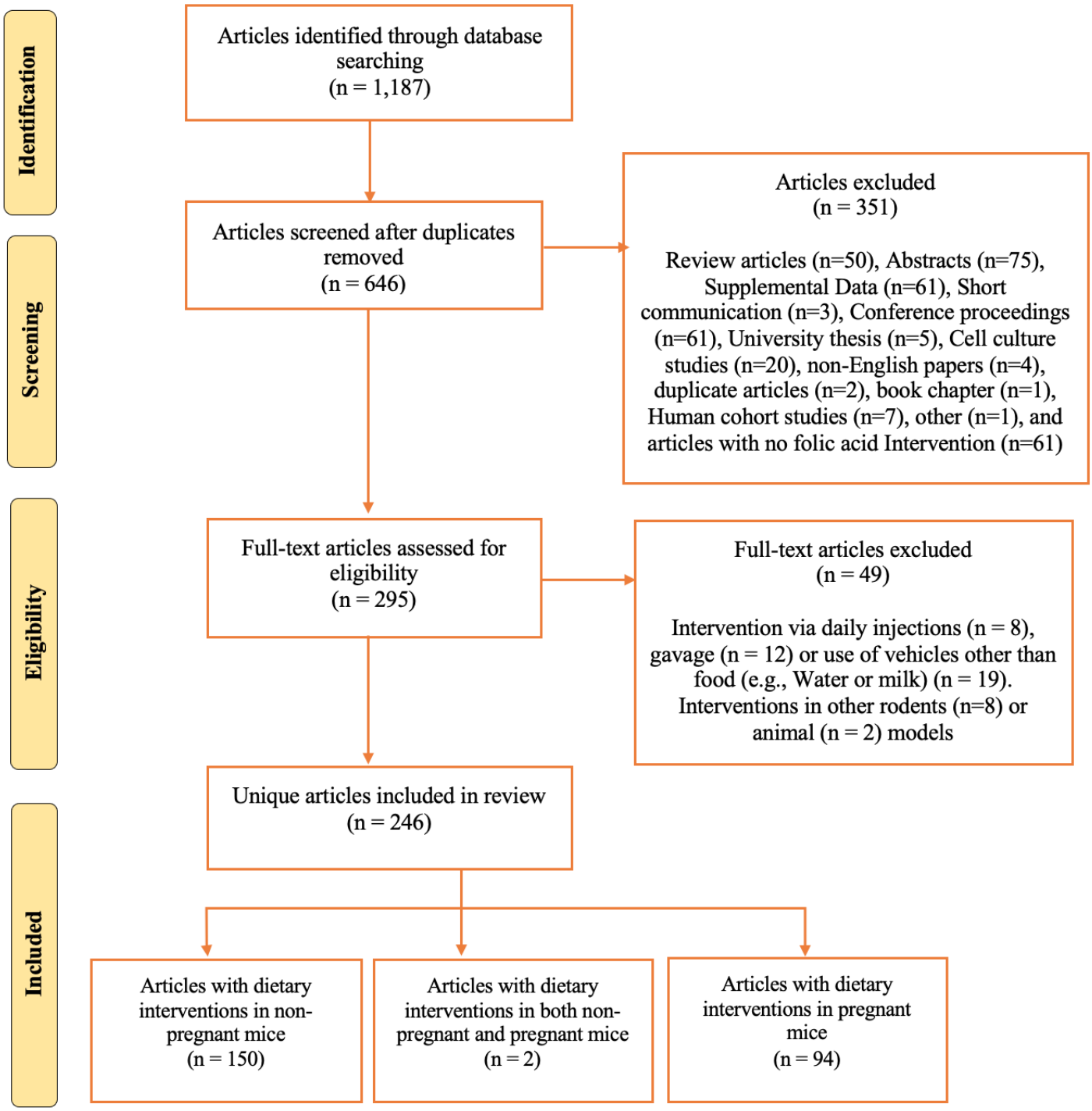

Figure 4: Study selection flow diagram. Flow chart of the identification of original peer-reviewed research articles that reported folic acid dietary interventions in mice included in the scoping review. Only English articles published between January 1, 2009 and May 3rd, 2019 were considered.

2.3.2 Selection of Studies

Literature search restrictions were used to focus on the last decade during which folate research experienced an exponential growth in publications. The inclusion criteria used to select published articles for analysis in this scoping review were articles that: (1) were original and peer-reviewed; (2) used a mouse model; (3) conducted a dietary folic acid intervention using food as a vehicle; (4) were in the English language and (4) published between January 2009 and May 2019.

2.3.3 Data Extraction and Charting

The selected articles were divided into two groups depending on whether the article described a study that examined folic acid interventions in pregnant or non-pregnant mice. Data from each article was extracted into a Microsoft Excel spreadsheet (Macintosh 16.30 version: Microsoft Corporation). If an article presented multiple independent experiments, each experiment was treated as an independent "Study". Two articles included both a 'non-pregnant' and a 'pregnant' mouse experiment; therefore, the two articles were included in both study groups. For studies in which experimental mice were exposed to two different interventions, each intervention dose and duration was considered individually.

Full references for each article were extracted including the article title, authors, journal, pages, country and publication year. Generic data extracted from each study included diseases and/or mechanisms under study, mouse strains, sex and age at start of intervention(s). Substrains of mice were grouped by strain. Additionally, nutrition-specific data extracted included the folic acid dose of the control and intervention(s), base diets and their folic acid content, intervention duration, and method of folate status verification. Folic acid content was recorded in mg/kg. Information on folic acid content in base diets was either obtained from the article (reported), from previously reported diet formulations when a reference was reported (declared), or manufacturer

website (declared) when the diet's catalogue number was provided. Closed-formula base diets and base diets reported only as standard rodent chow were grouped together as standard rodent chow. Unless otherwise stated in the article, when a standard rodent chow was used, we defined it as the control diet. Studies that used a standard rodent chow as a base diet but did not report ingredient formulation and the formulation could not be found on the manufacturer's website were defined as having an unreported folic acid content.

For pregnancy studies, data (when available) were extracted separately for the dams, sires and offspring, as applicable for the study design. Pregnancies whereby a live birth was achieved was classified as having an offspring endpoint. Studies whereby the dams were sacrificed during the pregnancy were classified as having an embryo endpoint. Multigenerational studies were treated as a single study when the dietary intervention was the same for all generations. Multigenerational studies in which different generations were given different dietary interventions, each generation was considered a unique "Study".

2.4 Results

The literature search was conducted and completed in May 2019 and identified 246 original research articles published between January 1, 2009 and May 3, 2019 in which a dietary folic acid intervention in mice was described. Of these articles, 152 articles described a study in non-pregnant mice, 94 articles described a study in pregnant mice, and 2 articles described studies in both pregnant and non-pregnant mice. The flow chart representing article identification and selection is in **Figure 4**. Review articles (n = 50), abstracts (n = 75), supplements (n = 61), short communications (n = 3), conference proceedings (n = 61), university theses (n = 5), cell culture studies (n = 20), articles with no FA intervention (n = 61), articles with folic acid interventions via

daily injections (n = 6), gavage (n = 12), or water or milk as a vehicle (n = 19) were excluded. Additionally, articles in humans (n=7) or those that used an animal model other than mice for their dietary folic acid intervention were excluded (n = 10; rats (n = 8), pigs (n =1) and monkeys (n = 1)). Finally, non-English papers (n = 4), a duplicate article (n = 2), a book chapter (n = 1) and an article whereby the full text could not be located (n = 1) were removed.

2.4.1 Health outcomes investigated

A wide variety of health outcomes and biochemical processes were investigated in the articles selected for this review. Articles were grouped into broad topic areas including cancer, cardiovascular diseases, congenital defects, gastrointestinal diseases, genetic instability, immune responses, liver diseases, neurodegeneration, urinary disorders as well various biochemical pathways (**Table 1**). Neurodegeneration (n = 36, 23.4%) and cardiovascular disease (n = 22, 14.3%) were the most common health outcomes examined in non-pregnant studies. In the pregnancy studies, congenital defects (n = 36; 37.9%), genetic instability (n = 23; 24.2%) and neurodegeneration (n = 15; 15.6%) were the most common outcomes examined.

Table 1: Health outcomes investigated.

Health outcome or biochemical processes	**No. of articles with studies in non-pregnant mice, *n* (%)**	**No. of articles with studies in pregnant mice, *n* (%)**
Biochemical Pathway	16 (10.4)	6 (6.3)
Cancer	31 (20.1)	6 (6.3)
Cardiovascular	22 (14.3)	3 (3.2)
Congenital defects	0 (0.0)	36 (37.9)
Gastrointestinal	7 (4.5)	2 (2.1)
Genetic Instability	16 (10.4)	23 (24.2)
Immune Response	5 (3.2)	1 (1.1)
Liver Disease	16 (10.4)	2 (2.1)
Neurodegeneration	36 (23.4)	15 (15.6)
Urinary disorders	5 (3.2)	2 (2.1)

2.4.2 Mouse strain

Most studies (99%) reported mouse strain. A total of 32 strains and 17 substrains were identified in the studies. Most studies used C57BL/6 (n = 184; 51%) or BALB/c (n = 48; 13%) mice. The percentage representation of commonly used mouse strains and low representations strains/substrains used in the studies is shown in **Figure 5**. Strains and substrains identified in the studies are presented in **Supplementary Tables 5.**

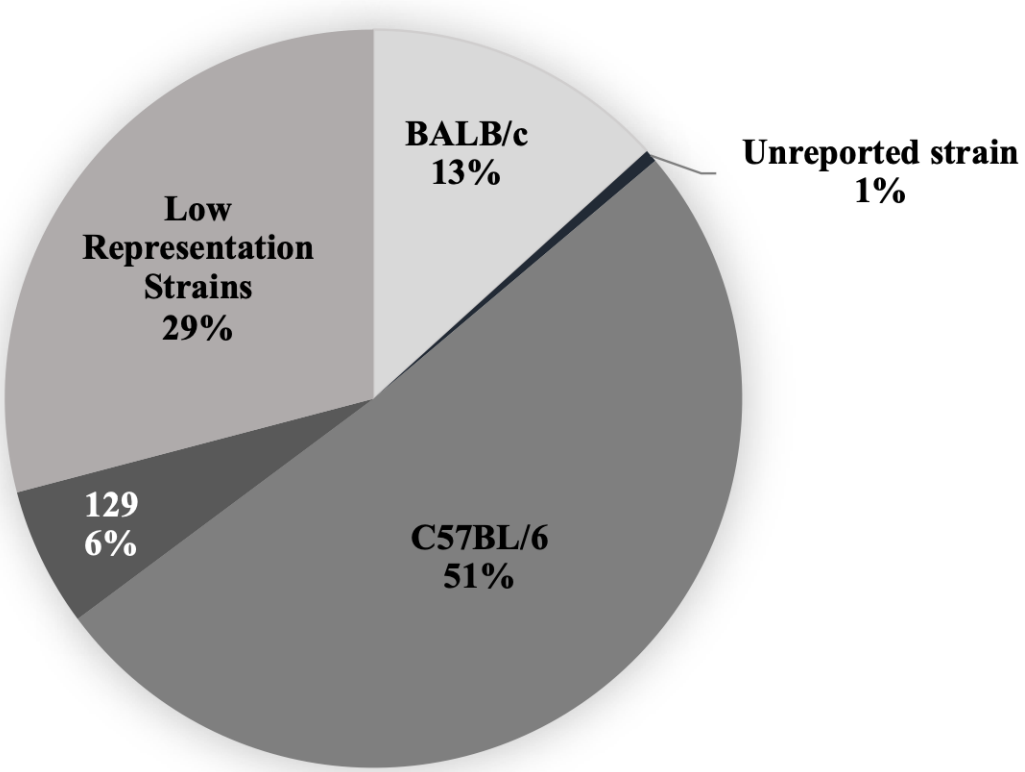

Figure 5: Mouse strains. Representation of mouse strains used in folate research studies published between January 2009 and May 2019. *Low representation strains are strains used in less than 5% of all the studies. These included the following strains: CD1, C3H, Swiss, AJ, WSB/EIJ, FVB, Kumming, Tg, CAST/EIJ, PWK/PHJ, Big Blue, CBA, LM/BC, ICR, Athymic nu/nu, SCID, SJL, SMV, DBA, NIH, Intercrossed Inbred, Intercrossed Outbred, 634Mui/J, Beige Nude XID, Mutamouse, SMV, LPT/LeJ, Curly Tail, CF1 and their substrains.

2.4.3 Sex

Among the 152 non-pregnant studied reviewed, all reported the sex of the mice. The majority of the studies used male mice (n = 87; 56.5%), followed by studies with both male and female mice (n = 48; 31.2%) and fewer used female mice (n = 19; 12.3%) as shown in **Figure 6, panel A**. For obvious reasons, the majority of pregnancy studies reported a dietary intervention in female mice (pregnant dams), although 7 studies examined the dietary effect in sires and their offspring. For the sex analysis of pregnancy studies, sex was identified based on the mice for which the endpoint was measured. Of the pregnancy studies, 54 studies examined embryos and 56 studied the live birth offspring in the postnatal period. Most studies examined both sexes in embryo (n = 51; 94.4%) analyses, but only about half of the time in live birth offspring (n = 30; 53.6%) analyses **(Figure 6, panel B)**. When embryo/live birth offspring analyses were performed in only one sex, it was more likely to be in males (n = 24/29; 82.8%).

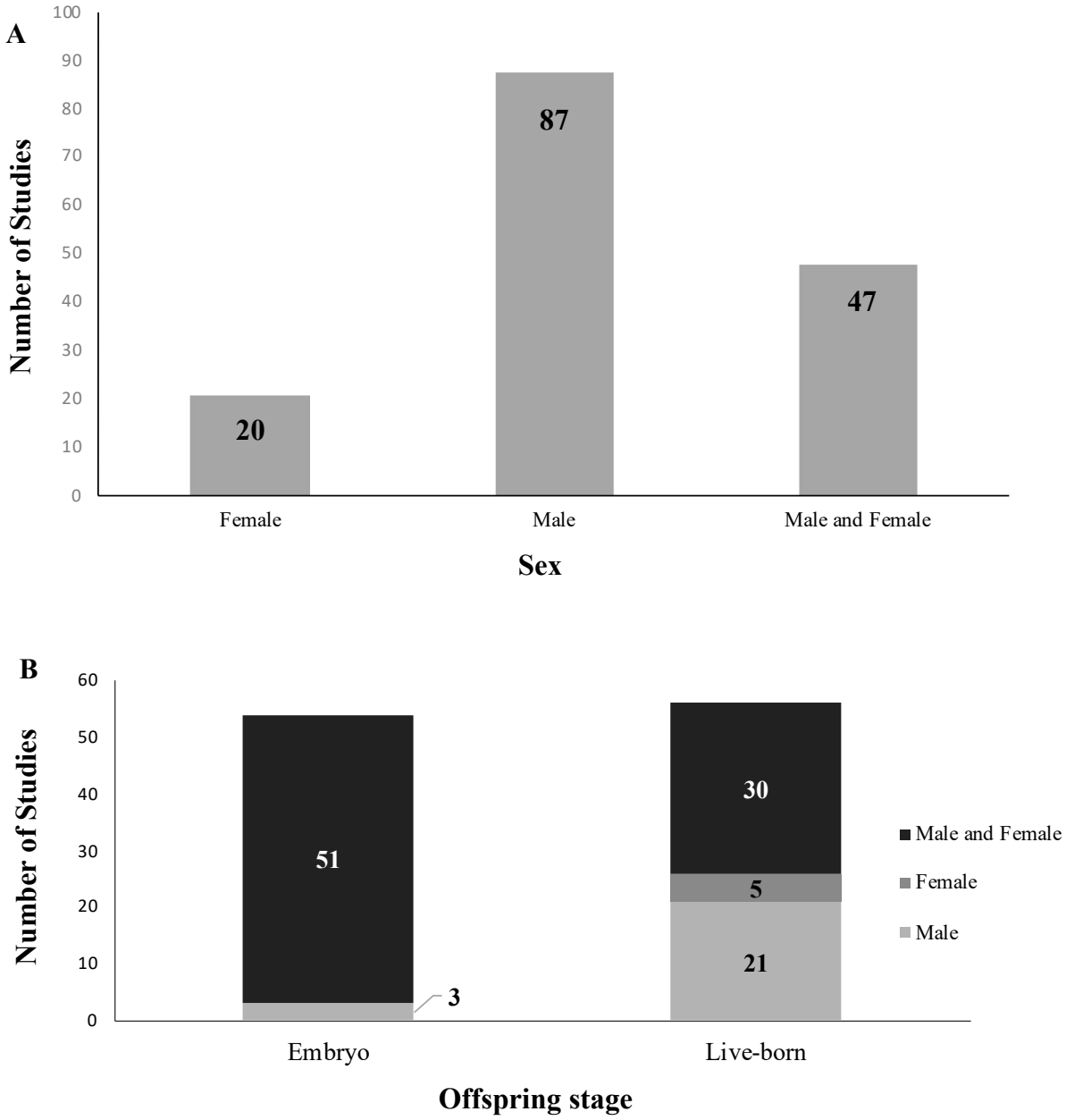

Figure 6: Mouse sex. Representation of mouse sex included in non-pregnant and pregnancy studies published between January 2009 and May 2019. **A)** Representation of mouse sex used in folate non-pregnant research studies. **B)** Representation of mouse sex of offspring analyzed in pregnancy studies.

2.4.4 Age

Forty-four studies (14.0%) did not report the age of the mice at the start of the dietary intervention. This was more common among pregnancy studies with 33 pregnancy studies (25%) compared to 11 non-pregnant studies (6%) not reporting age (**Figure 7, panel B and C**). The age at time of intervention among non-pregnancy studies varied substantially from 3 to 120 weeks (**Figure 7**, **panel A).** However, the majority of the studies (n = 120; 66.7 %) used younger mice aged 3-9 weeks. In the pregnancy studies, the age range of the dams and sires was smaller varying from 2 to 30 weeks**.** Similar to the non-pregnancy studies, the majority of the studies (n = 79; 59.8%) used younger mice aged 3-9 weeks at the start of the dietary intervention.

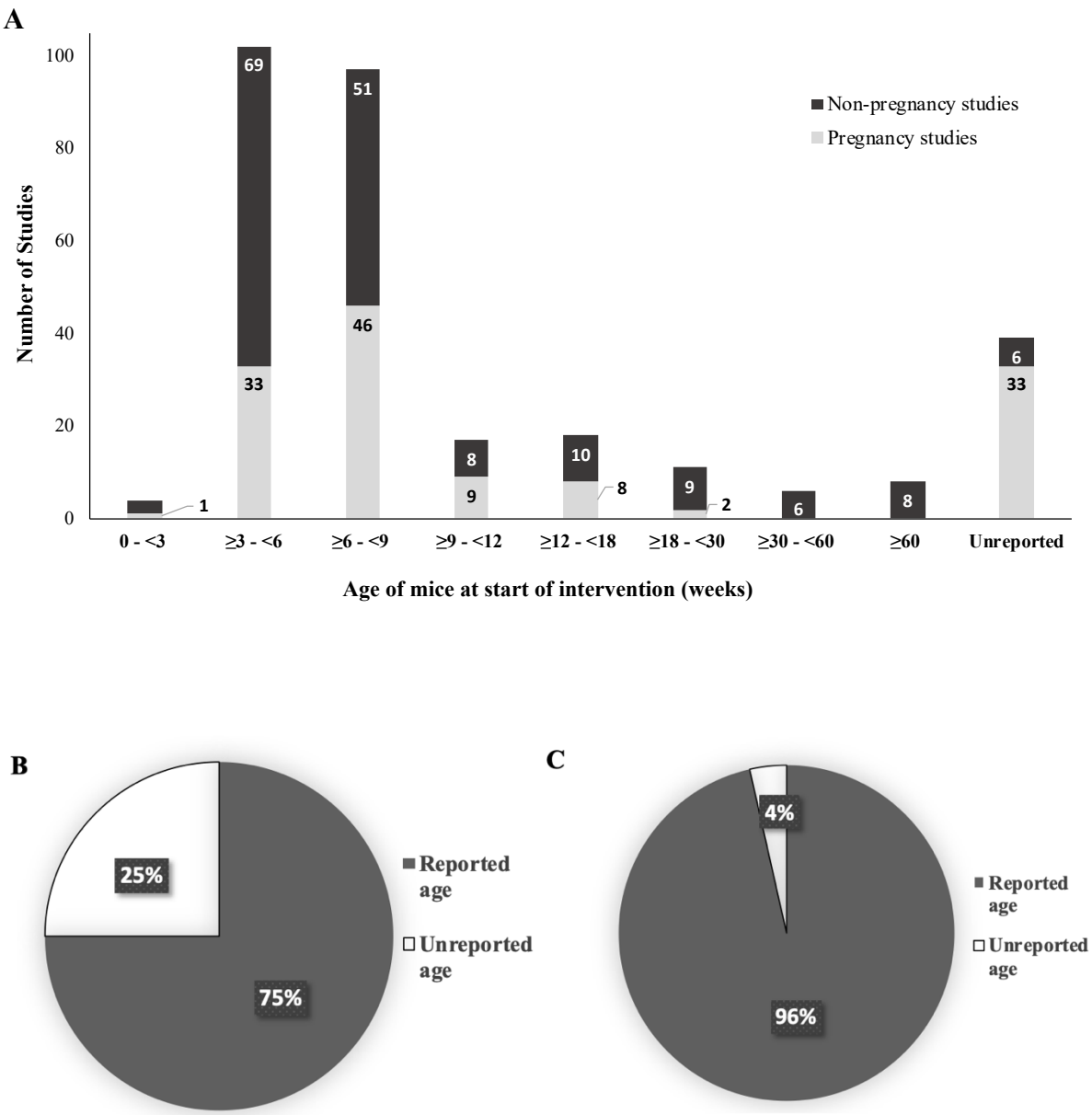

Figure 7: Mouse age. A) Representation of the age of the mice at the start of folate dietary intervention among studies published between January 2009 and May 2019. The unreported group consists of studies that did not indicate the age of the mice at the start of intervention. **B)** Percentage of non-pregnancy studies that reported the age of the mice at the start of intervention. **C)** Percentage of pregnancy studies reported the age of the mice at the start of intervention.

2.4.5 Folic acid intervention dose(s)

All studies reported at least one intervention dose, including control, deficient and/or supplemented intervention groups. Control diets ranged from 0 to 16 mg/kg of FA. This did not include one outlier study in the non-pregnant category that reported a 200 mg/kg FA control diet; it was assumed to be a typo but we could not verify with the authors. A control diet containing 2 mg/kg FA was most commonly used (n = 161, 74.8%) of studies. Deficient diets ranged from 0 to 1.99 mg/kg FA with the majority (n = 171, 94.5%) containing 0 mg/kg of FA. A broad range of supplemented diets was noted ranging from 0.17 – 60 mg/kg of FA with 32.7% of the studies containing 6 mg/kg of FA. The ranges of FA content of the deficient and supplemented diets overlapped with each other and the control diet (**Figure 8)**.

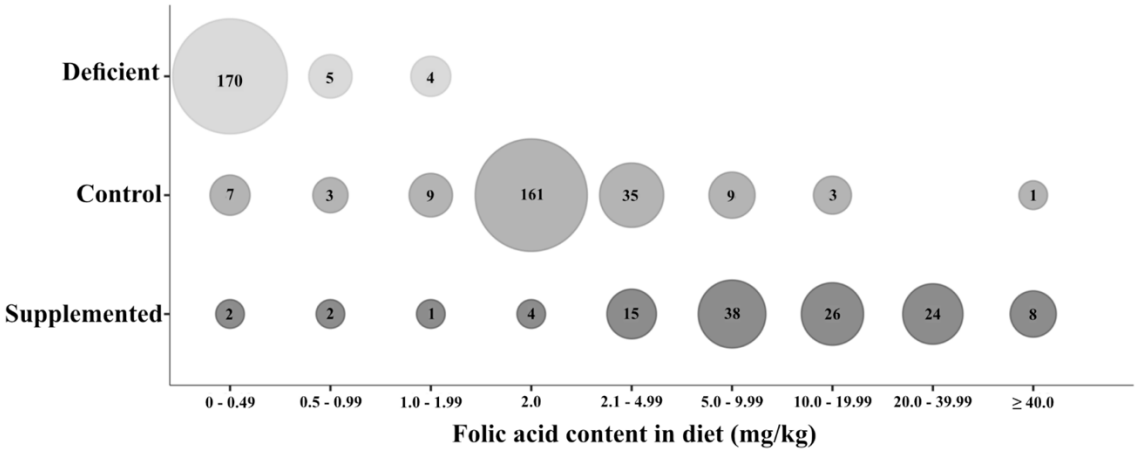

Figure 8: Folic acid dose included in the dietary intervention(s). Reported folic acid (FA) content of the control, deficient and supplemented diets used in both non-pregnancy and pregnancy mouse studies.

2.4.6 Base diet

Over half of the studies (n = 261; 60.3%) reported the use of a defined formulated base diet **(Figure 9, panel A)**. Specifically, the AIN-93 diet (70) was the most commonly used (n = 135; 31.2%), followed by the AIN-76 (71) (n = 31; 7.2%) and NIH-31 (72) (n = 17, 3.9%) diets. For analysis, closed-formula base diets were grouped together with diets reported as standard rodent chow. By this definition, eighty-three (43.7%) non-pregnancy studies used a standard rodent chow base diet, of which only 55 (66.3%) reported/had a declared FA content. Among the pregnancy studies, 89 (36.6%) used a standard rodent chow base diet, and of those, 79 (88.8%) reported/had a declared FA content (**Figure 9, panel B and C)**.

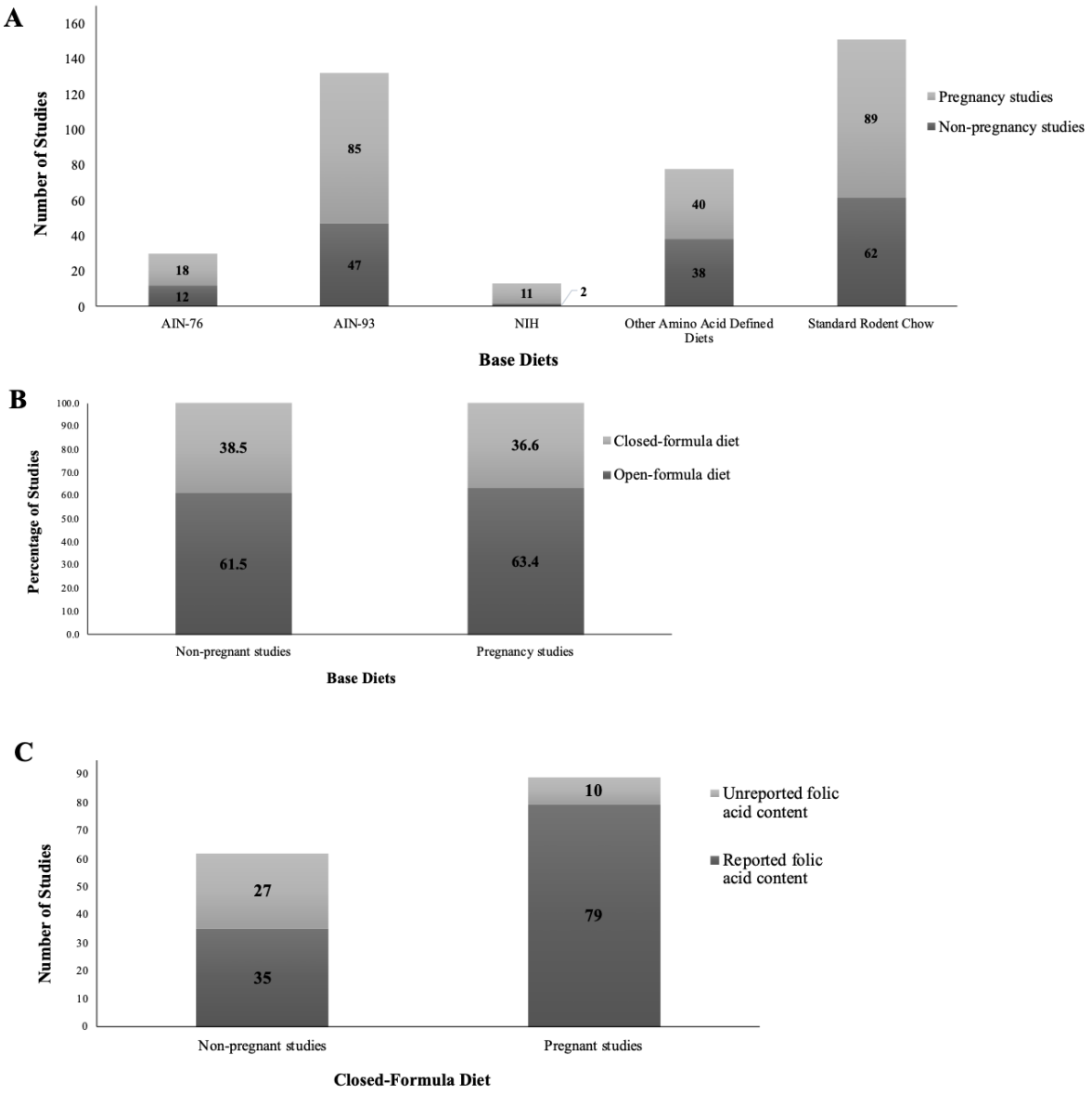

Figure 9: Base diets. A) Base diets reported in both non-pregnancy and pregnancy mouse studies. **B)** Percentage of studies that used an open-formula diet versus a closed-formula base diet in non-pregnancy and pregnancy mouse studies. **C)** Reported folic acid content (but not necessarily measured) in the closed-formula base diets used in non-pregnancy and pregnancy mouse studies.

2.4.7 Duration of intervention(s)

The duration of intervention among non-pregnant studies ranged from 2 days to 60 weeks (**Figure 10, panel A**). Six studies (2.8%) did not report the duration of intervention. Among the pregnancy studies, FA dietary intervention duration ranged from 2 – 21 weeks. However, the initiation of the FA dietary interventions relative to the pregnancy varied with some being initiated before mating, during pregnancy and/or during lactation, and with variable intervention durations (**Figure 10, panel B**). The majority of the studies initiated the dietary intervention before mating and maintained it throughout pregnancy (40.1%), followed by studies where the intervention started before mating and was continued until the pups were weaned at 3 weeks old (mating, gestation and lactation) (35.6%).

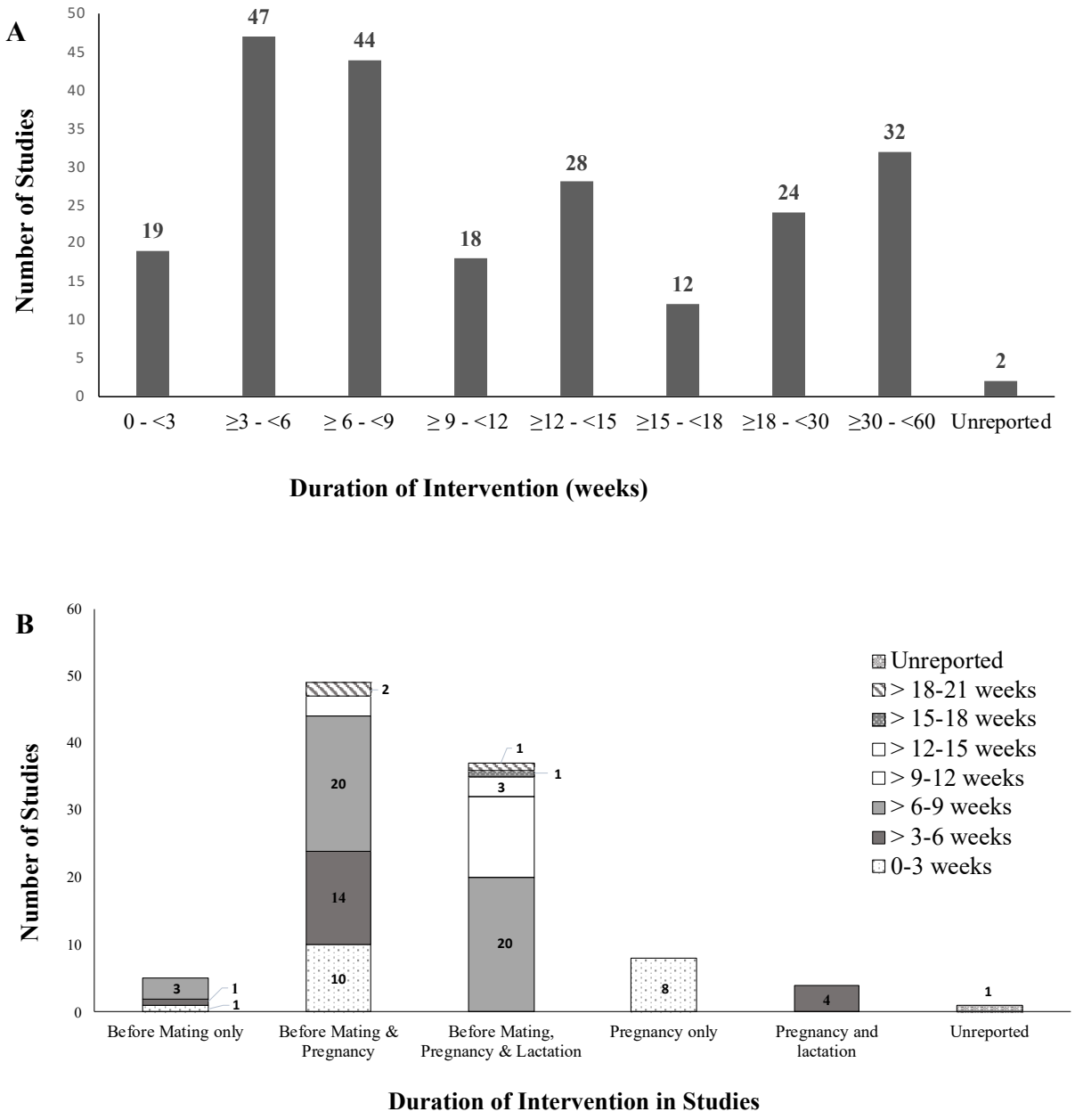

Figure 10: Duration of folic acid dietary interventions. A) Duration of folic acid dietary interventions reported in non-pregnancy mouse studies. **B)** Duration of folic acid dietary interventions reported in pregnancy mouse studies. Studies were grouped into 3-week intervals up until 18 weeks. Longer duration studies were grouped into 18-30 and greater than 30 weeks.

2.4.8 Folic acid intervention verified by biomarker

The total exposure to folic acid in a dietary intervention is dependent on both the FA content of the base diet and the intervention dose. Changes in folate status in response to dietary intake is one way that the exposure can be verified. Plasma/serum or tissue folate or plasma/serum homocysteine are biomarkers that can be used to verify folate exposure. About 40% of all studies did not verify folate status in exposed mice using one of these biomarkers. This included over a third of the non-pregnancy studies (n = 53; 34.9%) and almost half of pregnancy studies (n = 42; 43.8%) did not verify folate status in the mice (**Figure 11, panel A**). About 36.4% of the studies used a closed formula base diet for their intervention. Of these, only 20.8% verified the folate status in the mice. On the other hand, only 40.8% of the studies that used an open formula diet verified the folate status in the mice (**Figure 11, panel B**).

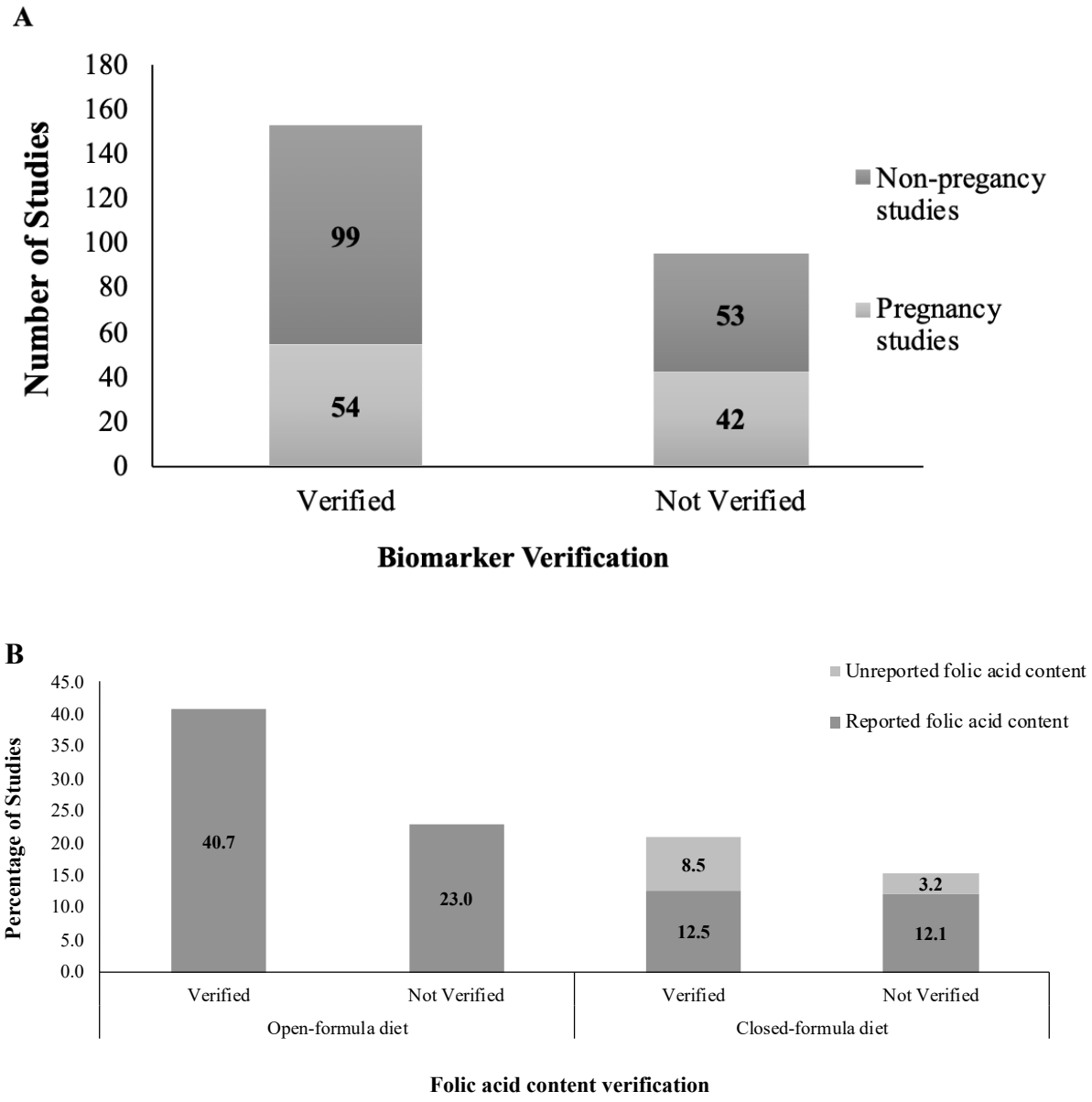

Figure 11: Folic acid exposure verification. A) Percentage of studies that used a biomarker to verify folate status of the mice used in folic acid intervention studies. Acceptable methods of folate status verification included circulating or tissue folate or circulating homocysteine analyses. **B)** Percentage of studies that reported folic acid content in the base diet and verified folate status of mice.

2.5 Discussion

We identified a high proportion of basic nutrition studies that did not report key generic and nutrition-specific details. While incomplete reporting in scientific research publications is not unique to nutrition science (63), it nonetheless needs to be addressed to minimize negative implications for reproducibility, ethical animal use, funding allocation, and translation to human health (73) (60,74). Animal models are vital for the identification and investigation of causal pathways that underpin health outcomes related to nutritional exposures, but the unique characteristics of the animal models used can influence the final experimental results (64). Similarly, the dietary intervention and how it is applied will also influence the outcome. Multiple systematic reviews investigating the reporting quality of animal-based studies, such as the 2009 report by Kilkenny et al. (63), highlight major reporting omissions including the hypothesis, sample size, basic animal characteristics (e.g., age, sex, strain), actions to reduce bias, and statistical methods. In addition to these generic reporting issues, we identified key nutrition-specific items that warrant proper reporting in basic nutrition research studies. These items include base diet source and composition, intervention dose, exposure verification, and intervention duration. Among the studies reviewed, 14% did not report at least one of the limited generic items we assessed and 41% did not report at least one nutrition-specific item, indicating that reporting omissions are common in basic nutrition research. Because we focused on a limited number of generic and nutrition specific items, this is likely an underestimate of underreporting in nutrition studies. It should be noted that this was not a full quality assessment of the studies, but rather a limited assessment of specific study reporting details in service of highlighting the lack of reporting of nutrition-specific details in basic nutrition studies. A full quality assessment is beyond the scope of this review.

The majority of studies reported the basic animal characteristics assessed in this scoping review, with 100%, 98% and 86% of studies reporting sex, mouse strain, and age at intervention, respectively. The frequency of reporting of these foundational details were better than previously reported for mouse studies, where only 65% and 68% of studies reported sex and age or weight of mice, respectively (63). It should be noted that ours was not a fulsome assessment of generic reporting items and likely represents a major underestimate of poor-quality reporting. For example, Kilkenny et al. found that ~45% of mouse studies reported the hypothesis, sex, strain, age and/or weight, and number of animals used, whereas >70% of studies demonstrated low quality reporting of experimental design and statistical analyses (63). Reporting of these seemingly simple but foundational details is particularly important because poor reporting can lead to erroneous conclusions and/or irreproducibility of experimental findings (66). For example, sex-differences account for differences in gonadal steroid hormones, body size, metabolic rate, epigenetic programming and gene expression (75–78), all of which can influence responses to nutritional interventions and health outcomes. Our study also highlighted the known publication bias towards the use of male animals in preclinical studies; over half (56.5%) of the non-pregnant studies were conducted in only male mice and only male live-born offspring were studied in over a third (37.5%) of pregnancy studies. The assumption that findings from single-sex (male) studies can be applied generally has been repeatedly reputed (75,79). Such publication bias inflates interventions and treatment outcomes that may be ineffective to the generalized population (66). Preclinical trials provide the foundations and guidelines for clinical trials, therefore, accounting for variations due to sex in preclinical trials is an effective way to avoid costly and potentially ineffective clinical trials (80). Recognizing the influence of sex on experimental outcomes, international agencies such as the National Institutes of Health (NIH), the Canadian Institutes of Health Research (CIHR) and

the European Union (EU) require the sex of animal models to be reported in publications and funding requests, and to justify the use of single-sex animals in their studies (75,80–82). These more recent policy changes may account for the consistent reporting of sex in the studies we surveyed. Time will tell whether they impact the bias towards the use of male animal models.

Animal strain-specific genetic and phenotypic variability can also impact metabolism and disease pathogenesis (80,83). Genomic diversity, and its manipulation through the use of transgenic tools, allows researchers to investigate the influence of individual genes on disease pathogenesis, intervention effectiveness, metabolic rates, susceptibility to disease and much more (74,83–85). Mouse strains are selected to study specific outcomes based on phenotypes defined by their genomic background (74). Strains can be more or less susceptible to a nutritional intervention and/or the development of specific health outcomes making this an important reporting detail (74). Almost all studies assessed in the scoping review reported mouse strain. It was noteworthy, however, that 3 major inbred mouse strains were used in 70% of folic acid research studies. Importantly, this is an underestimate of strain diversity because substrains of major strain types, which can be genetically and phenotypically distinct (86), were grouped together. The genetic and phenotypical differences can potentially influence how the mouse models respond to dietary interventions. The use of single strains or transgenic lines can limit the generalizability of findings to other strains, and by extension to human populations.

The age of mice at the start of intervention was less likely to be reported compared to sex and strain. Age varied substantially among studies (i.e., non-pregnant studies: 3 to 120 weeks; pregnancy studies: 2 to 30 weeks). Variability in age at intervention can be necessary and health outcome-dependent (i.e.; an aging study performed in older mice), but should be reported and rationalized given that metabolic rate, physiological response and disease pathogenesis are age-

dependent (87). Animal characteristics, including others not assessed in this scoping review, can interact to influence experimental outcome (88,89) making it essential that all details are reported consistently and transparently.

2.5.1 Reporting Nutrition Specific Items

Animal-based research allows for the assessment of controlled nutritional interventions on outcomes. However, this assumes we have an accurate assessment of the nutritional exposure. Two-thirds of the studies utilized an open-formula diet (63.7%), most often AIN-93. Open-formula defined diets were developed in the 1970s to address the growth, maintenance and developmental needs of rodents, to standardize laboratory animal diets, and to facilitate inter-study comparisons (70,71,90). Unlike closed-formula diets, the ingredients are purified and concentrations are declared and publicly available (90). Changes to the formulation can be made to meet specific experimental needs with few, if any, changes to other ingredients, which can enhance consistency in nutritional exposure within and between distinct study designs.

Surprisingly, over a third of the studies used closed formula diets (36.3%), which included studies that provided no details on the brand, manufacturer or folic acid content of the "standard rodent chow" employed. In contrast to open-formula diets, the ingredient list of closed-formula diets is often proprietary, only available upon request, and subject to change without public disclosure (90). Standard rodent chow can contain a variety of cereals and other ingredients resulting in variable concentrations of the food substance of interest (90,91). Diet formulation, and thereby specific food substance content, can be changed in response to fluctuations in ingredient costs, and seasonal or geographic variations in source materials (90). Variations in other intrinsic food substances in ingredients, such as endogenous phytoestrogens, may confound findings. The diet-health outcome relation investigated depends on the overall exposure from both the base diet

and the added intervention dose. For these reasons, if a closed formula diet is used, the absolute amount of exposure to a food substance may not be known and/or may vary considerably. For this review, when the catalogue number was provided in the study methods, we reached out to manufacturer for the ingredient list and FA content. Unfortunately, even that information was limited because it reflected the current formulation which may not be consistent with the diet used in the study. Overall, the use of closed formula diets hinders the ability to compare and reproduce basic nutrition studies. Ideally, investigators would use open-formula diets and declare information such as the manufacturer, catalogue number and lot number.

The prominent use of closed-formula diets in nutritional studies prompted us to investigate whether studies reported whether the FA exposure was verified by direct analysis of the diet or by assessing a biomarker of exposure in the mice. About two-thirds of studies verified the folate content of their diets and this was similar between closed and open-formula diets. A biomarker of exposure can be used to validate the nutritional exposure thereby confirming the relationship between the exposure and health outcome (92,93). Biomarkers can be used to evaluate exposure (e.g. dietary intake) or a functional response to an exposure (i.e. metabolism) (92,93). Serum folate is commonly used as an indicator of recent exposure while RBC (or other tissue) folate is an indicator of long-term folate status (11). Plasma homocysteine is a functional biomarker of folate status and is inversely correlated with circulating folate concentrations (11). Our findings showed that 44% of studies (34.9% of non-pregnant studies and 43.8% of pregnancy studies) did not verify folate status using a biomarker. Studies that lack folate exposure verification combined with the use of closed-formula diets (15.3%) would have a high degree of uncertainty in relating exposure to the experimental outcome of interest. Ideally, all studies would verify exposure by measuring

the food substance content of the intervention diet and/or analysing biomarkers of exposure in the animals.

Although not a reporting issue, we found a remarkable degree of variation and overlap among the definitions for what constituted a control, deficient and supplemented FA intervention. Open-formula diets generally define adequate folic acid as 2 mg/kg (70,71) and this was indeed the most commonly used control diet reported (63.9%). However, studies used a control diets containing 0 to 16 mg FA/kg. In addition, investigators routinely used the term FA deficient (0 to 2 mg FA/kg) or supplemented (0.17 to 60 mg FA/kg) to describe diets with FA contents that overlapped considerably with 2 mg/kg. The inconsistent definitions for "deficient", "control" and "supplemented" underscore the need for standardization. Even though a 2 mg FA/kg diet is commonly used as a control diet, it is unclear what the appropriate definition for deficient/excessive FA intake would be. The standardization of terms and definitions along with clear reporting of actual intervention doses would enable consolidation of findings across studies and improve reproducibility.

2.5.2 Recommendations

Improving the reporting quality of publications in nutrition science is one way to enhance accuracy, transparency and reproducibility of findings. Based on our scoping review, we propose a number of recommendations to address under- and/or misreporting of generic study design and implementation items and nutrition-specific items (Summarized in **Table 2**).

For the reporting of generic study design and implementation items, we recommend the use of the ARRIVE guidelines. The ARRIVE guidelines are currently recommended by the American Society for Nutrition journals, but only required for the American Journal of Clinical Nutrition. This reporting checklist outlines essential study design details that should be included in animal-

derived publications to facilitate accurate reporting and enhance reproducibility (63,68,73). The ARRIVE guidelines consist of two checklists, The Essential 10 and the complementary Recommended Set comprised of an additional 11 reporting items (94). The Essential 10 checklist addresses fundamental generic items including study design, sample size, inclusion/exclusion criteria, randomization, blinding, outcome measures, statistical methods, experimental animals, experimental procedures and results. The Recommended Set expands on the Essential 10 to address reporting of the abstract, background, objectives, ethics, housing and husbandry, animal care and monitoring, interpretation/implications, generalizability/translation, protocol registration, data access and declaration of interests. It is important to note that we limited our scoping review to basic experimental animal characteristics (strain, sex and age) as a proof-of-principle assessment of reporting quality in basic nutrition research. While the majority of studies in our review reported these basic details, we cannot ensure that the studies represent overall "high" quality reporting for generic study design items.

Requiring the use of the ARRIVE Guidelines will most certainly enhance the quality of reporting in basic nutrition research, but they do not address nutrition-specific issues. We recommend that a nutrition lens be applied to their interpretation. Examples include study design and experimental procedures. Study design focuses on the reporting of comparison groups and we would suggest that a rationale for the exposure levels be reported. For essential nutrients, an appropriate control exposure would be the level of intake required for adequacy, which could be supported by evidence of an intake-response relation between the nutrient intake and a health outcome. For non-essential food substances, a more nuanced approach would be needed to determine appropriate levels of exposure for comparison groups. Experimental procedures focus on the detailed methods used and the rationale for the approach taken. A number of studies

identified in our review used a route of exposure other than oral intake of diet. It would be important that a rationale be provided for exposing the animals to a food substance via a non-oral route and how it affects the interpretation of the results.

A rationale should also be provided for the timing of exposure and the duration between exposure and outcome assessment. Our scoping review saw a wide range of intervention durations used, ranging from 2 days to 60 weeks in non-pregnancy and 2 to 21 weeks in pregnancy studies. The timing/duration must be sufficient to plausibly affect a change on the outcome. For example, spermatogenesis takes 6-8 weeks, so studies examining male fertility should take into account whether the timing and duration of the nutritional intervention of interest would plausibly affect fertility parameters. The relevant duration will be outcome specific, and reporting its rational will improve reproducibility and accurate interpretation of results.

Details about the base diet, including manufacturer, catalogue and lot numbers, and nutrient composition should be declared. Lack of knowledge of total exposure (for example, base diet content plus supplement) prohibits the appropriate interpretation of results; conclusions and inferences to human health are impossible. In addition, study reproducibility is hindered if the composition of the diet is unknown. Preferably, open-formula diets should be used to better control the total exposure, minimize errors and increase reproducibility of findings. Ideally, the amount of the food substance should be verified either in the diet itself or through validated biomarkers of exposure in the animals. In cases where a closed-formula diet is utilized, the complete diet formulation should be included, food substance of interest level verified, and biomarker of exposure assessed. Other nutrition-specific considerations are included in Table 2. Animal-based nutrition research publications could use the ARRIVE checklist in tandem with our nutrition specific essential 6 checklist (**Supplementary Table 6**) as an add-on to further improve the

reporting quality of nutrition studies. Our nutrition-specific recommendations aim to enhance transparency and reproducibility of animal-based nutrition research.

For the purposes of our study, we did not evaluate the reporting of outcomes, but it should be noted that the use and clear reporting of a validated method for outcome ascertainment is critical for ensuring transparency and reproducibility. Surrogate markers that predict the outcome can be used to support these findings. As proposed by the Core Outcome Measures in Effectiveness Trials (COMET) initiative, using core outcomes in clinical trials allow for comparison and combination of data across studies (95,96). The core outcomes are defined as a 'standardized set of outcomes that should be measured as a minimum' (95). Similarly, verified outcomes and biomarkers should be considered in preclinical studies. The findings, effects and health outcomes studied in nutrition science is highly diverse, as observed in the suite of studies identified in this review. While a diversity of studies is imperative to fill knowledge gaps, it can also reinforce, undermine or introduce inconsistencies to the knowledge base depending on how outcomes are measured.

Table 2: Concepts that should be considered when reporting nutrition research that utilizes animal models.

TOPIC	CONCEPT	RECOMMENDATIONS
Experimental Animal Model	**Rationale for Use**	Provide rationale for model used including its relevance to the health outcome being investigated and the food substance under investigation.
	Characteristics	a. Report details about the species, strain, sex, developmental stage, age, and body weight of the animal model used. b. Provide brief details to explain choice of strain, sex, developmental stage, age, and body weight of animal model used. c. Comment on randomizations, blinding, statistical methods and actions to reduce bias.
	Outcome	a. Provide a rationale for investigating the relation between the food substance of interest and the health outcome. b. Report the use of a verified biomarkers of health status to certify experimental dietary intake-heath outcome investigation. c. Comment on the choice of tissues analyzed for health outcome. d. Provide a rationale for investigating non-oral interventions and how it affects the interpretation of results.
Nutritional Component	**Base Diet**	a. Use an open-formula diet to minimize experimental variability and improve precision and reproducibility. If a closed-formula diet is utilized, provide details of formulation (i.e., complete ingredient list and their relative concentrations), manufacturer and catalogue number and lot numbers with publication. The food substance of interest concentrations should be verified in the diet itself and/or through a validated biomarker of exposure in the animals. b. Provide details on method used to verify nutritional composition of diet. c. Comment on environmental factors that may influence dietary intake and act as confounding factors.
	Intervention Dose	a. Give full details on the concentration of dose used in the intervention, including that of the comparison diet (control), explain whether or not nutritional component was modified in the base diet or administered differently. Total exposure (base diet plus added amount, if applicable) should be reported. b. Provide a rationale for the doses used and relevance to adequate intakes (when established for an essential nutrient) or usual intakes for other food substances.
	Duration of Exposure	Provide a brief description to justify chosen duration of dietary intervention, including how it relates to the development of the outcome being measured.
	Verification of Exposure	a. Comment on the method used to verify exposure, such as the measurement of biomarkers of intake or status. b. Provide a rationale for the choice of tissues analyzed for the biomarkers of exposure.

2.6 Conclusions

Nutrition science publications provide the knowledge foundation that informs decisions made by the general public, research scientists, clinicians and policy makers (97). However, they can be influenced by trends and underlying stakeholder interests, and be used by the media to either promote or undermine the credibility and trust in scientific evidence (98). When publications are found to be conflicting, incomplete and/or irreproducible, they can erode the public's trust. Poor reporting of scientific findings contributes to these challenges. As our scoping review demonstrates, there is a great need for better reporting of animal-based nutrition research. The use of reporting guidelines such as ARRIVE to report basic animal characteristics and experimental designs, combined with consideration of nutrition-specific concepts, will improve the transparency, reproducibility and reliability of these publications.

CHAPTER 3: FOLIC ACID DOSE-RESPONSE STUDY IN MICE

3.1. Abstract

Folic acid, the synthetic form of folate is commonly used to supplement food in fortification programs. This essential group of enzyme co-factors are essential in nucleotide and methionine biosynthesis as well as various methylation reactions. Over time, clinical trials have provided essential information on the causal relationship between this nutrient and health outcomes. However, animal studies expand on this knowledge by identifying and investigating the causal mechanism governing these relationships. Knowledge translation of animal-derived data can be challenging when folate intake and identified health outcomes in mice cannot be accurately translated to human nutrition. The goal of this dose-response study was to identify folate-dependent physiological, functional and genomic endpoints that could be used as surrogate biomarkers of folate intake in mice while taking into account biological variations due to strain and sex. The findings of our study showed clear sex and strain differences for majority of the endpoints investigated (i.e., body weight, food consumption, plasma total folate, folate vitamers, CBC, plasma homocysteine and micronucleus frequency). To harmonize findings from mouse studies in the folic acid research field, we propose using biomarkers to define the folate intakes associated with adequacy, deficiency or supplementation. From the findings of our study, we proposed the appearance of elevated homocysteine concentrations (i.e., $\geq$ 3.88 umol/L) as a biomarker of low folic acid intake and elevated unmetabolized folic acid (i.e., $\geq$ 7.71 nmol/L) as a biomarker of high folic acid intake.

3.2. Introduction

Folates are a group of enzyme co-factors responsible for carrying out one-carbon metabolic reactions in the body. These one-carbon metabolic reactions play a critical role in purine, pyrimidine and methionine biosynthesis and mediate multiple methylation pathways (10,46,99,100). This essential water-soluble vitamin is primarily obtained through our diet. Foods such as legumes and fruits (especially citrus fruits) are rich in naturally occurring folates (10). The synthetic form of folate, known as folic acid (FA or PGA) is used to supplement foods such as pasta and white wheat flour in various countries such as Canada and the United States (101). Unlike the naturally occurring folates, FA is about 85% more bioavailable, less susceptible to oxidative cleavage and its monoglutamate structure allows for easier absorption (11,99). These characteristics makes the synthetic FA the ideal form of folate to use for fortification.

The one-carbon metabolic reactions that folate mediates play a role in gene regulation, transcription, DNA stability and repair (10,46,102). When these processes are hindered during embryogenesis due to folate deficiency, it can lead to devasting birth defects, namely NTDs. Food fortification programs were introduced with the goal of increasing the population's baseline folate intakes and ergo reduce the incidence of NTDs. Since the introduction of fortification programs in 1998, the prevalence of NTDs in Canada was reduced by 46% in 2007 (22). Nonetheless, concerns have been raised that over-consumption of FA may have adverse effects. As such, the challenge is to balance those potential health risks of over-consumption with the known health risks associated with deficiency. Identification of the optimal and safe FA intake range remains an important question.

Clinical trials provide essential information on the causal relationship between nutritional components and health outcomes, but do not necessarily elucidate mechanism. Animal studies

build on this knowledge by identifying and investigating the underlying causal mechanisms that give rise to these associations (103). However, the issue remains as to how to relate the intakes in the animals to those observed in humans.

Biomarkers provide us the ability to assess intake (exposure) and status. A number of accepted folate biomarkers are common to both mice and humans, including serum/plasma total folate (intake), RBC total folate (status), and plasma homocysteine (biomarker of metabolism). The different folate forms that make up total folate can also be measured as markers of exposure. Unlike in humans, the folate intakes associated with adequacy, deficiency or supplementation in mice have not been firmly established making it difficult to translate health outcome findings in mice to the human context. However, it might be possible to establish these relationships using functional biomarkers such as blood cell counts and RBC micronucleus frequencies, or markers of supraphysiological exposures such as the appearance of unmetabolized folic acid. A dose-response study allows us to both identify and validate surrogate biomarkers of folate exposure and to determine intakes associated with markers of inadequate or supplemental intakes.

The goal of this dose-response mouse study was to identify folate-dependent physiological, functional and genomic endpoints that could be used as surrogate biomarkers of folate exposure. Our literature review showed a wide variety of investigations on the various health effects associated with a wide range of, but inconsistent, folic acid doses; however, no comprehensive dose-response study has identified doses that are related to adequacy or over-exposure. Furthermore, the review also demonstrated the use of a large variety of mouse strains and bias in the use of mouse sex. These inconsistencies make it difficult to interpret the findings from mouse studies to humans. To address this knowledge gap, in this study, we used both male and female C57BL/6 and BALB/c mice, two of the most commonly used inbred mouse strains. As such, our

study investigated status and functional biomarkers of folate exposure in mice, while taking into account underlying sex and strain effects.

3.3. Methods

3.3.1. Animal Models and Study design

The mice in this study were cared for in accordance with the Guidelines of the Canadian Council on Animal Care (CACC) as described in the CACC Guide of the Care and use of Experimental Animals (104). The study was approved by the Health Canada Ottawa Animal Care Committee. Both male and female C57BL/6 and BALB/c mice (n = 80 sex/strain), the two most commonly used inbred mouse strains in folate research (see chapter 2) were purchased from Charles River Laboratories (Saint Constant, QC) at 3 weeks of age and housed at standard humidity and temperature with 12-hour light cycles. The specific substrains used was C57BL/6Ncrl and BALB/c AnNCrl. All the mice had *ad libitum* access to food and water.

Upon arrival, all mice were fed the 2 mg/kg FA-defined diet based on the AIN-93G formula (70) for 1 week during the acclimation period to establish a baseline folate status. After the acclimation period, the mice were fed one of the eight modified AIN-93G diets containing 0, 0.2, 0.5, 1, 2, 5, 20 or 40 mg of FA/kg (Dyets, Inc.; Bethlehem, PA). This range of FA content encompasses intakes that are reported in the literature (See chapter 2). There was a total of 20 mice (n = 10 male, n = 10 female) per strain per experimental diet for a total of 320 mice (20 mice x 8 diets x 2 strains). The power calculation used to determine the sample size was based on a previous experiment for RBC micronucleus assays: Alpha = 0.05, power 0.80, 1-sided, a 35% difference in means was assumed and a 30% standard deviation. The minimum sample size was determined to be 5. Because of the number of outcomes to be measured, only half of the full sample would be

available for MN analysis, therefore an n = 10 was required. The mice were fed their respective diets for 12 weeks before necropsy, a duration required to examine the impact of folate on functional endpoints including micronucleus formation in blood cells (105). The primary dietary exposure was the dose-response of folic acid. Primary outcomes investigated included the micronucleus frequency, total plasma folate, unmetabolized folic acid and homocysteine. Other secondary outcomes investigated included body weights, food consumptions, tissue weights, blood counts and other folate vitamers.

3.3.2. Necropsy and tissue collection

The body weight and food consumption for each mouse was recorded from week 1 until necropsy for a total of 12 weeks. Additionally, blood from cheek was sampled every 2 weeks starting at 4 weeks of age (baseline folate at the time the mice were switched to their experimental diets) to measure changes in plasma and RBC folate concentration over time. After 12 weeks on diet, the mice were killed under isoflurane anesthesia by cardiac puncture followed by cervical dislocation. Tissues collected at necropsy included colon epithelium, liver, kidneys, bone marrow, brain, spleen. Testes were also collected from male mice. All tissues were collected into 1.5 mL Eppendorf tubes, flash frozen in liquid nitrogen and stored at -80 ℃ until use.

Whole blood was drawn via cardiac puncture into a heparin coated syringe (5:1 ratio of blood to heparin). 100 μL of whole blood was aliquoted for micronucleus analysis, 700 μL for total folate, folate vitamers and homocysteine analyses and the remaining 200 μL was used for complete blood count (CBC) analysis. All blood samples, except CBC samples, were stored on wet ice (~4 °C) until further processing. The CBC samples were stored at room temperature until analysis. The 700 μL of whole blood collected was separated by centrifugation (5000 $\times$ g for 10 minutes at 4 °C) into RBC and plasma within an hour of collection. Plasma was divided for folate vitamer (150

μL), homocysteine (120 μL) and total folate (20 μL) analyses. Any extra plasma was added to the total folate. All blood samples, including the RBC pellets, were aliquoted to 1.5 mL Eppendorf tubes flash frozen and stored at -80 ℃.

The colon was dissected, the fecal pellets were squeezed out gently before being flushed with 5 mL of cold phosphate buffered saline (PBS). The colon was then cut opened and laid flat with the lumen side facing up on a glass plate. The epithelial layer was lightly scraped away from the underlying colon tissue using two glass sides, collected into 1.5 mL Eppendorf tubes, flash frozen in liquid nitrogen and stored at -80 ℃ until use.

Bone marrow was collected from the femur. The femur was detached from the hip bone and the tibia cut below knee joint. The femur and tibia were both scraped clean of muscle tissue using Kimwipes and the ball joint of the femur was cut off to expose the inside of the bone. The femur was placed cut-end down inside a 0.5 mL Eppendorf tube in which a small hole was created using a 18G needle at the bottom of the 0.5 mL Eppendorf tube, that was nested in a larger 1.5 mL Eppendorf tube. The nested tubes were centrifuged (~ 8000 x g) to collect bone marrow pellets that were resuspended in 200 μL of PBS. Finally, the resuspended bone marrow was evenly distributed into 4 separate 1.5 mL Eppendorf tube (~50 μL each) and flash frozen.

3.3.3. Plasma total folate analysis

Plasma total folate was measured for all necropsy samples (week 12) and a subset of samples (n = 5) at each time point (weeks 0, 2, 4, 6, 8 and 10) from each of the 32 (8 diets x 2 strains x 2 sex) experimental groups using the *Lactobacillus casei (L. casei)* microbiological assay as described by Molloy et al. (106). New *L. casei* (Lactobacillus rhamnosus from ATCC 7469) stock was first made in an 80% glycerol and stored in 1 mL cryogenic vials at -80 ℃ until use.

Fresh media was prepared according to the manufacturer's instructions (BD Difco Folic Acid Casei Medium 282210, lot# 0000434386) weekly and stored in the fridge for up to 5 days, wrapped in foil. The mouse plasma was diluted with 0.5 (w/v) sodium ascorbate (Sigma A-7631) for analysis. A plasma dilution of 30x was used for mice fed a 0-5 mg/kg FA diet while a 100x dilution was used for mice fed a 20-40 mg/kg FA diet. The folic acid standards (2, 5, 10, 25, 50, 75, 100 fmol/ul) used for our standard curve were prepared using serial dilution from a folic acid stock of 98.54 ug/ml. Plated samples were incubated overnight in a CO_2 incubator at 37 ℃ for ~ 16 hours. Absorbances were read using at a single wavelength of 550 nm. The *L. casei* bacteria used for this assay is dependent on folate for growth, therefore its growth can be correlated to the total folate content in the sample. The plasma total folate represents recent folate intakes (107), providing a biomarker for recent folate intake.

3.3.4. Plasma folate vitamer analysis

Folate vitamers analysis was performed by the Ontario Region Food Laboratories Division, Health Canada. The 6 folates vitamers analyzed included: unmetabolized FA (UMFA), 5-methyltetrahydrofolate (5-methylTHF), 5-formyltetrahydrofolate (5-FormylTHF), Tetrahydrofolate (THF), 5,10-methenyltetrahydrofolate (5,10-methenylTHF), and MeFox (an oxidation product of 5-methylTHF). Folate vitamers were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (108) using a Waters Xevo TQ-XS Mass Spectrometry coupled to the Waters Acquity UPLC system (Waters Limited, Mississauga ON). Plasma samples collected at necropsy were thawed and spiked with one of six internal standard mixtures followed by the addition of extraction buffer (at pH 5) and an incubation period of 4°C for 30 min. Sample extraction and cleanup was then performed by solid phase extraction (SPE) using a sodium acetate

elution buffer. The final extracts were then filtered through 0.2 μm syringe filter prior to LC-MS/MS analysis. Folate peaks were separated by a UPLC gradient mobile phase and detected by MS/MS in MRM mode. Quantitation was performed using external calibration standards based on the peak area ratio using internal standard. For quality control purposes, the samples were run with a calibration standard solution (~1 nmol/L FA) prepared on the same day. Quality control metrics for analysis was determined by percentage recovery and both intra and inter-assay coefficient variations. Folate vitamer concentrations that were below the limit of detection (LOD) were assigned a value of LOD/2 for analyses. Data collection and reduction was achieved using the TargetLynx XS software (MassLynx, V4.2). The sum of folate forms except MeFOX was reported as total folate. Combined non-methyl folates represented the sum of 5-formyl THF, THF and 5,10-methenylTHF.

3.3.5. Homocysteine analysis

The Advia Centaur XP (Siemens Healthcare Ltd) was used to perform homocysteine analysis. Plasma homocysteine was measured by the Nutrition Research Canadian Health Measures Survey Reference Laboratory following the manufacturer's instructions. All policies, procedures and processes conducted in the laboratory aligned with licensing and accreditation standards of clinical laboratories in Canada and the recommendations of the Institute for Quality Management in Healthcare (iQMH). The assay was verified prior to sample analysis using third party quality control materials (BioRad).

3.3.6. CBC analysis

The Sysmex® XT-2000*i* hematology analyzer (Sysmex Corporation, Kobe Japan) was used to analyse white blood cells and reticulocytes using fluorescent flow cytometry. Red blood cells and platelet counts were analyzed by the RBC detector using the Hydrodynamic Focusing method while hemoglobin was analyzed by the hemoglobin (HGB) detector based on a cyanide-free Sulfolyser hemoglobin detection method (109)(110). A total of 33 analytical parameters were assessed. White blood cell counts included: Total white blood cells (WBC), lymphocytes (LYMPH), monocytes (MONO), eosinophils (EO), basophils (BASO) and neutrophils (NEUT). Red blood cell counts included: red blood cells (RBC), reticulocytes (RET), HGB, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width with standard deviation (RDW-SD) and Immature Reticulocyte Fraction (IRF). Platelet counts included: platelets (PLT), mean platelet volume (MPV), platelet distribution width (PDW) and platelet large cell ratio (P-LCR).

3.3.7. Micronucleus analysis

The *In Vivo* MicroFlow® assay was used to measure micronucleated cells in whole blood. The whole blood was collected in a 1.5 mL Eppendorf tubes containing 350 μL of anticoagulant solution provided by Litron Laboratories (Rochester, NY). The whole blood-anticoagulant mixtures were stored at room temperature until fixation, stored at -80 ℃ for 48 hours before washing in and storing in long-term storage solution (LTSS; glycerol 5-10%) as per instructions provided in the *In Vivo* MicroFlow® kit (Litron Laboratories, Rochester NY). Samples stored in LTSS were sent to Litron Laboratories for analysis.

The *In Vivo* MicroFlow® assay uses a differential staining technique to measure the frequency of micronucleated cells in reticulocyte (RET) and mature normochromatic erythrocyte

(NCE) cell populations by flow cytometry. The micronucleus (MN) is a small secondary nucleus that can form spontaneously due to chromosomal damage or breakage (111). The MN develops in the cytoplasm outside the main nucleus (111). Its presence is most evident in RBCs which otherwise do not have a nucleus. The assay uses a propidium iodide DNA stain to identify the presence of micronuclei in cells from which MN frequency can be determined. The frequencies of micronucleated reticulocytes (MN-RET) and erythrocytes (MN-NCE) are indicators of chromosomal damage. To differentiate between RET and NCE cell populations, the assay uses an antibody against the cluster of differentiation 71 (CD71) transferrin receptors – surface proteins present on immature erythrocytes (RETs) but not on mature erythrocytes (NCE). The frequency of MN-RET and MN-NCE compared to RET and NCE without MN was determined by the presence of stained DNA in the cells by flow cytometry.

3.3.8. Statistical analysis

All statistical analyses were performed using SigmaPlot for Windows, version 13.0 (Systat Software, Inc.). For variables with repeated measures over time (i.e., body weight, food consumption and plasma total folates), main effects of diet, sex and strain were identified using a Two-Way Repeated Measure ANOVA. In cases where main effects of sex and strain were identified, the effect of diet over time was additionally assessed using a Two-Way Repeated Measure ANOVA within each strain/sex group (e.g., BALB/c females). For variables with a single endpoint measured at necropsy (i.e., Total body weight gained, homocysteine, folate vitamers, CBC, micronucleus, and tissue weight) a Three-Way ANOVA was used to identify main effects of diet, sex and strain. When a main effect of sex and strain were identified, a One-Way ANOVA analysis was used to identify differences among the diets within each strain/sex group. Differences in means were considered significant when $P \leq 0.05$.

3.4. Results

3.4.1. Body weights

The overall body weight gained by the mice differed significantly by sex ($p = <0.001$), strain ($p = <0.001$) and diet ($p = 0.009$) (**Figure 12**). As expected, female mice gained 65.0 % less body weight than male mice. BALB/c mice gained 32.7% less body weight than C57BL/6 mice. Even though significant diet differences were observed across strains and sexes, the total body weight gained within each sex/strain group showed no significant differences.

Similarly, when examining body weight gained over the course of the intervention period (**Figure 13**), the weight gained by the mice differed significantly by sex ($p = < 0.001$) and strain ($p = < 0.001$) but not by diet. Female mice gained weight at a slower rate than male mice every week ($p = < 0.001$). C57BL/6 mice gained weight significantly faster than BALB/c mice throughout the course of the intervention. Overall, no significant diet effect was observed within each sex/strain group. However, some inconsistent significant differences were identified among diets in BALB/c females and C57BL/6 females. Among BALB/c females, significant diet differences were observed at week 3 between the 40 mg/kg diet and the 0 mg/kg ($p = 0.002$), 0.2 mg/kg ($p = 0.035$), 1 mg/kg ($p = 0.005$), 2 mg/kg ($p = < 0.001$), 5 mg/kg ($p = 0.012$) and the 20 mg/kg ($p = 0.011$) diets. Among C57BL/6 females, the mean body weight of mice fed the 0.2 mg/kg diet was significantly more than mice fed 0 mg/kg at week 9 ($p = 0.020$) and week 12 ($p = 0.013$), mice fed 0.5 mg/kg at week 12 ($p = 0.031$), mice fed 1 mg/kg at week 12 ($p = 0.045$) and mice fed 40 mg/kg at week 5 ($p = 0.003$) and 12 ($p = 0.033$).

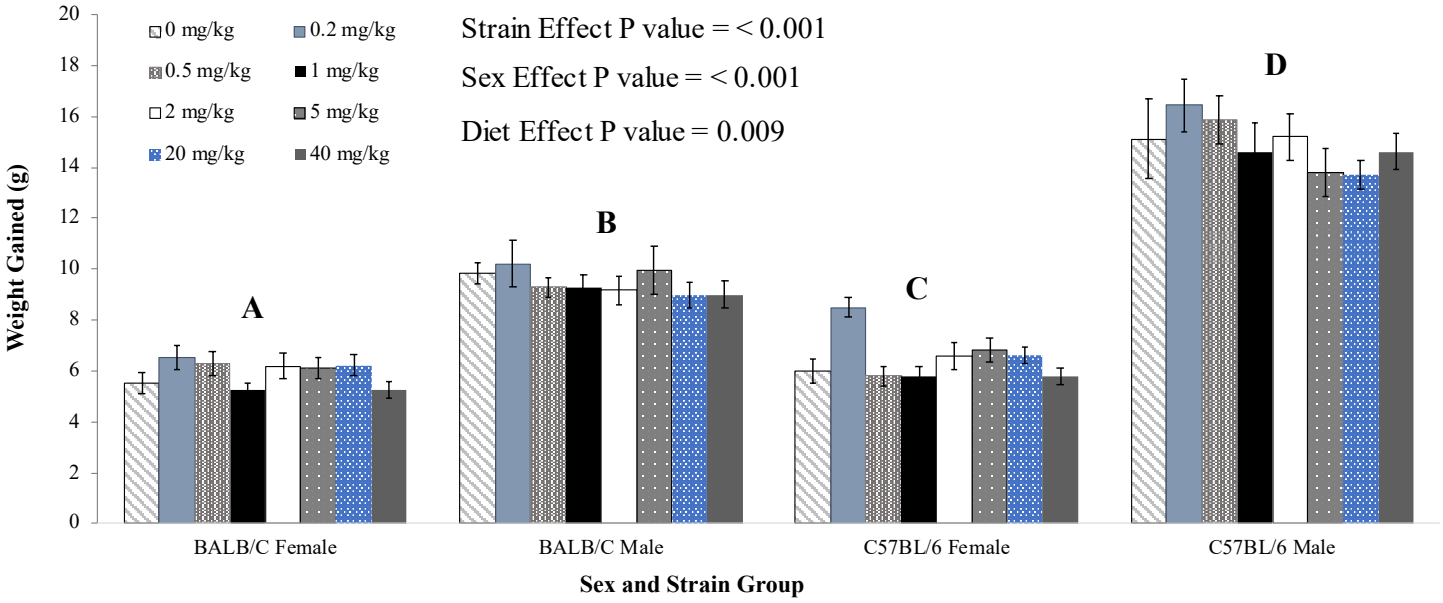

Figure 12: Total body weight gained by mice fed different folic acid diets over the duration of the study period. Differences in means among strain, sex and diets on total body weight gain were assessed by Three-Way ANOVA and Holm-Sidak post-hoc analysis. Differences in means among diet groups within each sex/strain group were assessed by One-Way ANOVA and Kruskal-Wallis post-hoc analysis. Sex/strain groups with different letters (A, B, C, D) are significantly different. Data are presented as mean ± SEM. Differences were considered significant if $p \leq 0.05$ (n = 10/group).

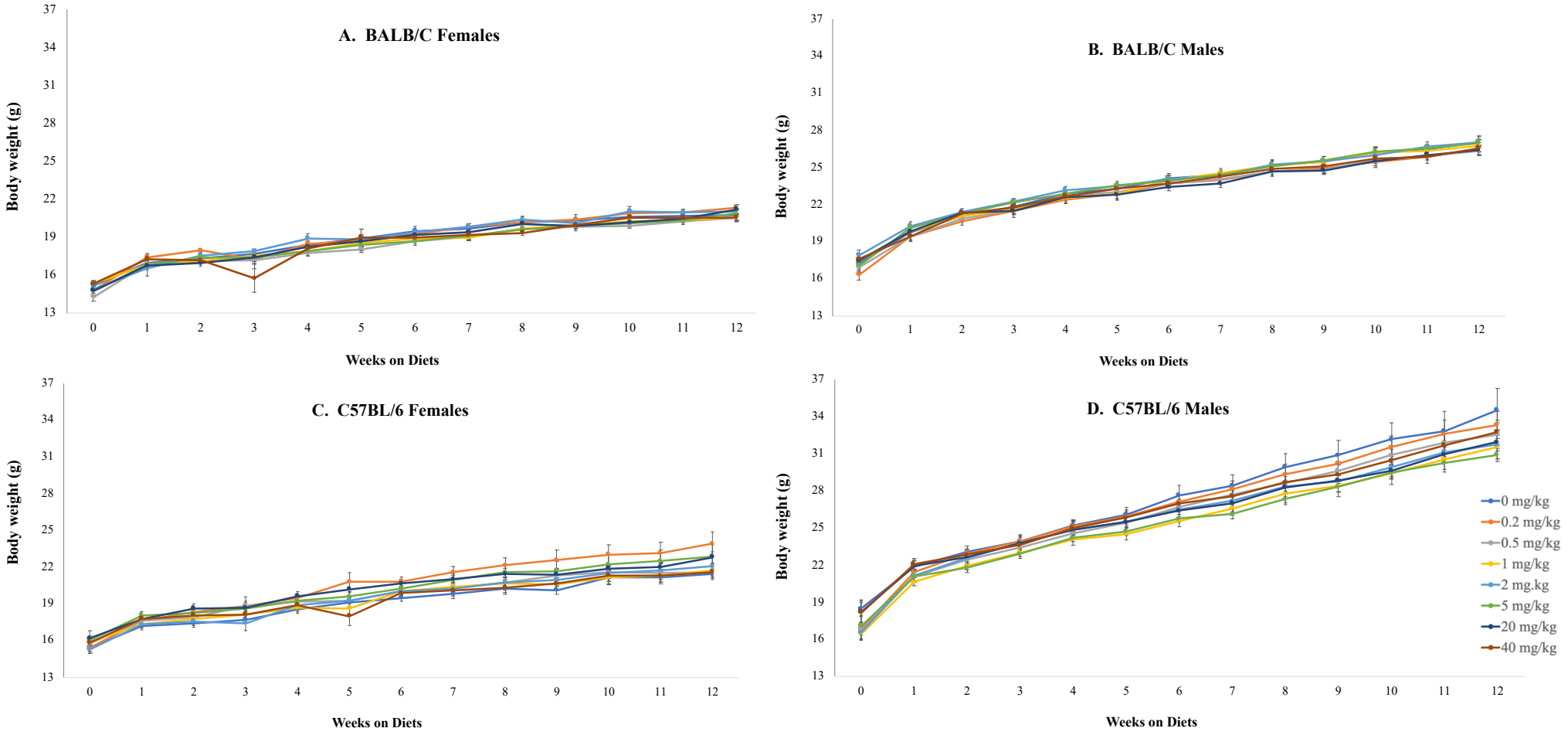

Figure 13: Body weights of male and female BALB/c and C57BL/6 mice fed different folic acid diets over time. Differences in the means among diet groups within each sex/strain group over time was assessed by a Two-Way Repeated Measures ANOVA and Holm-Sidak post-hoc analysis. **A)** Weight gained by BALB/c females **B)** Weight gained by BALB/c males **C)** Weight gained by C57BL/6 females **D**) Weight gained by C57BL/6 males. Data are presented as mean ± SEM. Differences were considered significant if $p \leq 0.05$ (n = 10/group).

3.4.2. Food consumption

The total food consumed by the mice differed significantly by sex ($p = <0.001$), strain ($p = <0.001$) and diet ($p = <0.001$). Female mice ate 13% less than male mice. BALB/c mice consumed 12% less food than C57BL/6 mice. Significant diet effects were also observed within each sex/strain group ($p = <0.001$ in BALB/c females; $p = 0.019$ in BALB/c males; $p = < 0.001$ in C57BL/6 females; $p = 0.014$ in C57BL/6 males) but the differences among the diets was inconsistent.

When examining the daily food consumption over the course of the intervention (**Figure 14**), similar trends were observed with significant differences in sex ($p = < 0.001$), strain ($p = <0.001$) but not diet, as assessed by Three-way ANOVA. Generally speaking, in BALB/c females ($p = 0.004$) consumed the least amount of food, followed by C57BL/6 females ($p = <0.001$), BALB/c males ($p = 0.037$) and finally C57BL/6 males ($p = 0.005$). While we did not observe a main effect of diet overall, within each sex/strain group a diet effect was observed but differences among the diets on any given week were inconsistent.

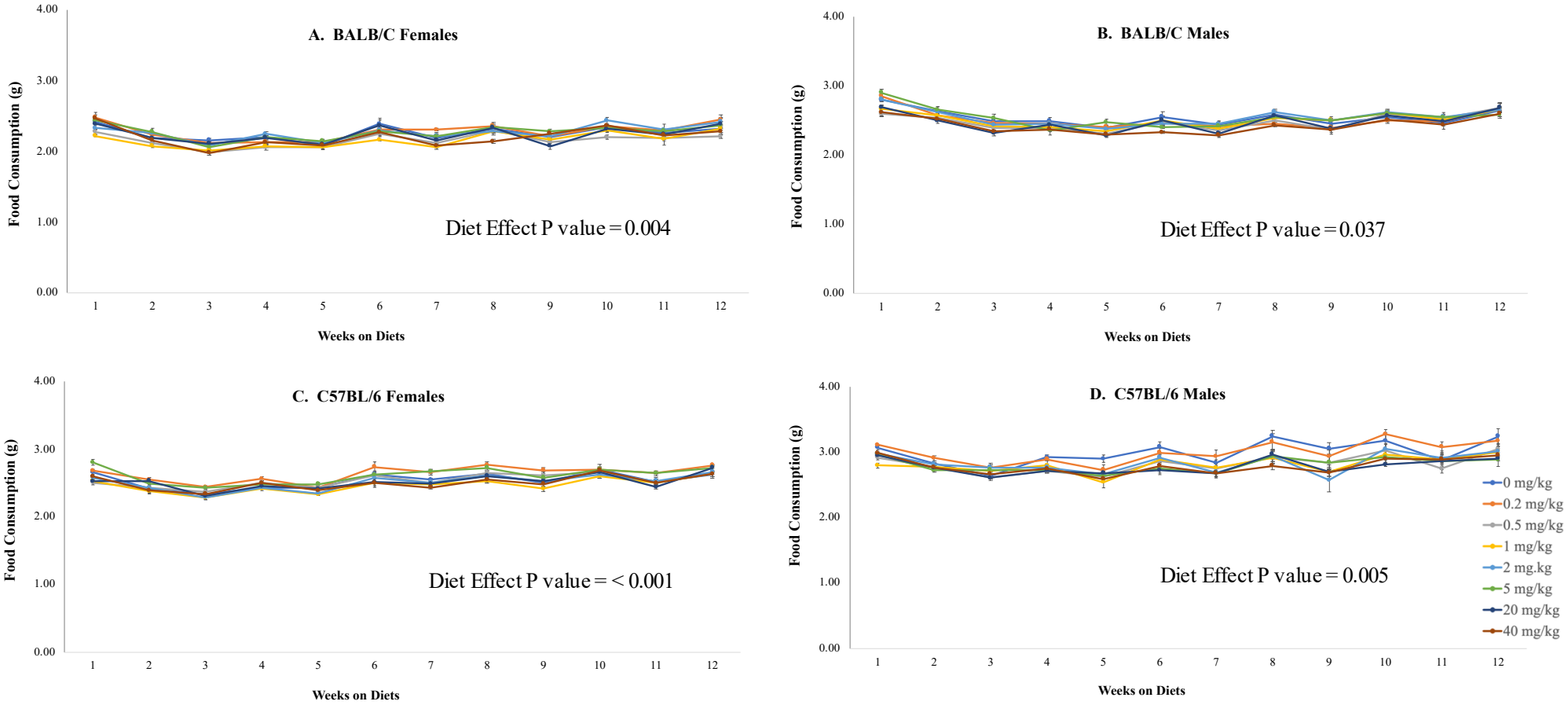

Figure 14: Daily mean food consumption of male and female BALB/c and C57BL/6 mice fed different folic acid diets over time. Differences in the means among diet groups within each sex/strain group over time was assessed by a Two-Way Repeated Measures ANOVA and Holm-Sidak post-hoc analysis. **A)** Food consumed by BALB/c females **B)** Food consumed by BALB/c males **C)** Food consumed by C57BL/6 females **D)** Food consumed by C57BL/6 males. Data are presented as mean ± SEM. Differences were considered significant if $p \leq 0.05$ (n = 10/group).

3.4.3. Tissue weights

A strain effect was observed for liver ($p = <0.001$) and brain ($p = <0.011$) weights but not the testes, kidney and spleen weights. Liver tissues weighed 19% more in C57BL/6 mice compared to BALB/c mice while brain tissues weighed 1% more in C57BL/6 mice. A sex effect was also observed for liver ($p = <0.001$), kidney ($p = <0.001$) and brain ($p = <0.001$) weights but not for the testes and spleen. The male mice had heavier liver (39%) and kidney (47%) tissues while female mice had heavier brain (1%) and spleen (1%) tissues. No diet effects were identified for any tissue weights. No significant diet differences were identified within sex/strain groups (**Table 3**).

Table 3: Tissue weights of BALB/c and C57BL/6 mice at necropsy. Differences in means among sex, strain and diet groups were assessed by Three-Way ANOVA and Holm-Sidak post-hoc analysis. Since significant sex and strain differences were observed for some tissue weights, data were evaluated within sex/strain groups for diet differences. Differences in means among the diets within each sex/strain group was assessed by One-Way ANOVA and Kruskal-Wallis on ranks post-hoc analysis (P values in table). Data are presented as mean ± SEM. Differences were considered significant if $p \leq 0.05$.

Strain/Sex	Tissue	0 mg/kg	0.2 mg/kg	0.5 mg/kg	1 mg/kg	2 mg/kg	5 mg/kg	20 mg/kg	40 mg/kg	P Value	Sample Size
BALB/C Females	Liver	0.8391 ± 0.0209	0.9132 ± 0.0212	0.8436 ± 0.0220	0.8415 ± 0.0166	0.9090 ± 0.0187	0.8795 ± 0.0232	0.8985 ± 0.0309	0.8568 ± 0.0249	0.073	n = 10
	Left Kidney	0.1161 ± 0.0027	0.1177 ± 0.0020	0.1130 ± 0.0031	0.1141 ± 0.0023	0.1225 ± 0.0020	0.1148 ± 0.0011	0.1165 ± 0.0045	0.1124 ± 0.0025	0.178	n = 10
	Spleen	0.0948 ± 0.0028	0.0942 ± 0.0026	0.0921 ± 0.0027	0.0919 ± 0.0018	0.0991 ± 0.0030	0.0900 ± 0.0024	0.0920 ± 0.0027	0.0920 ± 0.0027	0.384	n = 10
	Brain	0.4340 ± 0.0056	0.4434 ± 0.0046	0.4366 ± 0.0019	0.4348 ± 0.0041	0.4418 ± 0.0047	0.4347 ± 0.0050	0.4360 ± 0.0060	0.4343 ± 0.0026	0.817	n = 9-10
BALB/C Males	Liver	1.1756 ± 0.0339	1.2223 ± 0.0188	1.2053 ± 0.0326	1.1718 ± 0.0379	1.1818 ± 0.0220	1.1704 ± 0.0206	1.1749 ± 0.0232	1.2160 ± 0.0100	0.700	n = 10
	Left Kidney	0.1936 ± 0.0053	0.1839 ± 0.0063	0.1906 ± 0.0079	0.1951 ± 0.0071	0.1910 ± 0.0047	0.1764 ± 0.0025	0.1995 ± 0.0065	0.1934 ± 0.0056	0.167	n = 9-10
	Testes	0.1384 ± 0.0439	0.0892 ± 0.0026	0.0900 ± 0.0021	0.0933 ± 0.0032	0.0911 ± 0.0027	0.0929 ± 0.0021	0.0939 ± 0.0024	0.0898 ± 0.0015	0.571	n = 9-10
	Spleen	0.0826 ± 0.0016	0.0853 ± 0.0024	0.0905 ± 0.0066	0.0848 ± 0.0024	0.0852 ± 0.0012	0.0874 ± 0.0022	0.0934 ± 0.0052	0.0775 ± 0.0050	0.543	n = 10
	Brain	0.4344 ± 0.0024	0.4343 ± 0.0032	0.4257 ± 0.0032	0.4327 ± 0.0020	0.4333 ± 0.0025	0.4267 ± 0.0033	0.4341 ± 0.0025	0.4321 ± 0.0036	0.209	n = 9-10

Table 3 continued: Tissue weights of BALB/c and C57BL/6 mice at necropsy.

Strain/Sex	Tissue	0 mg/kg	0.2 mg/kg	0.5 mg/kg	1 mg/kg	2 mg/kg	5 mg/kg	20 mg/kg	40 mg/kg	P Value	Sample Size
C57BL/6 Females	Liver	0.9418 ± 0.0290	1.0156 ± 0.0554	0.9552 ± 0.0216	0.8830 ± 0.0588	0.9730 ± 0.0462	0.9749 ± 0.0461	1.0178 ± 0.0272	0.9348 ± 0.0280	0.215	n = 10
	Left Kidney	0.1161 ± 0.0029	0.1210 ± 0.0025	0.1196 ± 0.0040	0.1235 ± 0.0044	0.1163 ± 0.0025	0.1208 ± 0.0029	0.1218 ± 0.0026	0.1189 ± 0.0025	0.694	n = 10
	Spleen	0.0872 ± 0.0035	0.0943 ± 0.0037	0.0887 ± 0.0034	0.0897 ± 0.0051	0.0818 ± 0.0025	0.0887 ± 0.0026	0.0899 ± 0.0039	0.0820 ± 0.0029	0.242	n = 10
	Brain	0.4369 ± 0.0039	0.4413 ± 0.0047	0.4384 ± 0.0038	0.4416 ± 0.0068	0.4414 ± 0.0027	0.4468 ± 0.0037	0.4427 ± 0.0025	0.4355 ± 0.0077	0.823	n = 10
C57BL/6 Males	Liver	1.6139 ± 0.0859	1.5715 ± 0.0570	1.5634 ± 0.0421	1.5664 ± 0.0475	1.5248 ± 0.0457	1.4254 ± 0.0344	1.5245 ± 0.0292	1.5273 ± 0.0311	0.267	n = 9-10
	Left Kidney	0.2939 ± 0.1092	0.1820 ± 0.0034	0.1696 ± 0.0048	0.1708 ± 0.0055	0.1796 ± 0.0110	0.1675 ± 0.0051	0.1678 ± 0.0046	0.1793 ± 0.0058	0.202	n = 10
	Testes	0.0996 ± 0.0023	0.0985 ± 0.0035	0.1003 ± 0.0033	0.0981 ± 0.0028	0.0913 ± 0.0078	0.1036 ± 0.0037	0.1013 ± 0.0033	0.1005 ± 0.0024	0.489	n = 10
	Spleen	0.0948 ± 0.0037	0.0919 ± 0.0048	0.0986 ± 0.0039	0.0916 ± 0.0034	0.1053 ± 0.0089	0.0893 ± 0.0043	0.0875 ± 0.0029	0.0900 ± 0.0034	0.335	n = 10
	Brain	0.4414 ± 0.0034	0.4401 ± 0.0029	0.4349 ± 0.0032	0.4307 ± 0.0048	0.4344 ± 0.0061	0.4331 ± 0.0040	0.4369 ± 0.0036	0.4325 ± 0.0029	0.552	n = 9-10

3.4.4. Folate status

3.4.4.1. Plasma total folate

Over time, there were no significant sex or strain differences observed for plasma total folate concentrations over the course of the intervention period (**Figure 15**). The plasma total folate concentrations only differed significantly by sex and strain when comparing week 2 and 6. However, the plasma total folate in the mice differed significantly by diet ($p = < 0.001$). The plasma total folate concentrations differed significantly among all diet groups except when comparing the 2 mg/kg diet versus the 1 mg/kg diet, and 0.2 mg/kg diet versus the 0 mg/kg diet. As expected, higher folic acid content in the diet correlated with higher plasma total folate concentrations.

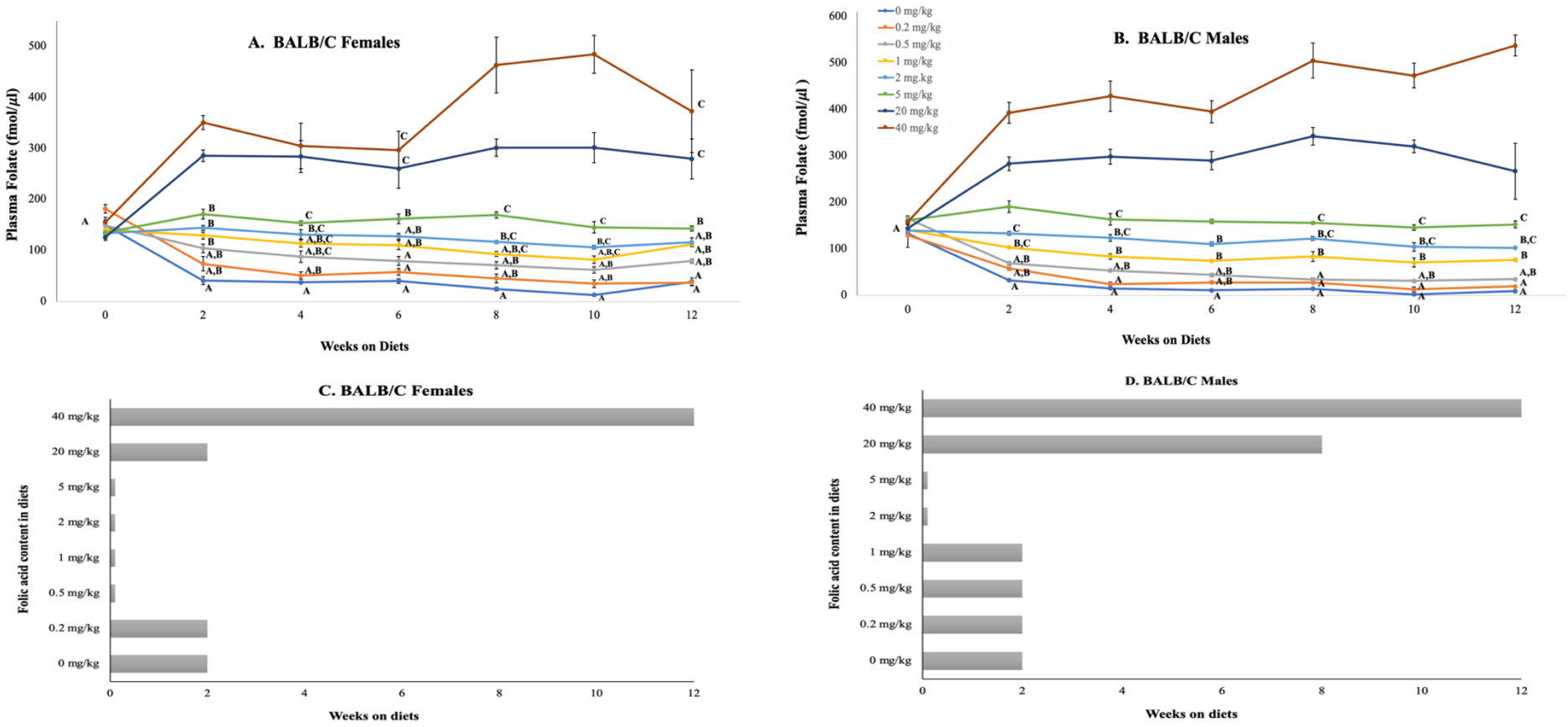

Figure 15: Plasma total folate of male and female BALB/c and C57BL/6 mice fed different folic acid diets over time. Differences in the means among diet groups within each sex/strain group over time was assessed by a Two-Way Repeated Measures ANOVA and Holm-Sidak post-hoc analysis. Plasma total folate of BALB/c females (**A**), BALB/c males **(B),** C57BL/6 females **(E)** and C57BL/6 males (**F**). Time taken for plasma folate changes to reach hemostasis in BALB/c females (**C**), BALB/c males **(D),** C57BL/6 females **(G)** and C57BL/6 males (**H**). Diets with similar letters (A, B, C, D) are not significantly different. Data are presented as mean ± SEM. Differences were considered significant if $p \leq 0.05$ ($n = 5$).

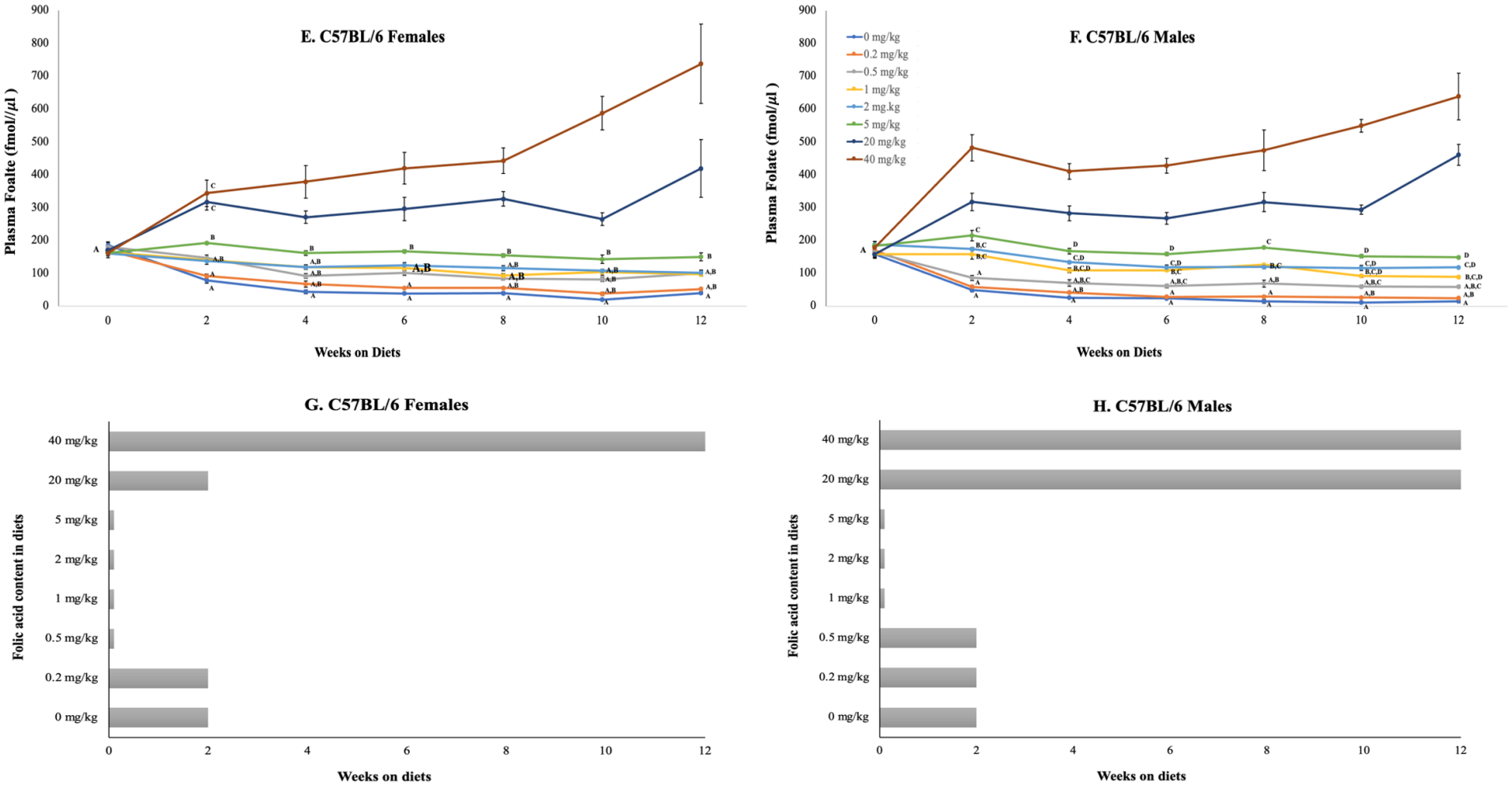

Figure 15 continued: Plasma total folate of male and female BALB/c and C57BL/6 mice fed different folic acid diets over time.

3.4.4.1. Folate vitamers

An analysis of the folate vitamers showed a significant strain effect for total folate ($p = < 0.001$), UMFA ($p = < 0.001$), 5-methylTHF ($p = < 0.001$) but not for combined nonmethyl folates (i.e., 5,10-methenylTHF, 5-formylTHF, THF) and MeFOX. C57BL/6 mice had higher total folate (48%), UMFA (74%), 5-methylTHF (123%) than BALB/c mice.

There were significant sex effects for all folate vitamers except for MeFOX. Male mice had higher total folate ($p = < 0.001$; 51%), UMFA ($p = < 0.001$; 86%), 5-methylTHF ($p = < 0.001$; 12%) and combined nonmethyl folates ($p = < 0.001$; 37%) than female mice.

All folate vitamer concentrations in the mice at necropsy differed significantly by diet ($p = < 0.001$). A further examination of the diet effect within each sex/strain group showed similar significant diet effects (**Table 4**). Exceptions included an insignificant diet effect for MeFOX in BALB/c female and C57BL/6 male mice.

Benchmark dose modeling (BMD) showed that UMFA concentrations increased at a benchmark dose response (BMDR) diets $\geq$ 1.87 mg FA/kg, 1.03 mg FA/kg, 1.02 mg FA/kg, 1.00 mg FA/kg for BALB/c male, BALB/c female, C57BL/6 female and C57BL/6 male mice respectively. This respectively corresponded to UMFA concentrations of 7.71 nmol/L, 11.53 nmol/L, 20.75 nmol/L and 18.43 nmol/L (**Figure 16**).

Table 4: Folate vitamers of BALB/c and C57BL/6 mice measured at necropsy. Differences in means among sex, strain and diet groups were assessed by Three-Way ANOVA and Holm-Sidak post-hoc analysis. Since significant sex and strain differences were observed for some folate vitamers, data was also evaluated within sex/strain groups for diet differences. Differences in means among the diets within each sex/strain group was assessed by One-Way ANOVA and Dunn's method on ranks post-hoc analysis. Data are presented as mean ± SEM. Differences were considered significant if $p \leq 0.05$. Values within a row that share a letter do not differ. Combined nonmethyl folates includes 5,10-methenylTHF, 5-formylTHF and THF.

Strain/Sex	Folate Vitamers (nmol/L)	0 mg/kg	0.2 mg/kg	0.5 mg/kg	1 mg/kg	2 mg/kg	5 mg/kg	20 mg/kg	40 mg/kg	P Value	Sample size
BALB/C Females	Total Folate	^{B} 20.11 ± 1.76	^{B,E} 21.99 ± 2.30	^{B,D} 33.86 ± 2.08	^{B,C} 51.60 ± 2.90*	^{A,C,D,E} 65.02 ± 4.95	^{A,C,D} 82.92 ± 2.67	^{A,C} 187.39 ± 23.32*	^{A} 303.37 ± 86.20	**< 0.001**	n = 9-10
	Combined Nonmethyl Folates	^{B} 2.60 ± 0.45	^{B} 2.65 ± 0.45	^{B,D} 3.99 ± 0.48	^{B,C} 6.91 ± 0.77*	^{B,C} 6.89 ± 0.64	^{A,C,D} 10.53 ± 0.81	^{A,C} 23.61 ± 3.11*	^{A} 50.57 ± 4.43	**< 0.001**	n = 9-10
	UMFA	^{B,C} 2.30 ± 0.34	^{B,C} 2.15 ± 0.35	^{B,C} 2.76 ± 0.25	^{B,C} 3.44 ± 0.53*	^{B,C} 4.62 ± 1.10	^{A,C} 12.51 ± 3.21	^{A} 96.62 ± 27.38*	^{A} 168.78 ± 84.06	**< 0.001**	n = 9-10
	5-MethylTHF	^{B} 15.42 ± 1.51	^{B}17.44 ± 1.76	^{B,C,D} 27.47 ± 1.75	^{B,C,D} 42.03 ± 2.89*	^{A,D} 54.19 ± 5.60	^{A,C} 60.99 ± 4.00	^{A,B} 69.50 ± 6.43*	^{A} 89.48 ± 2.86	**< 0.001**	n = 9-10
	MeFOX	0.45 ± 0.15	0.32 ± 0.11	0.57 ± 0.15	0.41 ± 0.11*	0.36 ± 0.06	0.47 ± 0.04	0.46 ± 0.06*	0.46 ± 0.05	0.534	n = 9-10
BALB/C Males	Total Folate	^{B} 8.06 ± 0.64	^{B,D} 10.67 ± 0.90*	^{B,C} 17.43 ± 1.08	^{B,C} 33.08 ± 1.71	^{A,C,D} 51.48 ± 2.02	^{A,C} 81.33 ± 7.19	^{A,C} 211.40 ± 22.61	^{A} 519.55 ± 73.64	**< 0.001**	n = 9-10
	Combined Nonmethyl Folates	^{B} 1.59 ± 0.35	^{B,D} 2.24 ± 0.45*	^{B,D} 2.57 ± 0.45	^{B,C} 5.44 ± 0.72	^{A,C,D} 8.13 ± 0.69	^{A,C} 15.62 ± 1.21	^{A,C} 38.83 ± 2.54	^{A} 77.92 ± 7.32	**< 0.001**	n = 9-10
	UMFA	^{B} 1.28 ± 0.21	^{B} 1.44 ± 0.24*	^{B} 1.60 ± 0.19	^{B,C} 4.18 ± 0.65	^{B,C} 4.95 ± 0.69	^{A,C} 24.53 ± 6.27	^{A,C} 109.39 ± 19.84	^{A} 446.98 ± 72.00	**< 0.001**	n = 9-10
	5-MethylTHF	^{B} 5.35 ± 0.41	^{B} 7.24 ± 0.44*	^{B,D} 13.54 ± 1.02	^{B,C} 24.04 ± 1.46	^{A,C,D} 39.21 ± 1.55	^{A,C,D} 42.72 ± 1.32	^{A,C} 67.19 ± 1.86	^{A} 103.06 ± 7.36	**< 0.001**	n = 9-10
	MeFOX	^{B,D} 0.18 ± 0.05	^{B,C,D} 0.23 ± 0.04*	^{A,D} 0.27 ± 0.07	^{A,C} 0.46 ± 0.02	^{A} 0.53 ± 0.05	^{A} 0.53 ± 0.05	^{A} 0.59 ± 0.10	^{A} 0.63 ± 0.09	**< 0.001**	n = 9-10

Table 4 Continued: Folate vitamers of BALB/c and C57BL/6 mice measured at necropsy.

Strain/Sex	Folate Vitamers (nmol/L)	0 mg/kg	0.2 mg/kg	0.5 mg/kg	1 mg/kg	2 mg/kg	5 mg/kg	20 mg/kg	40 mg/kg	P Value	Sample size
C57BL/6 Females	Total Folate	[B] 20.12 ± 1.51	[B,E] 28.02 ± 2.78	[B,D] 42.00 ± 1.36	[B,C] 50.61 ± 2.44	[A,C,D,E] 63.29 ± 4.66	[A,C,D] 90.02 ± 5.90	[A,C] 314.22 ± 123.78	[A] 393.69 ± 64.76	**< 0.001**	n = 9-10
	Combined Nonmethyl Folates	[B] 2.41 ± 0.17	[B] 2.57 ± 0.21	[B,D] 4.20 ± 0.28	[B,C] 5.53 ± 0.70	[B,C] 5.65 ± 0.54	[A,C,D] 11.15 ± 1.02	[A,C] 29.99 ± 4.19	[A] 50.41 ± 6.32	**< 0.001**	n = 9-10
	UMFA	[B] 2.59 ± 0.36	[B] 2.56 ± 0.24	[B] 3.43 ± 0.30	[B,C] 4.93 ± 0.63	[A,B] 5.87 ± 0.83	[A,C] 17.43 ± 7.48	[A,C] 217.20 ± 122.08	[A] 257.87 ± 61.97	**< 0.001**	n = 9-10
	5-MethylTHF	[B] 15.28 ± 1.33	[C] 23.12 ± 2.64	[D] 34.72 ± 1.28	[E,B] 40.69 ± 2.37	[A,C] 52.37 ± 5.03	[A,D] 62.65 ± 5.17	[A,E] 71.13 ± 3.99	[A] 91.38 ± 4.18	**< 0.001**	n = 9-10
	MeFOX	[B,C] 0.22 ± 0.05	[B,C] 0.24 ± 0.05	[C] 0.31 ± 0.06	[C] 0.36 ± 0.05	[A] 0.49 ± 0.04	[C] 0.42 ± 0.05	[C] 0.44 ± 0.04	[C] 0.44 ± 0.05	**0.001**	n = 9-10
C57BL/6 Males	Total Folate	[B] 12.14 ± 1.26	[B,E] 14.31 ± 0.60	[B,D] 30.18 ± 2.05	[B,C] 46.52 ± 1.39	[A,C,D,E] 66.18 ± 3.24	[A,C,D] 107.97 ± 9.48	[A,C] 460.05 ± 69.88	[A] 1206.90 ± 242.16	**< 0.001**	n = 9-10
	Combined Nonmethyl Folates	[B] 2.14 ± 0.21	[B,C] 2.02 ± 0.17	[B,C,D] 3.67 ± 0.34	[B,C,D,E] 6.39 ± 0.42	[A,B,C,D,E] 7.86 ± 0.80	[A,D,E] 15.79 ± 1.49	[A,E] 46.86 ± 6.27	[A] 83.90 ± 9.40	**< 0.001**	n = 9-10
	UMFA	[B] 2.15 ± 0.25	[B,C] 2.04 ± 0.35	[B,C,D] 3.62 ± 0.46	[B,C,D,E] 6.08 ± 0.87	[A,B,C,E] 10.63 ± 1.71	[A,D,E] 40.51 ± 9.13	[A,E] 339.15 ± 66.04	[A] 1013.44 ± 233.05	**< 0.001**	n = 9-10
	5-MethylTHF	[B] 8.03 ± 1.01	[B,C] 10.45 ± 0.43	[B,C,D] 23.24 ± 1.65	[B,C,D,E] 34.81 ± 1.19	[A,D,E] 48.65 ± 2.78	[A,D,E] 53.50 ± 2.18	[A,E] 79.88 ± 3.48	[A] 119.87 ± 4.69	**< 0.001**	n = 9-10
	MeFOX	0.25 ± 0.05	0.32 ± 0.03	0.31 ± 0.04	0.35 ± 0.12	0.61 ± 0.18	0.31 ± 0.11	0.53 ± 0.14	0.52 ± 0.12	0.668	n = 9-10

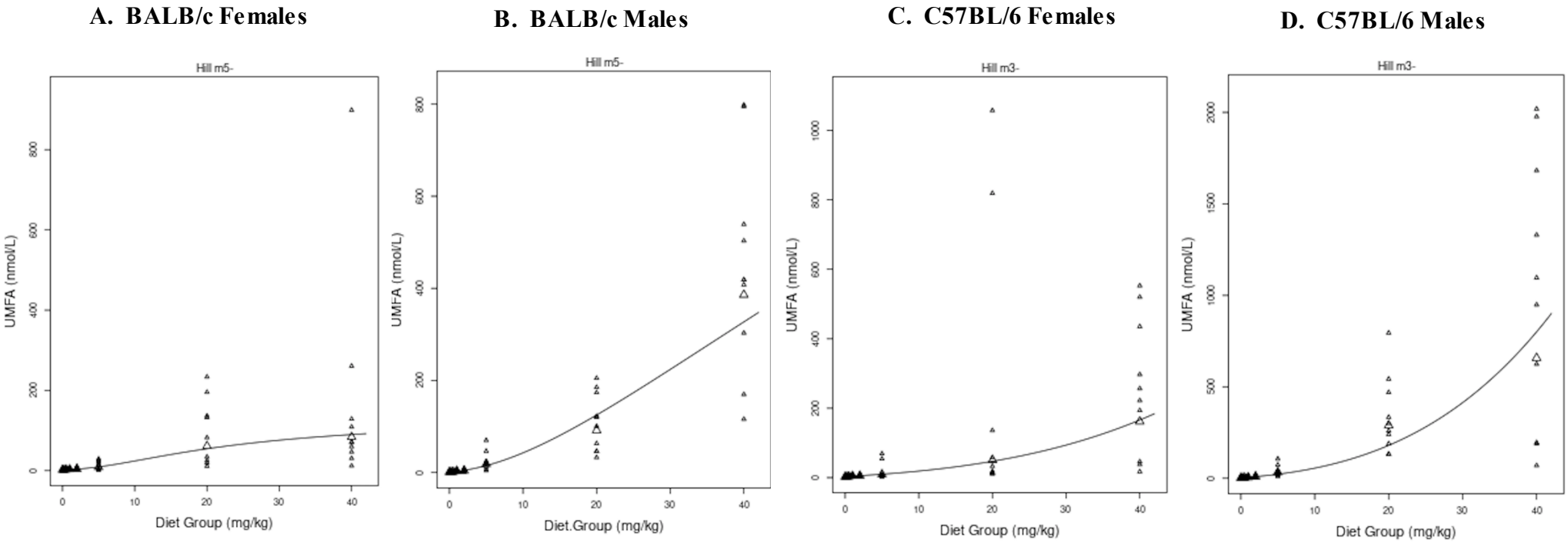

Figure 16: Benchmark Dose (BMD) response modeling of unmetabolized folic acid (UMFA) concentrations in mice fed different folic acid diets measured at necropsy (n = 5). BMD approach was applied as a continuous response of UMFA with increasing folic acid content in diets. The Hill function model was fitted to the mean responses to determine folic acid content in diets upon which Hcy concentrations are elevated.

3.4.5. Functional and genomic instability

3.4.5.1. Homocysteine

Homocysteine concentrations in mice differed significantly by sex ($p = <0.001$), strain ($p = <0.001$) and diet ($p = < 0.001$) (**Figure 17**). C57BL/6 mice had 27% higher homocysteine concentrations than BALB/c mice. Female mice also had 24% higher homocysteine concentration than male mice. As expected, the homocysteine concentrations were lower as the folic acid content in the diets increased.

When examining the homocysteine concentrations within the sex/strain groups, only BALB/c male mice showed significant diet differences ($p = < 0.001$). The significant difference in mean was observed when comparing the 0 mg/kg diet to all other diets ($p = 0.010$ when compared to 0.2 mg/kg; $p = 0.003$ when compared to 0.5 mg/kg; $p = < 0.001$ when compared to 1 mg/kg; $p = < 0.001$ when compared to 2 mg/kg; $p = < 0.001$ when compared to 5 mg/kg; $p = < 0.001$ when compared to 20 mg/kg and $p = < 0.001$ when compared to the 40 mg/kg diet).

BMD modeling showed that Hcy concentrations were higher in mice fed diets containing ≤ 0.95 mg FA/kg (3.88 umol Hcy/L) for BALB/c female mice and ≤ 0.53 mg FA/kg (4.01 umol Hcy/L) for BALB/c male mice. BMDR could not be determined for C57BL/6 mice. BMD analysis further showed that female mice had higher Hcy threshold/baseline concentrations than male mice (**Figure 18**).

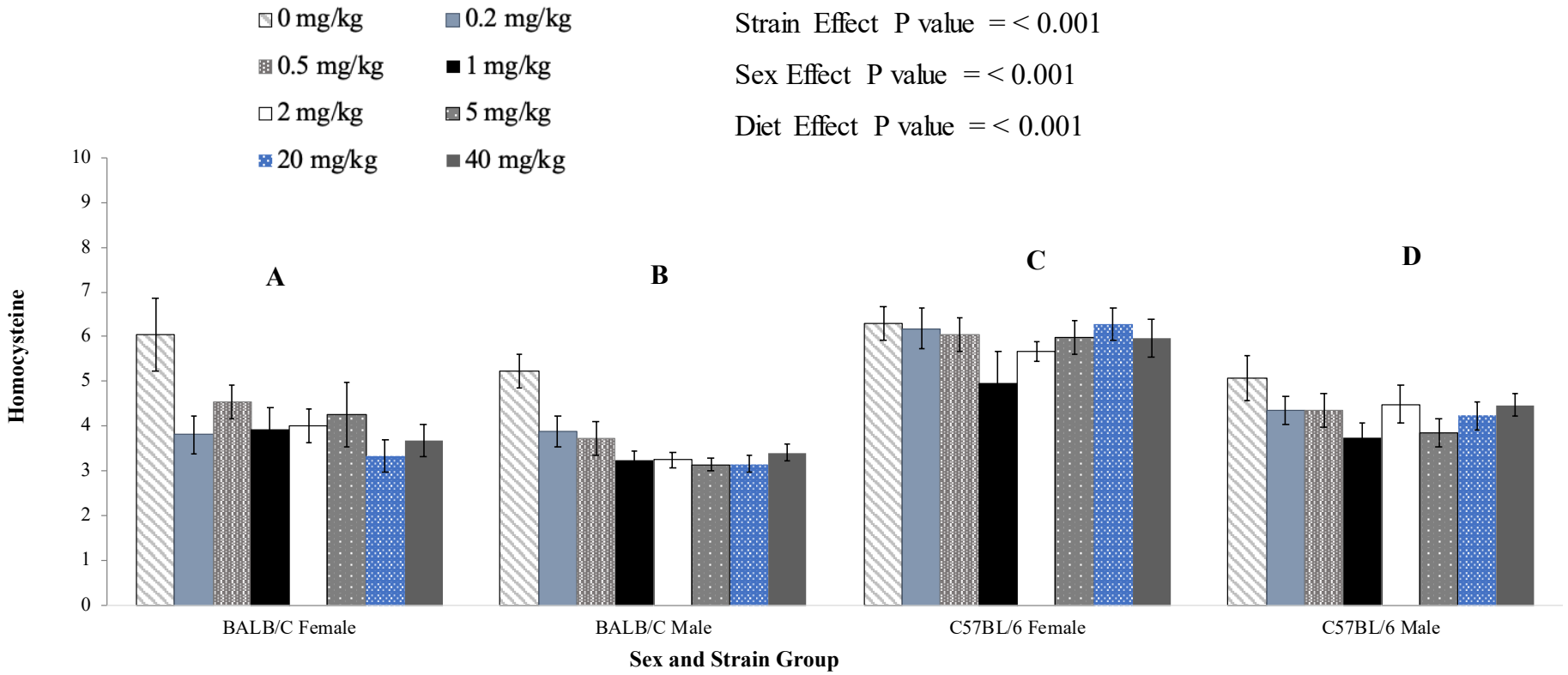

Figure 17 : Homocysteine concentrations in mice fed different folic acid diets measured at necropsy. Differences in means among strain, sex and diets on homocysteine concentrations were assessed by Three-Way ANOVA and Holm-Sidak post-hoc analysis. Differences in means among diet groups within each sex/strain group were assessed by One-Way ANOVA and Kruskal-Wallis post-hoc analysis. Sex/strain groups with different letters (A, B, C, D) are significantly different. Data are presented as mean ± SEM. Differences were considered significant if $p \leq 0.05$ ($n = 5$).

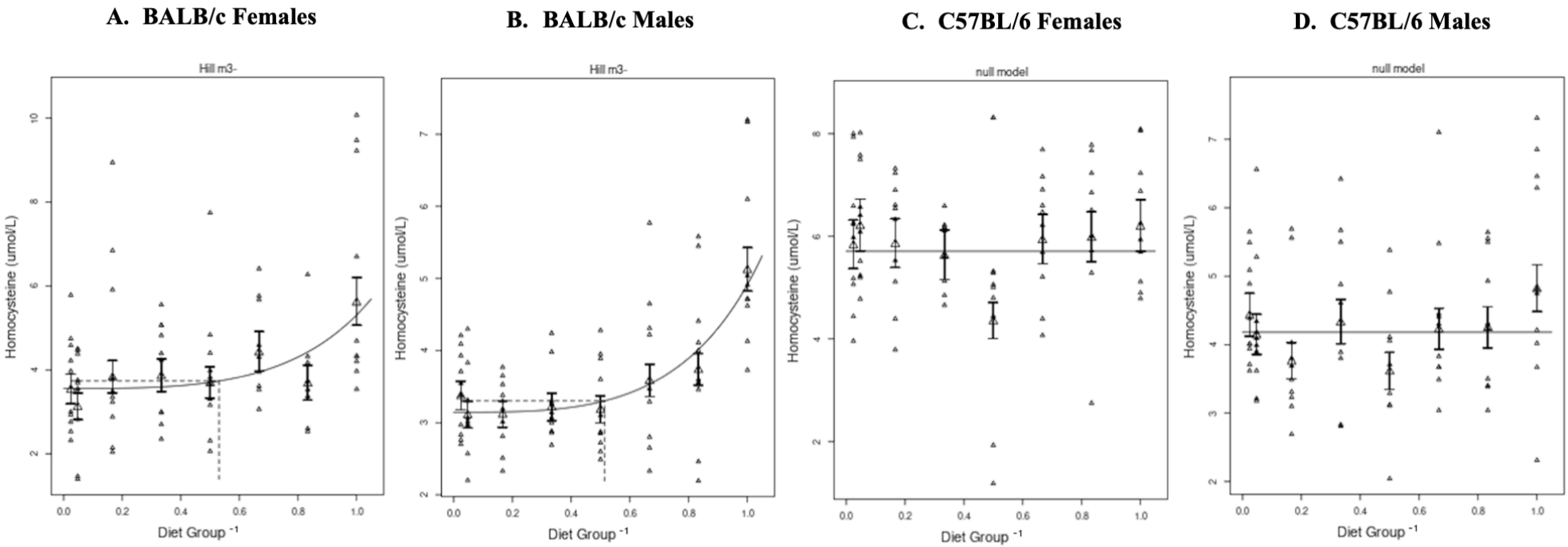

Figure 18: Benchmark Dose (BMD) modeling of homocysteine concentrations in mice fed different folic acid diets measured at necropsy. BMD approach was applied as a continuous response of Hcy with increasing folic acid content in diets. The Hill function model was used to fit the Hcy response to folic acid content in diets. FA content in diets were transformed for analysis (n = 5).

3.4.5.2. CBC analysis

White blood cell counts. Significant strain differences were observed for WBC (p = < 0.001), LYMPH (p = < 0.001), MONO (p = < 0.001), EO (p = < 0.037), BASO (p = < 0.001) cell counts. C57BL/6 mice had higher WBC (5%), LYMPH (21%), MONO (60%), EO (14%) and BASO (136%) counts than BALB/c mice.

Significant sex differences were also observed for LYMPH (p = < 0.001), MONO (p = 0.007), BASO (p = 0.003) and NEUT (p = < 0.001) cell counts. Male mice had higher MONO (37%) and EO (72%) counts than female mice. On the other hand, female mice had a higher LYMPH (20%) and BASO (79%) cell counts.

No main diet effect was observed in the white blood cell counts. Further analysis of the diet effect within a sex/strain groups showed a significant difference among diets of LYMPH (p = 0.034) count in BALB/c female mice, which were lower in mice fed 5 mg/kg diet compared to the 0 mg/kg diet. A diet effect was also observed of BASO (p = 0.049) cell counts in BALB/c females and WBC (p = 0.034) in C57BL/6 males but post hoc analysis could not identify groups that differed (**Table 5**).

Red blood cell counts. A significant strain effect for all red blood cell counts, except for %RET, was identified. C57BL/6 mice had higher MCV (4.40% difference; p = < 0.001) and IRF (7.92% difference; p = < 0.001) than BALB/c mice. BALB/c mice had higher RBC (8.99% difference; p = < 0.001), HBG (13.4% difference; p = < 0.001), MCH (2.97% difference; p = 0.026), MCHC (7.57% difference; p = < 0.001), RDW-SD (6.63% difference; p = < 0.001) and RET (4.86% difference; p = 0.048) than C57BL/6 mice.

A significant sex effect for all red blood cell counts was identified, except for the absolute count /percentage of RET, MCH and MCHC components. Female mice had a higher MCV (1.29% difference; p = < 0.001) than male mice. On the other hand, male mice had a higher RBC (10.37% difference; p = < 0.001), HBG (7.74% difference; p = < 0.001), RDW-SD (4.61% difference; p = < 0.001), and IRF (2.32% difference; p = < 0.024) than female mice.

No overall significant main diet effect was identified when examining red blood cell count. However, further analysis within the sex/strain groups showed significant difference among diets for some red blood cell counts. Among the C57BL/6 male mice, IRF (p = 0.015) was lower in mice fed the 0.2 mg/kg diet than the 0 mg/kg diet. HGB count (p = 0.044) also showed a diet effect but no specific differences were identified by post hoc analysis. Similarly, MCH (p = 0.31) and MCHC (p = 0.012) counts in C57BL/6 female mice showed a diet effect but not specific differences were identified (**Table 6**).

Platelet counts. Overall, all platelet endpoints showed significant strain (p = < 0.001) and sex (p = < 0.001) effects. The single exception was the mean differences of the PDW that showed no sex effect. C57BL/6 mice had a higher mean number of PLT (p = < 0.001) than BALB/c mice. BALB/c mice had higher PDW (12.02% difference; p = < 0.001), MPV (6.640% difference; p = < 0.001) and P-LCR (66.5.4% difference; p = < 0.001) than C57BL/6 mice.

Male mice had a higher absolute count of PLT (9.90%-12.15% difference; p = < 0.001) than female mice. Female mice had higher MPV (2.48% difference; p = < 0.001) and P-LCR (10.25% difference; p = < 0.001) than male mice.

No significant diet effect was observed when platelet endpoints were evaluated. Similarly, no diet effect was found when the analysis was carried out within individual sex/strain groups (**Table 7**).

Table 5: White blood cell counts of BALB/c and C57BL/6 mice at necropsy. Differences in means among sex, strain and diet groups were assessed by Three-Way ANOVA and Holm-Sidak post-hoc analysis. Since significant sex and strain differences were observed for some white blood cell components, data was also evaluated within sex/strain groups for diet differences. Differences in means among the diets within each sex/strain group was assessed by One-Way ANOVA and Kruskal-Wallis on ranks post-hoc analysis. Data are presented as mean ± SEM. Differences were considered significant if $p \leq 0.05$. Values within a row that share a letter do not differ. WBC: white blood cell; LYMPH: lymphocyte, MONO: monocyte; EO: Eosinophil; BASO: Basophil.

Strain/Sex	WBC Components	0 mg/kg	0.2 mg/kg	0.5 mg/kg	1 mg/kg	2 mg/kg	5 mg/kg	20 mg/kg	40 mg/kg	P Value	Sample size
BALB/C Females	WBC (10^9/L)	5.32 ± 0.36	4.56 ± 0.33	4.56 ± 0.35	4.28 ± 0.22	4.31 ± 0.66	3.21 ± 0.44	5.10 ± 1.44	3.76 ± 0.38	0.096	n = 5-8
	LYMPH# (10^9/L)	^{A}4.18 ± 0.27	^{A}3.72 ± 0.26	^{A}3.88 ± 0.32	^{A}3.31 ± 0.10	^{A}3.47 ± 0.59	^{B}2.51 ± 0.40	^{A}2.92 ± 0.30	^{A}3.01 ± 0.31	**0.034**	n = 4-8
	MONO# (10^9/L)	0.24 ± 0.06	0.20 ± 0.06	0.13 ± 0.04	0.16 ± 0.07	0.18 ± 0.03	0.16 ± 0.04	0.61 ± 0.45	0.18 ± 0.04	0.963	n = 4-8
	EO# (10^9/L)	0.15 ± 0.02	0.14 ± 0.02	0.10 ± 0.02	0.10 ± 0.01	0.09 ± 0.16	0.08 ± 0.01	0.09 ± 0.03	0.10 ± 0.02	0.132	n = 4-8
	BASO# (10^9/L)	0.01 ± 0.002	0.00 ± 0.00	0.00 ± 0.002	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.002	0.00 ± 0.00	**0.049**	n = 5-8
	NEUT# (10^9/L)	0.76 ± 0.07	0.50 ± 0.05	0.63 ± 0.04	0.61 ± 0.09	0.57 ± 0.08	0.46 ± 0.04	1.48 ± 0.98	0.48 ± 0.06	0.061	n = 4-8
BALB/C Males	WBC (10^9/L)	3.74 ± 0.28	4.12 ± 0.48	4.74 ± 0.37	3.54 ± 0.40	3.46 ± 0.51	4.16 ± 0.45	3.84 ± 0.48	3.47 ± 0.49	0.463	n = 9-10
	LYMPH# (10^9/L)	2.62 ± 0.20	3.00 ± 0.37	3.25 ± 0.35	2.56 ± 0.30	2.65 ± 0.42	3.06 ± 0.36	2.62 ± 0.30	2.66 ± 0.46	0.812	n = 9-10
	MONO# (10^9/L)	0.15 ± 0.03	0.17 ± 0.03	0.26 ± 0.05	0.11 ± 0.02	0.13 ± 0.04	0.16 ± 0.02	0.15 ± 0.03	0.11 ± 0.02	0.152	n = 9-10
	EO# (10^9/L)	0.08 ± 0.02	0.08 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.940	n = 9-10
	BASO# (10^9/L)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.000	n = 9-10
	NEUT# (10^9/L)	0.88 ± 0.13	0.87 ± 0.15	1.16 ± 0.25	0.79 ± 0.13	0.60 ± 0.09	0.86 ± 0.12	1.00 ± 0.22	0.62 ± 0.04	0.153	n = 9-10

Table 5 continued: White blood cell counts of BALB/c and C57BL/6 mice at necropsy.

Strain/Sex	WBC Components	0 mg/kg	0.2 mg/kg	0.5 mg/kg	1 mg/kg	2 mg/kg	5 mg/kg	20 mg/kg	40 mg/kg	P Value	Sample size
C57BL/6 Females	WBC (10^9/L)	4.78 ± 0.47	4.97 ± 0.61	4.92 ± 0.42	4.35 ± 0.63	5.27 ± 0.73	5.10 ± 0.46	5.04 ± 0.41	4.47 ± 0.93	0.964	n = 4-7
	LYMPH# (10^9/L)	4.11 ± 0.41	4.23 ± 0.52	4.20 ± 0.37	0.43 ± 0.20	4.59 ± 0.61	4.30 ± 0.42	4.27 ± 0.34	3.89 ± 0.85	0.995	n = 4-7
	MONO# (10^9/L)	0.21 ± 0.02	0.21 ± 0.02	0.20 ± 0.02	0.20 ± 0.03	0.24 ± 0.08	0.25 ± 0.01	0.24 ± 0.02	0.21 ± 0.05	0.796	n = 4-7
	EO# (10^9/L)	0.11 ± 0.02	0.11 ± 0.02	0.12 ± 0.02	0.11 ± 0.01	0.10 ± 0.03	0.11 ± 0.01	0.09 ± 0.01	0.10 ± 0.02	0.982	n = 4-7
	BASO# (10^9/L)	0.00 ± 0.002	0.00 ± 0.002	0.00 ± 0.002	0.00 ± 0.002	0.00 ± 0.003	0.00 ± 0.002	0.00 ± 0.002	0.00 ± 0.002	0.715	n = 4-7
	NEUT# (10^9/L)	0.35 ± 0.05	0.42 ± 0.07	0.39 ± 0.06	0.39 ± 0.06	0.34 ± 0.04	0.44 ± 0.09	0.44 ± 0.73	0.27 ± 0.04	0.571	n = 4-7
C57BL/6 Males	WBC (10^9/L)	6.54 ± 0.68	6.28 ± 0.72	6.14 ± 0.52	5.18 ± 0.80	6.63 ± 1.10	4.15 ± 0.71	4.44 ± 0.40	4.46 ± 0.44	**0.034**	n = 8-10
	LYMPH# (10^9/L)	4.11 ± 0.38	3.75 ± 0.45	3.94 ± 0.60	3.71 ± 0.59	2.94 ± 0.52	3.08 ± 0.54	3.02 ± 0.50	2.72 ± 0.42	0.324	n = 7-10
	MONO# (10^9/L)	0.66 ± 0.17	0.59 ± 0.13	0.46 ± 0.06	0.38 ± 0.10	0.77 ± 0.15	0.40 ± 0.11	0.31 ± 0.11	0.40 ± 0.08	0.115	n = 8-10
	EO# (10^9/L)	0.15 ± 0.02	0.11 ± 0.03	0.13 ± 0.02	0.13 ± 0.03	0.09 ± 0.02	0.09 ± 0.02	0.10 ± 0.02	0.08 ± 0.02	0.252	n = 8-10
	BASO# (10^9/L)	0.00 ± 0.002	0.00 ± 0.001	0.00 ± 0.001	0.00 ± 0.00	0.00 ± 0.001	0.00 ± 0.001	0.00 ± 0.001	0.00 ± 0.001	0.300	n = 8-10
	NEUT# (10^9/L)	1.61 ± 0.56	1.83 ± 0.64	1.61 ± 0.41	0.97 ± 0.22	2.26 ± 0.60	0.87 ± 0.15	1.01 ± 0.36	1.26 ± 0.26	0.190	n = 7-10

Table 6: Red blood cell counts of BALB/c and C57BL/6 mice at necropsy. Differences in means among sex, strain and diet groups were assessed by Three-Way ANOVA and Holm-Sidak post-hoc analysis. Since significant sex and strain differences were observed for some red blood cell components, data was also evaluated within sex/strain groups for diet differences. Differences in means among the diets within each sex/strain group was assessed by One-Way ANOVA and Holm-Sidak or Dunn's method post-hoc analysis. Data are presented as mean ± SEM. Differences were considered significant if $p \leq 0.05$. Values within a row that share a letter do not differ. RBC: red blood cell; RET: reticulocyte; HGB: hemoglobin; MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, RDW-SD: red cell distribution width, IRF: immature reticulocyte fraction.

Strain/Sex	RBC Components	0 mg/kg	0.2 mg/kg	0.5 mg/kg	1 mg/kg	2 mg/kg	5 mg/kg	20 mg/kg	40 mg/kg	P Value	Sample size
BALB/C Females	RBC (10^{12}/L)	9.94 ± 0.39	9.68 ± 0.19	8.57 ± 1.58	10.11 ± 0.14	9.84 ± 0.18	9.82 ± 0.22	8.03 ± 1.6	9.97 ± 0.08	0.632	n = 5-8
	RET# (10^{9}/L)	467.3 ± 45.2	462.0 ± 24.6	481.5 ± 27.8	427.6 ± 14.2	434.1 ± 23.0	423.6 ± 13.7	443.6 ± 28.2	423.6 ± 22.1	0.697	n = 5-8
	RET (%)	4.70 ± 0.41	4.78 ± 0.26	4.75 ± 0.28	4.23 ± 0.12	4.43 ± 0.27	4.32 ± 0.13	4.67 ± 0.47	4.25 ± 0.22	0.711	n = 5-8
	HGB (g/L)	153 ± 3	148 ± 2	128 ± 24	153 ± 2	150 ± 2	150 ± 2	138 ± 8	153 ± 1	0.191	n = 5-8
	MCV (fL)	43.5 ± 0.3	43.7 ± 0.2	43.9 ± 1.1	43.5 ± 0.4	43.6 ± 0.1	43.1 ± 0.3	43.2 ± 1.8	43.6 ± 0.3	0.700	n = 5-8
	MCH (pg)	15.5 ± 0.3	15.3 ± 0.1	15.2 ± 0.2	15.2 ± 0.07	15.3 ± 0.1	15.3 ± 0.2	14.5 ± 0.5	15.3 ± 0.1	0.462	n = 5-8
	MCHC (g/L)	336 ± 7	349 ± 3	348 ± 4	349 ± 3	350 ± 3	356 ± 3	348 ± 2	352 ± 2	0.735	n = 5-8
	RDW-SD (fL)	28.3 ± 0.3	28.0 ± 0.3	27.1 ± 0.9	28.2 ± 0.4	28.1 ± 0.2	27.9 ± 0.2	27.0 ± 1.3	28.1 ± 0.2	0.925	n = 5-8
	IRF (%)	43.3 ± 1.7	43.0 ± 1.8	42.7 ± 1.9	46.9 ± 1.2	46.0 ± 1.4	45.6 ± 0.9	45.1 ± 1.1	42.9 ± 0.6	0.321	n = 5-8
BALB/C Males	RBC (10^{12}/L)	10.55 ± 0.009	10.32 ± 0.11	10.61 ± 0.14	10.65 ± 0.07	10.54 ± 0.10	10.52 ± 0.09	10.33 ± 0.13	10.12 ± 0.29	0.433	n = 9-10
	RET# (10^{9}/L)	485.0 ± 12.8	472.9 ± 11.1	492.4 ± 21.2	475.9 ± 12.7	463.0 ± 6.47	471.8 ± 13.8	535.2 ± 40.5	462.4	0.815	n = 9-10
	RET (%)	4.60 ± 0.13	4.58 ± 0.09	4.63 ± 0.15	4.47 ± 0.12	4.40 ± 0.09	4.48 ± 0.12	5.22 ± 0.47	4.58 ± 0.11	0.763	n = 9-10
	HGB (g/L)	157 ± 1	156 ± 2	160 ± 2	160 ± 1	157 ± 2	158 ± 2	154 ± 2	152 ± 4	0.189	n = 9-10
	MCV (fL)	43.2 ± 0.2	43.3 ± 0.2	43.0 ± 0.2	43.0 ± 0.2	42.9 ± 0.2	43.4 ± 0.2	43.2 ± 0.3	43.4 ± 0.2	0.877	n = 9-10
	MCH (pg)	14.9 ± 0.03	15.1 ± 0.1	15.0 ± 0.1	15.0 ± 0.1	14.9 ± 0.04	15.0 ± 0.05	14.9 ± 0.05	15.0 ± 0.1	0.423	n = 9-10
	MCHC (g/L)	345 ± 1	349 ± 2	350 ± 2	349 ± 2	348 ± 2	346 ± 2	344 ± 2	346 ± 1	0.217	n = 9-10
	RDW-SD (fL)	28.1 ± 0.2	28.2 ± 0.1	29.1 ± 0.4	28.6 ± 0.1	28.5 ± 0.1	28.2 ± 0.1	29.0 ± 0.3	28.1 ± 0.2	0.075	n = 9-10
	IRF (%)	47.0 ± 0.6	47.1 ± 0.9	45.8 ± 1.1	44.7 ± 0.5	45.6 ± 0.8	45.0 ± 1.0	48.0 ± 1.3	46.1 ± 0.9	0.197	n = 9-10

Table 6 continued: Red blood cell counts of BALB/c and C57BL/6 mice at necropsy.

Strain/Sex	RBC Components	0 mg/kg	0.2 mg/kg	0.5 mg/kg	1 mg/kg	2 mg/kg	5 mg/kg	20 mg/kg	40 mg/kg	P Value	Sample size
C57BL/6 Females	RBC (10^{12}/L)	7.53 ± 1.47	8.14 ± 1.22	9.05 ± 0.19	8.30 ± 1.24	9.64 ± 0.14	9.38 ± 0.15	9.55 ± 0.14	7.27 ± 1.64	0.201	n = 4-7
	RET# (10^{9}/L)	450.3 ± 31.9	372.3 ± 60.6	468.1 ± 32.3	418.8 ± 71.8	438.1 ± 15.2	470.3 ± 35.6	455.8 ± 28.9	471.2 ± 57.1	0.752	n = 4-7
	RET (%)	5.04 ± 0.44	4.47 ± 0.29	5.19 ± 0.38	4.69 ± 0.45	4.56 ± 0.22	5.01 ± 0.34	4.78 ± 0.32	5.26 ± 0.38	0.752	n = 4-7
	HGB (g/L)	112 ± 21	119 ± 19	135 ± 2	120 ± 19	140 ± 2	139 ± 2	138 ± 3	108 ± 25	0.465	n = 4-7
	MCV (fL)	46.1 ± 0.8	46.0 ± 0.6	45.1 ± 0.2	45.5 ± 0.8	45.1 ± 0.2	46.3 ± 0.7	44.8 ± 0.1	46.7 ± 0.9	0.084	n = 4-7
	MCH (pg)	17.7 ± 2.8	13.9 ± 0.8	14.9 ± 0.1	13.6 ± 1.0	14.5 ± 0.1	14.8 ± 0.2	14.5 ± 0.1	14.0 ± 1.1	**0.031**	n = 4-7
	MCHC (g/L)	381 ± 51	304 ± 20	330 ± 1	302 ± 25	323 ± 3	321 ± 1	323 ± 3	301 ± 28	**0.012**	n = 4-7
	RDW-SD (fL)	25.2 ± 0.5	25.2 ± 0.6	25.3 ± 0.2	25.4 ± 0.7	25.4 ± 0.1	26.1 ± 0.3	25.7 ± 0.1	24.8 ± 0.9	0.698	n = 4-7
	IRF (%)	52.4 ± 0.7	46.4 ± 1.9	48.4 ± 1.9	49.3 ± 2.2	45.1 ± 1.3	51.5 ± 0.6	49.6 ± 1.9	47.7 ± 2.1	0.143	n = 4-7
C57BL/6 Males	RBC (10^{12}/L)	9.74 ± 0.19	10.17 ± 0.13	9.70 ± 0.11	9.52 ± 0.26	9.52 ± 0.11	8.84 ± 0.88	9.68 ± 0.12	9.83 ± 0.14	0.102	n = 8-10
	RET# (10^{9}/L)	445.4 ± 21.2	449.7 ± 15.1	435 ± 32.2	434.7 ± 18.2	458.4 ± 18.5	393.2 ± 43.1	457.6 ± 13.4	450.3 ± 38.0	0.893	n = 7-10
	RET (%)	4.61 ± 0.28	4.42 ± 0.14	4.48 ± 0.31	4.58 ± 0.19	4.82 ± 0.18	4.31 ± 0.23	4.73 ± 0.14	4.57 ± 0.36	0.894	n = 7-10
	HGB (g/L)	141 ± 3	147 ± 2	141 ± 2	138 ± 4	138 ± 1	127 ± 12	139 ± 2	142 ± 2	**0.044**	n = 8-10
	MCV (fL)	44.9 ± 0.3	44.7 ± 0.3	44.9 ± 0.2	44.9 ± 0.2	45.2 ± 0.2	45.2 ± 0.4	44.8 ± 0.2	44.7 ± 0.2	0.882	n = 8-10
	MCH (pg)	14.5 ± 0.1	14.4 ± 0.1	14.5 ± 1	14.5 ± 0.2	14.5 ± 0.1	15.4 ± 1.1	14.3 ± 0.1	14.4 ± 0.1	0.554	n = 8-10
	MCHC (g/L)	322 ± 2	323 ± 3	322 ± 2	322 ± 2	321 ± 1	339 ± 20	321 ± 2	323 ± 1	0.957	n = 8-10
	RDW-SD (fL)	27.5 ± 0.5	27.2 ± 0.2	27.2 ± 0.4	27.0 ± 0.3	28.6 ± 0.7	26.4 ± 0.6	27.0 ± 0.3	27.5 ± 0.4	0.363	n = 8-10
	IRF (%)	^{A}51.9 ± 1.5	^{B}46.0 ± 1.4	^{AB}49.8 ± 0.9	^{AB}51.0 ± 0.6	^{AB}51.1 ± 0.8	^{AB}47.9 ± 1.3	^{AB}47.2 ± 1.6	^{AB}49.4 ± 1.2	**0.015**	n = 7-10

Table 7: Platelet counts of BALB/c and C57BL/6 mice at necropsy. Differences in means among sex, strain and diet groups were assessed by Three-Way ANOVA and Holm-Sidak post-hoc analysis. Since significant sex and strain differences were observed for some platelet cell components, data was also evaluated within sex/strain groups for diet differences. Differences in means among the diets within each sex/strain group was assessed by One-Way ANOVA and Kruskal-Wallis on ranks post-hoc analysis. Data are presented as mean ± SEM. Differences were considered significant if $p \leq 0.05$. PLT: platelets; MPV: mean platelet volume; PDW: platelet distribution width; P-LCR: Platelet large cell ratio.

Strain/Sex	Platelets Components	0 mg/kg	0.2 mg/kg	0.5 mg/kg	1 mg/kg	2 mg/kg	5 mg/kg	20 mg/kg	40 mg/kg	P Value	Sample size
BALB/C Females	PLT (10^9/L)	967 ± 46	952 ± 45	978 ± 33	825 ± 127	978 ± 21	928 ± 40	1050 ± 176	987 ± 25	0.881	n = 5-8
	MPV (fL)	7.0 ± 0.1	6.9 ± 0.1	7.0 ± 0.08	7.0 ± 0.2	6.8 ± 0.07	7.0 ± 0.08	7.0 ± 0.1	6.8 ± 0.08	0.726	n = 5-8
	PDW (fL)	8.0 ± 0.2	7.9 ± 0.3	7.8 ± 0.3	8.2 ± 0.3	7.6 ± 0.1	7.9 ± 0.2	7.8 ± 0.5	7.7 ± 0.1	0.601	n = 5-8
	P-LCR (%)	7.9 ± 0.8	7.4 ± 0.9	7.8 ± 0.5	8.2 ± 1.3	6.7 ± 0.4	7.8 ± 0.5	7.7 ± 0.7	7.2 ± 0.5	0.921	n = 5-8
BALB/C Males	PLT (10^9/L)	995 ± 51	1017 ± 31	1005 ± 46	1013 ± 26	1027 ± 25	1029 ± 33	1057 ± 41	1068 ± 29	0.874	n = 9-10
	MPV (fL)	6.8 ± 0.1	6.7 ± 0.1	6.7 ± 0.1	6.7 ± 0.1	6.7 ± 0.1	6.7 ± 0.1	6.8 ± 0.1	6.7 ± 0.1	0.662	n = 8-10
	PDW (fL)	7.8 ± 0.1	7.5 ± 0.1	7.9 ± 0.2	7.6 ± 0.1	7.6 ± 0.1	7.7 ± 0.1	7.8 ± 0.1	7.6 ± 0.1	0.546	n = 8-10
	P-LCR (%)	6.8 ± 0.3	6.4 ± 0.5	6.7 ± 0.5	6.6 ± 0.3	6.4 ± 0.4	6.5 ± 0.5	7.1 ± 0.3	6.1 ± 0.3	0.807	n = 8-10
C57BL/6 Females	PLT (10^9/L)	1193 ± 38	1105 ± 158	1290 ± 57	1045 ± 157	1295 ± 56	1197 ± 62	1248 ± 59	1146 ± 76	0.698	n = 4-7
	MPV (fL)	6.6 ± 0.1	6.4 ± 0.1	6.5 ± 0.1	0.5 ± 0.1	6.4 ± 0.1	6.4 ± 0.1	6.5 ± 0.1	6.5 ± 0.1	0.453	n = 4-7
	PDW (fL)	6.9 ± 0.2	6.6 ± 0.2	7.1 ± 0.02	6.9 ± 0.1	7.0 ± 0.1	6.9 ± 0.1	7.1 ± 0.1	6.7 ± 0.2	0.142	n = 4-7
	P-LCR (%)	4.0 ± 0.6	3.2 ± 0.3	4.1 ± 0.2	3.5 ± 0.2	3.3 ± 0.1	3.6 ± 0.1	3.8 ± 0.4	3.3 ± 0.4	0.440	n = 4-7
C57BL/6 Males	PLT (10^9/L)	1371 ± 96	1259 ± 67	1369 ± 61	1378 ± 77	1458 ± 83	1194 ± 127	1370 ± 38	1367 ± 57	0.578	n = 7-10
	MPV (fL)	6.3 ± 0.03	6.4 ± 0.04	6.3 ± 0.1	6.3 ± 0.03	6.3 ± 0.1	6.4 ± 0.1	6.2 ± 0.02	6.2 ± 0.03	0.062	n = 8-10
	PDW (fL)	6.9 ± 0.1	7.0 ± 0.1	6.9 ± 0.1	6.8 ± 0.1	6.9 ± 0.1	6.9 ± 0.1	6.8 ± 0.1	6.9 ± 0.1	0.285	n = 8-10
	P-LCR (%)	3.5 ± 0.1	3.6 ± 0.1	3.6 ± 0.2	3.3 ± 0.1	3.5 ± 0.2	4.0 ± 0.3	3.2 ± 0.1	3.3 ± 0.1	0.075	n = 8-10

3.4.5.3. Micronucleus frequency

Similar to what was identified using CBC analysis, the percentage of immature red blood cells at necropsy (%RET) did not differ by strain, sex or diet either. We did, however, notice that CBC-determined concentrations were 78-98 % higher than when measured using the micronucleus assay. A significant sex ($p = < 0.001$) and strain ($p = < 0.001$) effects were observed for the frequency rate of %MN-RET and % MN-NCE. BALB/c mice had 25% more MN-RET cells and 41.6% more MN-NCE cells than C57BL/6 mice. Male mice had 21% more MN-RET cells and 16 % more MN-NCE cells than female mice. No diet effect was observed across all mouse groups or within each sex/strain group (**Table 8**).

Table 8: Micronucleus frequencies of BALB/c and C57BL/6 mice at necropsy. Differences in means among sex, strain and diet groups were assessed by Three-Way ANOVA and Holm-Sidak post-hoc analysis. Since significant sex and strain differences were observed for micronucleus frequencies, data was also evaluated within sex/strain groups for diet differences. Differences in means among the diets within each sex/strain group was assessed by One-Way ANOVA. Data are presented as mean ± SEM. Differences were considered significant if $p \leq 0.05$. No significant differences were observed within sex/strain groups. RET: reticulocytes; MN-RET: micronucleated reticulocytes; MN-NCE: micronucleated erythrocytes.

Strain/Sex	Miconucleus Frequency	0 mg/kg	0.2 mg/kg	0.5 mg/kg	1 mg/kg	2 mg/kg	5 mg/kg	20 mg/kg	40 mg/kg	P Value	Sample Size
BALB/C Females	%RET	2.21 ± 0.22	1.98 ± 0.16	2.12 ± 0.12	1.97 ± 0.04	2.20 ± 0.20	1.92 ± 0.11	2.17 ± 0.12	1.82 ± 0.10	0.434	n = 5
	%MN-RET	0.41 ± 0.03	0.37 ± 0.01	0.36 ± 0.02	0.35 ± 0.01	0.44 ± 0.04	0.37 ± 0.01	0.36 ± 0.04	0.38 ± 0.04	0.378	n = 5
	%MN-NCE	0.24 ± 0.01	0.24 ± 0.004	0.23 ± 0.01	0.23 ± 0.01	0.25 ± 0.02	0.24 ± 0.01	0.23 ± 0.01	0.23 ± 0.01	0.484	n = 5
BALB/C Males	%RET	2.04 ± 0.08	2.42 ± 0.20	2.31 ± 0.14	2.28 ± 0.14	1.96 ± 0.07	2.24 ± 0.07	2.70 ± 0.34	2.22 ± 0.09	0.110	n = 5
	%MN-RET	0.37 ± 0.02	0.38 ± 0.04	0.42 ± 0.02	0.41 ± 0.03	0.39 ± 0.01	0.39 ± 0.02	0.42 ± 0.02	0.38 ± 0.03	0.712	n = 5
	%MN-NCE	0.23 ± 0.01	0.24 ± 0.01	0.26 ± 0.01	0.24 ± 0.003	0.23 ± 0.01	0.23 ± 0.01	0.25 ± 0.01	0.23 ± 0.01	0.388	n = 5
C57BL/6 Females	%RET	2.08 ± 0.07	2.72 ± 0.20	2.16 ± 0.18	2.43 ± 0.52	2.10 ± 0.15	1.93 ± 0.11	2.53 ± 0.31	1.89 ± 0.12	0.199	n = 5
	%MN-RET	0.25 ± 0.02	0.23 ± 0.02	0.22 ± 0.01	0.25 ± 0.02	0.24 ± 0.02	0.21 ± 0.01	0.26 ± 0.02	0.24 ± 0.01	0.603	n = 5
	%MN-NCE	0.13 ± 0.01	0.12 ± 0.01	0.13 ± 0.004	0.12 ± 0.002	0.14 ± 0.004	0.12 ± 0.01	0.13 ± 0.01	0.12 ± 0.004	0.539	n = 5
C57BL/6 Males	%RET	1.93 ± 0.14	1.76 ± 0.04	2.23 ± 0.30	2.25 ± 0.06	1.90 ± 0.28	1.97 ± 0.07	2.12 ± 0.08	2.21 ± 0.25	0.753	n = 5
	%MN-RET	0.40 ± 0.02	0.36 ± 0.02	0.38 ± 0.02	0.35 ± 0.05	0.36 ± 0.01	0.38 ± 0.01	0.34 ± 0.02	0.38 ± 0.02	0.224	n = 5
	%MN-NCE	0.18 ± 0.01	0.18 ± 0.01	0.19 ± 0.01	0.18 ± 0.01	0.18 ± 0.01	0.19 ± 0.01	0.19 ± 0.01	0.19 ± 0.01	0.904	n = 5

3.5. Discussion

Where many human studies can demonstrate associations, or in the case of clinical trials causal relationships, studies in animal models such as mice allow us to investigate the causal mechanisms relating the folate pathway to a health outcome. However, even though folate biomarkers in humans have been established, the corresponding equivalents in animal models have yet to be identified. Consequently, this limits the translation of animal-based folate research to inform nutritional risk assessments. Our study aimed to bridge this gap by conducting a dose-response analysis of FA intake in mice using diets that encompassed the range observed in the literature. Using both sexes and two of the mostly commonly used inbred mouse strains in folate research, we investigated both traditional status biomarkers and candidate functional biomarkers of folate status. The vast majority of the endpoints investigated in our study showed clear sex and strain effects. Plasma total folate concentrations and folate vitamers in mice progressively increased, whereas homocysteine decreased, as the FA contents in the diets increased. The candidate functional biomarkers of status, RBC micronucleus and white and red blood cell counts, were not related to FA intake.

3.5.1. Biomarkers of FA Intake

Plasma total folate. Mayeux et al. describes biomarkers as "cellular biochemical or molecular alterations that are measurable" (103). In nutritional research, this could indicate nutritional status in terms of intake/exposure and/or the functional/metabolic responses (92). For folates, one of the traditional biomarkers of exposure is plasma/serum total folate. Plasma or serum total folate concentrations are great indicators of recent intakes. On the other hand, RBC folate concentrations provide information on long-term storage and accumulation in tissue (7,11). It should be noted that

in rodents fed a consistent diet, daily variation in intake is minimal as compared to humans, and therefore plasma total folate is more likely to represent usual intakes in rodents. Analysis of recent intake as assessed by plasma total folate allows for a quick identification of dose-response and dietary patterns over time (7,11). In clinical terms, it can be used as an early indicator of changes in folate status, therefore allowing early intervention and prevent the onset of adverse health effects. Similar to what is outlined in the literature for both humans and animals, plasma total folate concentrations progressively increased as the FA content in the diets increased. Our data further validated plasma total folate concentration as a biomarker of folate exposure in mice as it is observed in humans. Additionally, as a biomarker of exposure, plasma total folate concentrations also quantitively allows us to evaluate the diet and time intervention folate-responsive health effects start to appear.

Within 2 weeks of the intervention, the plasma total folate concentrations in mice fed diets $\leq$ 5 mg/kg had fallen and plateaued. This trend was shorter than the 6 to 8-week time period observed for low FA intakes in humans and the 8 to 10-week period previously described in human FA supplementation studies (7,112). The BALB/c male mice fed the 20 mg/kg diet was the only group that showed similar trends to those observed in humans. However, this trend was not detected in the 40 mg/kg diets. Our data showed that plasma total folate concentrations continued to rise and did not plateau even by the 12-week intervention time period. Our study provides insight on how long plasma concentrations can take to stabilize and the importance of the duration of interventions when considering study designs. Underlying causes of observed health effects identified *in vivo* may fluctuate and change during a dietary intervention. We can only accurately associate intakes with health effects if we understand the status at the time of disease pathogenesis. The results of

our data showed that the duration of intervention required for animal study designs to investigate health effects of folate status varied based on the FA content of the diets.

Unmetabolized folic acid. Concerns have been raised that FA can cause adverse effects; however, there are no biomarkers for high or excessive intake of FA. It has been proposed that unmetabolized FA in circulation could negatively impact folate metabolism. FA has been shown to have an inhibitory effect on TS and MTHFR *in vitro*. As FA is consumed, its monoglutamate form is metabolized to DHF then THF by the DHFR enzyme primarily in the liver (49,105). High FA intakes can oversaturate the DHFR enzyme and result in the appearance of unmetabolized FA in plasma and tissues (113). In humans, for example, this occurs with intakes of FA around 0.4 mg (114,115). Additional *in vitro* studies have also shown that DHF, which might accumulate because of increased FA intake, can also trigger a negative feedback mechanism that prevents the regeneration of both THF and DHF downstream via the direct inhibition of TS and MTHFR in the folate cycle. We hypothesized that the appearance of unmetabolized FA or its increase in response to diet is a marker of supraphysiological FA intake.

The *L. casei* microbiological assay is considered the 'gold standard' for determining folate status (11). However, this assay is unable to distinguish between the different folate forms in its determination of total folate because L. casei are able to use all folate forms to grow. In contrast, LS-MS/MS analysis is able to quantitatively measure the different folate forms in plasma. As such, LC-MS/MS analysis of folate vitamers allowed us to determine when UMFA appears and/or increases in the plasma. We show that concentrations of UMFA significantly increased at diets ≥ 5mg/kg diets in all the mice. This concentration jump in the plasma can be used as an indicator of

the DHFR enzyme saturation levels in mice models and therefore as an indicator of high FA intakes.

High FA intakes has been associated with a number of adverse health outcomes. The presence of unmetabolized FA in circulation is associated with concerns of the adverse health outcomes such as the propagation of existing tumor cells and impaired natural killer (NK) cells in humans (10,44). Previous studies have identified an inverse U-shaped relationship between the total folate intake and NK cytotoxicity whereby both low and high FA intakes were associated with impaired mechanisms in humans. Specifically, the study conducted by Troen et al. further demonstrated a significant inverse linear association between unmetabolized FA in plasma and NK cytotoxicity in post-menopausal women (44,49). Furthermore, it has also been proposed that high FA intakes can increase the availability of nucleotides and methyl groups via the folate pathway which could facilitate that growth of existing tumors (10). Furthermore, the UL of 1000 ug of FA/day was established due to the concern of masking or exacerbating vitamin B12 deficiency.

Despite some associations with adverse effects in humans, there are no plasma total folate or unmetabolized FA cut-offs for high/excessive FA intake. At one point, a high folate cut-off of 45.3 nmol/L of plasma total folates was proposed for humans. However, this was based on the highest calibrator used in the radioimmunoassay (116,117), not on an adverse health effect (116,117). This limitation highlights an existing knowledge gap of a cut-off intake concentration associated with an adverse health effect that can be used as an indicator of over-consumption of FA in humans and animal models. We propose using the appearance and increase of UMFA in circulation as a biomarker of high exposure, as it likely results from supraphysiological intakes. In our study, we saw elevated concentrations of UMFA at an estimated FA intake $\geq$ 1.00 mg/kg (range: 1.00 – 1.87 mg/kg). This correlated with an estimated UMFA concentration $\geq$ 7.71 nmol/L (range: 7.71 –

20.75 nmol/L) in our mice models. The appearance of elevated UMFA concentration in plasma could potentially be a better indication of high/excess FA intakes. Equivalent determinations in humans could also be determined to establish a plasma concentration associated with high FA intakes.

3.5.1. Functional biomarkers of folate

Macrocytic anemia. In 1968, a plasma total folate cut-off concentration of 6.8 nmol/L was proposed as an indicator of folate deficiency (7,116). This cut-off value was based on an increased risk for developing macrocytic anemia. Macrocytosis is a condition whereby the RBC are abnormally enlarged and oval shaped (24,26). Macrocytic anemia can result from folate and/or vitamin B_{12} deficiency (26). Unlike humans, rodents generally do not exhibit these characteristic enlarged RBC cells unless folate/vitamin B12 deficiency occurs simultaneously with protein restriction, an observation confirmed in this study. As a result, macrocytic anemia cannot be used as a biomarker of health effect due to folate deficiency in mice. In our dose-response study, we investigated if FA intakes influenced other blood cell phenotypes. Even though strain and sex differences exist, no consistent diet effect was identified when analysing the size or number of blood cell components. These findings further highlighted the need to identify and validate other biomarkers of health effect due to folate deficiency in mouse models.

RBC micronuclei. The presence of imbalanced intracellular nucleotide pools increases the likelihood of DNA mutations and chromosome strand breaks (24). For instance, when thymidylate synthesis is impaired and uracil is misincorperated into the DNA strand, DNA repair mechanisms attempt to correct the error by excising uracil. However, the lack of thymidylate to fill in the gap

leaves single strand breaks in the chromosome and depending on the extent of uracil misincorporation, double strand DNA breaks can occur (24,25). If chromosome instability occurs, micronuclei can form. As erythroblasts proliferate and differentiate to RET in the bone marrow, they lose their nucleus but may retain small fragments of DNA in the cytoplasm (111,118). This small cluster of chromosomal fragments, known as the micronucleus, can be retained as the cells differentiate to NCE. Due to this, the micronucleus frequency in immature (RET) or mature (NCE) RBCs can be used as an indicator of DNA fragmentation due to folate deficiency. In a previous study, mice fed a FA deficient diet (0 mg/kg) had a 1.3 fold increased MN-NCE than those fed a control diet (2 mg/kg) (105). On the other hand, another study showed that a FA supplemented diet (6 mg/kg) diet dot not affect the micronucleus frequency (119). Our aim with a dose-response study was to determine the FA intake that the micronucleus frequency started to increase. Despite results from previous studies, no diet effect was observed in the micronucleus frequency of both RET and NCE cells in this study. Our micronucleus data differed from the literature and findings from previous studies that have shown that low FA diets are induce increased micronucleus frequencies (105,118). We were unfortunately, unable to validate the use of micronucleus frequency as a functional biomarker of low FA intake.

Plasma Homocysteine. In 2005, an alternative cut-off plasma total folate concentration value for folate deficiency (10 nmol/L) was proposed based on population-based observations that homocysteine increases with lower folate intake (116,120,121). There is an inverse relationship between Hcy and folate at plasma total folate concentrations < 15 nmol/L in humans (122). This is related to the role of folate in the remethylation of homocysteine to form methionine. 5-methyl THF is converted to Hcy which is then methylated to methionine via a vitamin B_{12}-dependent

reaction catalyzed by MTR (8,26). In other words, increased plasma Hcy concentrations can be an indication of impaired MTR activity and subsequently methionine synthesis. THF is also regenerated from 5-methyl THF concurrently (7). As a metabolic intermediate of the folate pathway, Hcy acts as an effective functional biomarker of folate status.

Mice also demonstrate higher homocysteine on lower FA diets (123), our study aimed to find the FA intake dose associated with a rise in homocysteine in mice. Using benchmark dose modeling (PROAST), we found that Hcy concentrations continued to decrease with increasing FA intakes and plateaued at an estimated intake dose of 0.53 mg/kg in BALB/c females and 0.95 mg/kg in BALB/c males. Unfortunately, even though previous studies have shown that plasma homocysteine in C57BL/6 mice was significantly higher in mice fed deficient diets, this was not observed in our study (124). A null model was produced when analysing C57BL/6 mice. This indicated that there was either no change in slope or a relationship could not be modeled to determine a benchmark dose. Therefore, Hcy cannot be accurately used as an indicator of low FA intake in C57BL/6 mice. It is unclear why a dose-response change in Hcy concentrations was not observed in the C57BL/6 mice. However, these mice eat more and had a higher folate status than BALB/c mice, but they also exhibited higher Hcy concentrations. One theory that might explain these differences is a difference in the activity of the DHFR enzyme. Strain-specific differences could be assessed to explore whether it results in differences in folate metabolism.

3.6. Conclusion

In this study, we determined the mouse-equivalent cut of values associated with both high and low FA intake. We found that Hcy concentrations started to increase when FA intakes were ≤ 0.53 mg/kg and 0.95 mg/kg in BALB/c female and male mice respectively. We propose using this

as the lower limit cut off value for adequate FA intakes for BALB/c mice. The equivalent diet dose upon which Hcy concentrations started to increase could not be determined using BMD modeling. Furthermore, we also propose using significantly elevated concentrations of unmetabolized FA (UMFA) in plasma as an indicator of high FA intake. Our study showed that this occurred with estimated FA intakes $\geq$ 1.87 mg/kg, 1.03 mg/kg, 1.02 mg/kg and 1.00 mg/kg in BALB/c female, BALB/c male C57BL/6 female and male mice respectively. Additionally, the use of multiple analytical methods to identify indicators of high and low FA intake can go a long way in harmonizing data derived from mouse models in the folate research field. Accordingly, this would improve the translation of animal-derived data to human nutrition.

CHAPTER 4: DISCUSSION

4.1. Study context and implication to human nutrition

Adequate folate status is vital for growth, development and maintenance of health in both humans and animals. Its synthetic form, FA, is mainly obtained via fortified foods or prenatal supplements. However, like many essential nutrients, there are concerns over the adverse health effects associated with inadequate folate intakes and the potential for adverse effects from excess FA consumption. The purpose of our study was to evaluate animal-based research available to inform FA nutritional risk assessments and to address some of the challenges that hinder their knowledge translation to human nutrition.

4.1.1. Reporting animal-based research

Folic acid research in animal models has allowed us to examine the relationship between FA intakes and adverse health effects associated with both inadequate and excess intakes. Animal-derived data play an important role in the elucidation of the specific mechanisms of action linked to FA intakes. Evidence derived from these studies can inform the U-shaped nutritional risk assessment curve. Nutritional risk assessments are important for establishing guidelines on safe nutrient intakes upon which public health nutrition policies are based.

Our ability to accurately and transparently perform nutritional risk assessment is based on both the quantity and quality of available data. As seen in our study, multiple health effects and their relationships to folate status have been investigated using mouse models. The dietary intervention and how it is applied will also influence the outcome of these investigations. One of the surprising findings of our scoping review was the number of studies that used a closed formula

diet (39.7%) as their base diets. Unlike open-formula diets, closed-formula diets or "standard rodent chow" can vary significantly in formulation without notification from the manufacturer (90,91). In fact, other than basic information such as source materials and macronutrient proportions, little is often known about their composition. Simple differences such as changing the geographic source of the ingredients can introduce variation that may contribute to total nutrient exposure and confounding thereby limiting their reproducibility (90). To avoid these limitations, we strongly recommend the use of open-formula diets for dietary interventions investigating health outcomes associated with intakes. Open-formula diets, such as the AIN-93G diet used in our dose-response study, will limit confounding variables, improve transparency, reproducibility and therefore give confidence to the proposed causal associations with health effects.

Additionally, important basic details such as age of the mice and the duration of the dietary intervention should be considered when designing a study and interpreting findings. Often the age of the mice at the start of intervention is chosen based on the intended health outcome under investigation (e.g., older mice would be chosen for aging studies), and the rationale for the choice of particular details should be reported. The lack of reporting of age in 14% of the studies reviewed raises concerns on how the findings from these studies are to be interpreted and utilized when planning further research and/or performing nutritional risk assessments. Metabolic rate, physiological response and disease pathogenesis are animal characteristics that are age-dependent (87). For example, in a nutritional intervention such as our own, the metabolic rate and nutrient requirements of a growing mouse would be significantly different to that of an older mouse. In the context of a FA dietary intervention study, growing mice would potentially require higher FA intakes to meet their metabolic needs. This is apparent when examining the rapid decline in plasma total folate in our mice fed lower than adequate diets early on in the study. Similarly, a rationale

should also be provided for the timing of exposure and the duration between exposure and outcome assessment. Namely, if the timing/duration must be sufficient to plausibly affect a change on the outcome. For instance, spermatogenesis takes 6-8 weeks, so studies examining male fertility should take into account whether the timing and duration of the nutritional intervention of interest would plausibly affect fertility parameters. Studies that investigate the effect of FA intake on initiation and/or progression of tumors, which can take weeks to months in rodents, should report the rationale for the proposed timing and duration of the dietary intervention. In our study, the mice were fed their respective diets for a total of 12 weeks. We chose this duration because this was the time it took to examine the full impact of folate, if any, on functional endpoints including micronucleus formation in blood cells as previously reported in the literature (7). Reporting these details in publications improves transparency and our ability to accurately repeat findings. Furthermore, it also allows data derived from these studies to accurately inform nutritional risk assessments.

4.1.1. Mouse sex and strain differences

Even though generic animal characteristics (i.e., sex, strain and age) were generally well reported in 86% of studies reviewed, serious publication bias in the literature was also highlighted. The findings from our study showed that over half (56.5%) of the non-pregnant studies were conducted in only male mice and only male live-born offspring were studied in over a third (37.5%) of pregnancy/offspring studies. The assumption that findings from these single-sex (in this case, male) studies can be generalized is erroneous and has been repeatedly refuted (75,79). The findings of our own dose-response study indicated clear and consistent sex differences across the majority of our endpoints. Based on these findings, we recommend using both sexes to

investigate and validate health effects associated with folate and any other nutrient intake. This will ensure that studies account for any sex effects that influence responses to nutritional intake. This includes, but is not limited to differences in gonadal steroid hormones, body weight, food consumption, tissue weights, metabolic rate, epigenetic programming and gene expression (75–78).

Similarly, significant strain differences were observed across the majority of our endpoints. Different mouse strains can be more or less susceptible to a nutritional intervention and/or the development of specific health outcomes (74). C57BL/6 mice generally ate 12% more than BALB/c mice and gained 32.7% more weight. This translated to the measured plasma total folate concentrations. C57BL/6 mice had higher plasma folate concentrations at necropsy than BALB/c mice. Accordingly, Hcy plateaued to baseline at a higher concentration (5.71 umol/L and 4.18 umol/L for female and male mice respectively) for C57BL/6 mice than what was observed in BALB/c mice (4.01 umol/L and 3.88 umol/L for male and female mice respectively). With a higher folate status, homeostasis with Hcy was achieved at a higher concentration, and therefore a lower diet was required for elevated Hcy to be observed. These differences highlight the importance of considering genomic diversity while investigating nutritional interventions and the importance of reporting strain in publications. For the purpose of our dose-response study, we used only the two most commonly used strains in folate research to determine the generalizability of our findings to other studies

4.1.2. Biomarkers of folate deficiency

There is a need to establish biomarkers of folate intake, status and associated health effects in animal models to facilitate comparisons between findings in mice and humans. From our

scoping review, the 'control' diets identified in articles ranged from 0 to 16 mg/kg FA with the majority (63.9%) containing 2 mg/kg FA. However, this range often overlapped with the content from diets considered as "deficient" or "supplemented" in other studies. Additionally, we also showed that 41.2% of the studies reviewed did not report verifying the folate status in their mouse models using a biomarker. This poses a challenge when associations of folate with health outcomes are proposed solely based on the reported FA content of the diets. We propose the use of biomarkers as indicators of FA intake and verifiers of folate status. Specifically, we propose using elevated Hcy concentrations (i.e., $\geq$ 3.88 umol/L) in mouse models as an indicator of folate deficiency, which in our study was associated with an estimated intake of $\leq$ 0.95 mg/kg diet.

Establishing elevated Hcy concentration as a functional marker of deficiency in mice has multiple benefits. Primarily, it functions as an indicator of the increased risk of developing an adverse health effect associated with folate deficiency. In both humans and mouse models, elevated Hcy is an indicator of impaired MTR activity, limited supply of 5-methyl THF and reduced methionine synthesis and THF recapitulation (7). Diminished subsequent nucleotide synthesis can have dire consequences including the development of megaloblastic anemia, induction of DNA mutation and altered genome methylation patterns (33,42,116). In humans at the population level, Hcy concentrations increase when plasma total folate concentrations are < 10 nmol/L (122). Below this cut-off concentrations, there is an increased risk of developing adverse effects. The use of functional biomarkers of deficiency associated with a health effect has benefits. Used as a deficiency biomarker, it can be used to validate proposed health claims related to low FA intakes/folate status. It can also be used to harmonize and standardize the use of the term "deficiency" in mouse studies that use a FA intervention.

4.1.3. Biomarkers of Over Supplementation

The total FA intake among the Canadian population, and more specifically women of childbearing age via food fortification and prenatal supplementation, in addition to the naturally occurring folates in our diets, has raised concerns over the potential for adverse health effects due to high intakes. Multiple health effects have been hypothesised including promoting the progression of existing tumors, increased DNA mutations, and reducing NK cell cytotoxicity (10,44). However, a definition for "high intake" has yet to be established.

Furthermore, the UL of 1000 ug of FA/day recommended is based on the concern of vitamin B_{12} masking with FA supplementation. Vitamin B_{12}-deficiency, which results in impaired MTR activity and low THF production, is usually clinically diagnosed with the appearance of megaloblastic anemia. Because FA is reduced to THF by the enzyme DHFR, its consumption can bypass this methyl trap and treat megaloblastic anemia. However, other vitamin B12 related pathologies, namely neurodegeneration, are not rectified by FA and can progress and possibly even exacerbated (6,42). Left untreated, vitamin B_{12}-deficiency can lead to irreversible neurological degeneration and cognitive decline (6). Nonetheless, this value does not reflect an adverse health effect specifically associated with high folate intake. Even though generalized, this UL does not necessarily apply to individuals with no vitamin B_{12}-deficiency.

Similarly, it is not clear what level of dietary FA should be considered high in mouse models. Our review of the literature showed a broad range of FA content considered as "supplemented". Ranging from 0.17 to 60 mg/kg of FA, this range markedly overlapped with diets defined as "control" (0 to 16 mg/kg of FA). We propose the use of elevated unmetabolized FA (UMFA) as a biomarker of high folate intake. Although elevated UMFA has not been definitively associated with a specific adverse outcome, it does represent a FA exposure that is likely supraphysiological.

Previous studies have shown that high FA intakes can oversaturate the DHFR enzyme and result in the appearance of UMFA in plasma and tissues (113). Furthermore, increased DHF concentrations due to high FA intakes have also been hypothesised to have an inhibitory effect on TS and MTHFR (49,105). In our mouse models, we found that the concentration of UMFA significantly increased above 7.71 nmol/L (range: 7.71 nmol/L – 20.75 nmol/L) when our mouse models consumed diets with FA content ≥ 1.00 mg/kg (range: 1.00 – 1.87 mg/kg). We currently do not have a human-equivalent value correlated with a significant UMFA concentration increase in plasma, as its concentration remains fairly low even among women consuming prenatal supplements. However, two Canadian pregnancy cohort studies revealed ubiqutous unmetabolized FA in circulation (125) and a recent study among women consuming very high dose FA supplements (4-5 mg) demonstrated that at this intakes UMFA is higher compared to women consuming lower doses (126). With a defined upper cut-off value, researchers are in a better position to investigate various health effects associated with supraphysiological UMFA concentration and therefore high FA intakes. In animal models, such as the mice used in our study, it will also allow us to further investigate the causal mechanisms of actions related to elevated UMFA concentrations in circulation due to high FA intakes.

4.2. Strengths and limitations

One of the main strengths of this study was our ability to use multiple FA diets in our dietary intervention. Informed by the findings of our scoping review, we were able to encompass most of the diets investigated in the literature. Additionally, our study design also allowed us to take blood samples over the course of the intervention which enabled us to follow the progressive effect of chronic FA intake for each of the diets. Our use of both sexes and the two most commonly used

mouse strains used in folate research (also identified in the scoping review) highlights the consistent sex and strain effect of FA intake on various endpoints and improves the generalizability of our findings.

Additionally, we also used different analytical methods to evaluate FA intake and status. These analytical methods included the *L. casei* microbiological assay, LC-MS/MS, complete blood counts and micronucleus detection by flow cytometry. This allowed us to investigate simultaneously multiple biomarkers of folate intake, status and functional mechanisms. Plasma total folate concentration was used as our main biomarker of FA intake, as it is a marker of recent exposure. We also investigated different folate forms using LC-MS/MS, an analysis that has only been performed in mice in a handful of studies. This allowed us to examine the different folate forms in circulation and their response to different levels of intake.

On the other hand, the use of plasma total folate concentrations as a biomarker of FA intake has its limitations. Hemolyzed plasma samples could result in inflated total folate concentrations due to folate released from RBCs (7,11).

Our scoping review only focused on a limited number of generic (i.e., sex, strain, age) and nutritional specific item (i.e., base diet composition, intervention doses, duration, and exposure verification). This is likely an underestimation of the underreporting of important study design details in nutrition studies, ranging from housing to statistical analysis. We also only reviewed major strain types reported in the studies identified in the review. A limitation of this is that even though substrains can be genetically and phenotypically distinct, they were grouped together to streamline our results (86). As a result, the biodiversity of mouse models used in the folate research is likely underestimated in this study. A next step would be to validate the proposed reporting

criteria against the studies included in this scoping review. Finally, the scoping review was limited to only a 10-year period so does not reflect the totality of reporting in FA-related research in mice.

4.2. Future directions

Red blood cell total folate concentration is an important biomarker used for assessing long term folate status. At the population level, the WHO recommends a minimum RBC folate concentration of 226.5 nmol/L (100 ng/mL) to prevent the development of megaloblastic anaemia and a concentration of 906 nmol/L (400 ng/mL) for maximal protection against NTDs (41). As a next step, we will evaluate RBC folate status to determine the FA dose at which tissue saturation is reached and concentrations plateau. Correlated with the increased concentration of unmetabolized folate in circulation, this data would further inform the upper limit cut-off value of folate intake. Additionally, since RBC folate represents long-term folate status, analysis of RBC concentrations across time can also help us determine when saturation occurs.

Furthermore, we will also measure folate vitamers by LC-MS/MS in liver to determine the dose at which UMFA is measurable in tissues. In humans, UMFA does not accumulate in tissues to an appreciable degree, so it is important to determine whether it does in mice at the doses we have used in our study. Such analysis could also be used to inform sex and strain differences observed in our mouse models.

4.3. Conclusion

Our ability to perform accurate and efficient nutritional risk assessments for folic acid depends on the quality and relevance of the available evidence. The findings outlined in this thesis shows that more work is needed to improve the reporting frequency of key generic and nutritional specific items in basic nutrition mouse-based publications. We recommend the use and requirement of

reporting guidelines to improve the reporting quality of nutrition research, and specifically animal-based research publications. We have also identified candidate biomarkers, namely homocysteine and UMFA, to use in future studies to determine inadequate and excessive exposure to FA. These biomarkers can be used to harmonized proposed health effects associated with varying FA intakes.

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Statement of Contribution

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Chapter 2: Poor reporting quality in basic nutrition research

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