Moderate Vitamin B6 Deficiency and Sulfur Amino Acid Metabolism in Male and Female Rats

By

Frida Hernandez Garcia.

This thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

As partial fulfillment of the requirements of the degree of

Master of Science

Food and Human Nutritional Sciences

University of Manitoba

Winnipeg, Manitoba, Canada

R3T 2N2

Abstract

Homocysteine, cysteine, and methionine are sulfur amino acids. Methionine converts to cysteine in the transsulfuration pathway, utilizing homocysteine as an intermediate. Research indicates that vitamin B6 deficiency may impair the regulation of cellular homocysteine concentrations and decrease cysteine synthesis. Previous studies on B6 deficiency focused solely on male rats overlooking sex-specific differences. This study aims to demonstrate that moderate vitamin B6 deficiency intake in rats affects methionine plasma levels, leading to reduced conversion into cysteine within the transsulfuration pathway compared to the adequate vitamin B6 group. Thus, moderately deficient rats are expected to exhibit decreased B6 plasma levels, with sex contributing to specific differences. Forty-six seven-week-old female and male Sprague-Dawley rats were randomly assigned to receive either a moderate (0.7 mg/day) or adequate (7.0 mg/day) B6 diet for five-weeks. Plasma SAA, B6, estrogen, progesterone, and 1-carbon metabolites in plasma, and enzyme expression of CBS and CGL in the liver were determined. A significant diet effect was observed in 5-MTHF, GSH, choline, glycine, and cystine, with lower plasma levels in the moderate B6 diet group. Conversely, cystathionine and GSSG showed higher plasma levels in the same diet group. Furthermore, a sex effect was evident, with significantly higher plasma levels in male groups for 5-MTHF, methionine, DHFR, vitamin B12 and B2, acetylcholine, choline glutamic acid, SAM, glycine, serine, and GSSG. On the contrary, total cysteine, estrogen, and betaine were significantly higher in female groups. Finally, a sex-by-diet interaction was observed in CBS enzyme expression, with males exhibiting higher expression levels in the adequate diet group compared to the moderate deficiency group, while females showed higher expression levels in the moderate

B6 deficient diet group. This study underscores the importance of including both sexes in nutrition research and emphasizes the importance of maintaining an adequate intake of B6 to support optimal functioning of the transsulfuration pathway and metabolism.

Acknowledgments

I express my deepest gratitude to my advisor Dr. James House from the Department of Food and Human Nutritional Sciences at the University of Manitoba, who gave me the opportunity to work in his lab and provided me with invaluable knowledge and experience throughout my master's program. It has been a challenging path, but I am proud of what I have achieved.

Also, I would like to thank my committee members, Dr. Heather Blewett and Dr. Karmin O. For their valuable feedback and orientation throughout my program. I am grateful for your patience and because you always encouraged me to have critical thinking in my research.

I am happy for the great lab peers that I have because they were always ready to support me when needed, various important tasks would not have been accomplished without their kind assistance. In addition, I extend my acknowledgment to Mingyan, and Jason who helped me so much providing their time, suggestions, and knowledge in the lab. Thank you all for your patience and support.

Finally, I want to massively thank my family, Adriana, Luisa, and Luis for their emotional support, and for always being my cheerleaders. I am deeply grateful for always listening to me, for caring about me, and for all your love. You, the women of my life, were and continue to be one of my best motivations to achieve my goals. I want to thank God for holding my hand and for never letting me fall.

Dedication

I dedicate my thesis to my family and, to me.

To my beloved mom Adriana García

To my precious sister Luisa Hernández

To my dad Luis Hernández who is in heaven

I would not have been able to do it without you, I love you.

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List of Abbreviations

AdoMet: Adenosyl-L-methionine

B6-A: B6 adequate

B6-D: B6 deficient

CAD: Coronary Artery Disease

CBS: Cystathionine β-synthase

CCHS: Canadian Community Health Survey

CGL: Cystathionine-*y*-lyase

CRP: C- Reactive protein

CSE: Cystathionine y-lyase

CVD: Cardiovascular Disease

DHFR: Dihydrofolate Reductase

EAR: Estimated Average Requirement

EDTA: Ethylenediaminetetraacetic acid

GABA: Gamma-Aminobutyric Acid

GCS: y-Glutamyl cysteine synthetase

GPX: Glutathione peroxidase

GSSG: Glutathione disulfide

HPLC: High-Performance Liquid Chromatography

IL-1: Interleukin-1

IP: Intraperitoneal

LPS: Lipopolysaccharide

MAT: Methionine adenosyltransferase

MS: Methionine synthase

Mg: Milligrams

Nanomolar: nmol

PA: Pyridoxal Phosphate

4-PA: 4-Pyridoxic Acid

PAPS: 3'-Phosphoadenosine-5'-Phosphosulfate

PLP: Pyridoxal 5'-Phosphate

PMP: Pyridoxamine 5'phosphate

Pg/mL: Picograms/millilitre

RDA: Recommended Daily Allowance

SAA: Sulfur Amino Acid

SAH: S-Adenosylhomocysteine

SAHH: S-Adenosylhomocysteine hydrolase

SAM: S-adenosylmethionine

SBD-F: Ammonium 7-fluorobenzo-2-oxa-1, 3-diazole-4-sulfonate

TCEP: Tris 2-carboxyethyl phosphine

TNF-α: Tumor Necrosis Factor Alpha

5-MTFH: 5-methyltetrahydrofolate

Tx: Treatment

mL: Millilitres

μL: Microliters

Chapter 1 General Introduction

1.1 Introduction.

Biological sex serves as a fundamental factor contributing to variations in physical traits among various species. Distinct nutritional requirements and variations in nutrient digestion and utilization exist between males and females, leading to divergent health outcomes over the course of life. As personalized nutrition gains prominence in both scientific inquiry and clinical applications, it becomes crucial to grasp the foundational aspects of sex disparities in nutritional research (Chen et al., 2022). In this context, it is important to examine pivotal studies exploring sexual dimorphism in nutrition research, encompassing variations in nutrient intake and metabolism, divergent responses in nutrient-restricted conditions based on sex, and distinctions in the interactions between diet and the gut microbiome in relation to sex. Within each of these domains, emphasis is placed on factors originating from sex hormones, and sex-specific genetics. Studies including only males lack the opportunity of understanding how sex differences impact and affect metabolism.

Nutritionally speaking, while all amino acids are important for protein synthesis, the essential amino acid methionine plays an additional important role within the 1-carbon metabolism that involves a network of interconnected metabolic pathways. Notably the methionine and folate cycles, which play crucial roles in cellular activities. These cycles supply one-carbon units, also known as methyl groups, essential for synthesizing DNA, polyamines, amino acids, creatine, and phospholipids. S-adenosylmethionine (SAM), a powerful donor of aminopropyl and methyl groups within these cycles, acts as the primary substrate for methylating DNA, associated proteins, and RNA (Clare et al., 2019).

Methionine is a substrate of SAM (Allis & Jenuwein, 2016), and SAM is involved in methylation reactions which help to increase growth in cells, and preserves the phospholipid bilayer within cell membranes. In addition, SAM-dependent methylation reactions promote the maintenance of numerous hormones and neurotransmitters which influence homeostasis and neuronal activity. High concentrations of SAM can be found in the liver and the brain as SAM is reported to be a major methyl donor acting as an antidepressant, maintaining liver health, preventing injury and hepatocellular carcinoma (Lu & Mato, 2012).

Differences in sulfur amino acid (SAA) metabolism have been noted in older adults compared to young adults, showing an approximately 50% slower glutathione (GSH) synthesis rate in older adults relative to their younger counterparts (Sekhar et al., 2011). This reduced GSH synthesis rate was found to be significantly correlated with higher levels of oxidative stress markers. This improvement corresponded with a significant decrease in oxidative stress marker concentrations (Paoletti et al., 2023). These findings imply an increased demand for amino acid substrates for GSH synthesis with advancing age. However, whether there may be sex disparities in SAA metabolism between both sexes remains unclear. Nonetheless, sexual dimorphisms in muscle protein synthesis have been reported, with women exhibiting higher rates of muscle protein synthesis in the fasted state and displaying greater resistance of protein synthesis to dietary protein intake (Smith et al., 2008).

On the other hand, vitamin B6 takes part in regulating homocysteine blood levels and in the production of cytokines, which are among the principal mediators of chronic inflammation. In addition, vitamin B6 is essential for activating lymphocytes that comprise

a vital part of the inflammatory response and in the activity of the immune system. In its active form, vitamin B6 is present as Pyridoxal 5'-Phosphate (PLP) and acts as a coenzyme in various enzymatic reactions involving amino acids, including reactions in the transsulfuration pathway. Within this pathway, the sulfur from methionine eventually becomes part of the amino acid cysteine (Doke et al., 1998).

An excessive or deficient intake of certain micronutrients can have critical impacts on body functions that may work differently in both males and females (de Torre-Minguela et al., 2017). Future research in model animal species should explore the effects of dietary disruptions to 1-carbon metabolism simultaneously in both sexes, mirroring the human experience more accurately Additionally, an overlooked aspect in the field is the sex-specific outcomes, which seem to differ across dietary interventions, genetic strains, and species (Aiken & Ozanne, 2013).

Continuing research in the nutrition field will eventually lead to a clearer understanding of the role of food and nutrients in metabolism, 1-carbon metabolism sulfur amino acids, and sex-specific differences that may accelerate the usage of personalized nutrition approaches to enhance human health. By developing new practices and nutrition approaches depending on the sex of the patient is one future goal this study looks to help achieve by directing attention to the importance of hormones and metabolism differences in nutrition research.

Chapter 2 Literature Review

2.1 Vitamin B6.

2.1.1 Main sources and Bioavailability.

B6 is a water-soluble vitamin, extensively present in foods including fish, nuts, beans, vegetables, grains, meat, and fruits. Vitamin B6 can be found in some multivitamin preparations and in supplements for children and adults, and it is added to foods like energy bars and powders. Pyridoxine alcohol and pyridoxamine are some of the active vitamers that represent the generic B6, as the primary active coenzyme form of this vitamin are PLP and pyridoxamine 5'phosphate (PMP). Furthermore, vitamin B6 is found as PMP and PLP in animal tissues mainly (Institute of Medicine, 2005a) and its basic form can be found in meats as active esters. Pyridoxine is mainly find in plant sources, but is less bioavailable and, thus, is most commonly found in multivitamins to prevent a low intake (Brown et al., 2021a). Vitamin B6 bioavailability is determined by the fraction of dietary vitamin B6 that is absorbed and metabolically utilized (Nguyen & Gregory, 1983). It has been indicated that plasma PLP is a superior indicator of the status of vitamin B6 due to its sensitivity of nutriture to this vitamin in contrast to other biochemical parameters (Li & Lumeng, 1981).

In a mixed diet, usually there is a bioavailability of approximately 75% of vitamin B6 (Bowling, 2011). A fact to consider is that food preparation tends to affect vitamin B6 bioavailability and food processing methods lead to an interchange of B6 vitamers. For instance, heating foods have been proved to reduce vitamin B6 content (Gregory, 1997). Even though the bioavailability of vitamin B6 in foods can differ, once the vitamin is absorbed it moves through a series of processes to become biologically active.

2.1.2 Vitamin B6 Intake and Requirements for Adults.

Dietary reference intakes are crucial to guide people to meet their nutritional demands to prevent future deficiencies which are linked to risk for several diseases. It is recommended that the population meet the estimated average requirement (EAR) to assure an adequacy of nutrients intake during their period of life. By developing new practices and nutrition approaches depending on the sex of the patient. The EAR is defined as, ‘‘The daily nutrient intake value that is estimated to meet the requirements in half the healthy individuals within a group’’ (Health Canada, 2010). The recommended daily allowance (RDA) is established on the EAR values plus 2 standard deviations. The RDA is ‘‘the average daily consumption amount that is adequate to meet the nutrient requirements of closely 97% to 98% of the healthy individuals in a specific life-stage and gender group’’. The RDA and EAR values for vitamin B6 for adults are reported in Table 1 (Health Canada, 2010).

The data assessed and collected from the US NHANES 2003-2004 has suggested that vitamin B6 consumption from food only averages around 1.9 mg/day. Yet, intakes from food and supplements containing vitamin B6 of below 2 mg/day seem to be related to high proportions of low vitamin B6 status in all age groups (Morris et al., 2018). Nevertheless, individuals following a very limited diet as vegetarians and vegans might have to improve their vitamin B6 intake by eating fortified foods or by taking supplements to meet their demands. There are numerous foods rich in vitamin B6 content, some of them are listed in Table 2 in milligrams (mg) per serving (U.S. Food and Drug Administration HHS, 2016).

Table 1. Health Canada 2010 EAR & RDA Values for B_6 in Humans

Health Canada 2010 EAR & RDA Values for B6 EAR mg/day RDA mg/Day	EAR mg/day	RDA mg/day
Men ages: 14-50 years old	1.1	1.3
Men ages: 51 years and older	1.4	1.7
Women ages: 14-18 years old	1.0	1.2
Women ages: 19-50 years old	1.1	1.3
Women ages: 51 years and older	1.3	1.5
Women in pregnancy: 18-50 years old	1.6	1.9

Health Canada 2010 Dietary Reference Intakes Retrieved from http://www.hc-sc.gc.ca/fn-an/nutrition/reference/table/index-eng.php

Table 2. Vitamin B6 Rich Foods

Food	**Milligrams (mg) per serving**	**% Daily Value**
1 cup Chickpeas, canned	1.1	65
3 ounces Beef liver	0.9	53
3 Ounces Salmon	0.6	35
3 Ounces Chicken Breast	0.5	29
1 medium Banana	0.4	25
½ cup Spinach	0.1	6
3 ounces Tuna	0.9	53
Fortified breakfast cereals	0.4	25

U.S. Food and Drug Administration 2016 Food Labeling: Revision of the Nutrition and Supplement Facts Levels Retrieved from: https://www.federalregister.gov/documents/2016/05/27/2016-11867/food-labeling-revision-of-the-nutrition-and-supplement-facts-labels

2.1.3 Vitamin B6 status in North America.

By analyzing food intake or by measuring plasma PLP values, vitamin B6 status can be estimated. Traditionally, there has been a lack of epidemiological data reporting Canadian population food intake. The Canadian Community Health Survey (CCHS) was launched to address this issue, and represents the initial national survey to evaluate Canadian's eating habits since the 1970's (Health Canada, 2017). The CCHS survey that took place in 2015 included 24,000 people across Canada looking to examine the population through a 24-hour diet recall. However, certain sub-populations were not included in this analysis, including the army, indigenous reserves, and remote locations. Also, due to changes in food formulations by manufacturers, addition of new food products or nutrients, and continuous update of nutritional values within the database, these changes contribute to modifications of nutrient intake between the surveys of 2004 and 2015 that should be considered when interpreting final results. The percentage of adults aged 19 and over with a usual intake of vitamin B6 below the EAR in both sexes per province are as the following: Manitoba 12%, Ontario 14.3%, Saskatchewan 15.1%, and Quebec 7.9% to mention some examples. Data from 2015 shows that adults with a lower intake of vitamin B6 than the EAR from all of Canada is equal to 12%, whereas males are 6.2%, and females 17.7% (Health Canada, 2019). As observed in the survey, adult females between 19-50 years old tend be at more risk of a vitamin B6 deficiency.

Vitamin B6 is crucial for all stages of life including pregnancy in which vitamin B6 status brings concerns given that a shortage of plasma PLP is related to elevating the risk of miscarriage and preterm birth (Ronnenberg et al., 2007). Vitamin B6 can be found in meat and thus meat intake can be an important contributor of this vitamin to the diet. A study

conducted in 2004 revealed the average daily meat intake in men between ages 14-75 years old to be around 200 g/day. Whereas 1 in 4 men consumed above 300 g/day, while females averaged 200 g/day or even less (Garriguet, 2007). These findings suggest on average, females met their meat serving needs, while a significant fraction of men over ingested this food. Hence, the study indicates that men consumed more meat sources of vitamin B6 than women, a case similarly noted in a nutritional survey conducted by (Starkey et al., 2001).

2.1.4 Measurement of Vitamin B6 Status.

Although vitamin B6 deficiency is rare, insufficiency may result from conditions including the current extensive food processing that can deplete foods of vitamin B6. Moreover, people who suffer from alcoholism, malabsorption, protein-energy deficiency, rare inborn errors of metabolism, and the presence of concurrent intake of certain drugs could present with vitamin B6 insufficiency. Although categorizing vitamin B6 status/insufficiency is difficult, scientists have attempted to group individuals based on plasma PLP concentrations. Individuals are considered B6 vitamin deficient or moderately deficient if their plasma PLP concentrations are less than 20 nmoles/L or less than 30 nmoles/L, respectively (Leklem, 1990). In addition, the EAR for adults both sexes between the ages of 14-50 years old are 1.1 mg/day, any intake below the average requirement would suggest a vitamin B6 insufficiency.

Consequently, understanding how to measure vitamin B6 status is meaningful. Levels of vitamin B6 are commonly analyzed through plasma, urine, blood cells, or indirectly through the use of the methionine loading test (Institute of Medicine, 2005b). Another way to measure B6 status is by analyzing the PLP-dependent activity of

transsulfuration enzymes as Cystathionine β-synthase (CBS) or by measuring the levels of the key transsulfuration substrate, homocysteine, vitamin B12, and folate. A relation between higher plasma homocysteine and lower CBS activity can indicate a depletion of B6 levels that impair the transsulfuration pathway (Cabrini et al., 2005). Furthermore, ratios of 3-hydroxykynurenine and kynurenic acid showed a significant connection with PLP since it is involved in the kynurenine pathway. In conclusion, low plasma PLP concentrations are associated with high values of hydroxykynurenine and kynurenic acid, therefore, these ratios can be used as a marker of intracellular PLP availability and B6 status (Ulvik et al., 2013).

4-Pyridoxic acid (4-PA) in urine and PLP in tissues and plasma are direct measurements of vitamin B6 status. The excretion of PA can indicate the degree of intake of B6 and its subsequent digestion and absorption (Hansen et al., 1996). Vitamers of vitamin B6 in plasma can be assessed via reversed-phase high-pressure chromatography and electrochemical coulometry detection (Marszall et al., 2009). These are short-term indicators since they can be easily affected by the diet. Moreover, the preceding work has shown that the excretion of more than 3 μmoles/day of 4-PA is related to sufficient B6 status in humans. Typically, plasma B6 concentrations below 20 nmoles/L indicate a vitamin B6 insufficiency in humans (Spinneker et al., 2007). The total measurement of vitamin B6 that includes all forms of vitamers is performed utilizing high-performance liquid chromatography (HPLC) (Bisp et al., 2002). This method has been applied considering the remarkable ability to quantify and separate vitamin B6 with a superior sensitivity (Talwar et al., 2003a).

2.2 Factors Influencing Vitamin B6 Status.

2.2.1 Main Factors Affecting B6 Levels.

Populations with alcohol addiction, protein-energy malnourishment, and obesity have been shown to exhibit lower plasma PLP levels. In addition, people undergoing malabsorption syndromes (celiac, inflammatory bowel disease, and bariatric surgery), can also present with a low PLP concentration. Prenatal women suffering from preeclampsia and eclampsia showed reduced plasma PLP concentrations (Brown et al., 2021b). Furthermore, groups with insufficient consumption of vitamin B6 or individuals with an excessive metabolic need may develop a functional deficiency of vitamin B6. Those with autoimmune conditions, including rheumatoid arthritis have higher vitamin B6 catabolism, leading to greater demands for dietary supplementation of B6 (Joyce et al., 2018). Moreover, there is evidence that patients with renal impairment, patients who underwent kidney transplantation, and those receiving dialysis are at greater risk of vitamin B6 deficiency. The physiopathology underlying this is the minimal serum PLP levels in such patients due to increased catabolism of vitamin B6, resulting in a greater requirement for dietary B6 (Joyce et al., 2018). Crucial for clinical nutrition and toxicology is the fact that treatments utilizing isoniazid for tuberculosis, penicillamine for cystinuria, and levodopa for Parkinson's disease act as vitamin B6 antagonists. Similarly, certain antiepileptic drugs may interfere with vitamin B6 metabolism (Echaniz-laguna et al., 2018).

PLP has a vital role in neurotransmitter metabolism, specifically on the inhibitory transmitter of synthesis Gamma-Aminobutyric Acid (GABA). Unsurprisingly inborn errors lead to a deficiency of PLP to manifest as B6-responsive epilepsy generally in the early

stages. This complication alters brain PLP reception resulting in the accumulation of metabolites that inactivate PLP (Mills et al., 2006).

As for dietary factors that may affect vitamin B6 status, there are some antagonists and anti-pyridoxine factors present in food that may further exacerbate a moderate vitamin B6 deficiency within the population that consumes these products. For instance, flaxseed contains the anti-pyridoxine factor 1-amino D-proline in the form of a peptide called linatine. There are nutrients found in flaxseeds that are linked to enhancing health including, phytoestrogens and omega-3 fatty acids, reason why the ingestion of this food has increased over the time (Mayengbam et al., 2015a).

Previous studies among vegetarians have shown lower or almost similar serum levels of PLP in contrast to non-vegetarians (Wegmüller et al., 2015). A potential reason for the discordant data among vegetarians may be due to vitamin B6 status biomarkers, particularly PLP, but also 4-PA. These biomarkers happen to be impacted by a series of factors that include smoking, obesity, and inflammation (Ueland et al., 2016). A study conducting a project to examine dietary and health habits led by the Centers for Disease Control and Prevention recruited 5,000 participants from 15 provinces of the United States. Data available came from two 24-h recalls applied per participant and a detailed set of questions on shellfish and fish consumption to evaluate if self-perceived vegetarians ingested animal products. Some individuals were categorized into smokers, non-smokers, former smokers, drinkers, non-drinkers, heavy drinkers, and their physical activity level by moderate/vigorous and none. Finally, several serum parameters served as covariates: CRP (inflammation), cotinine (active and passive smoking), albumin, creatinine (kidney

functionality), glycosylated hemoglobin (sugar metabolism), and alkaline phosphate (kidney and liver function).

The results indicated the mean age for meat-eaters to be 48 years old, while for vegetarians to be 32 years old, this last group exhibited lower serum alkaline phosphatase against ‘meatarians’. The vitamin B6 dietary mean intake among vegetarians was 1.7 mg/day, in contrast to meat-eaters with 1.8 mg/day. Evaluations performed after a linear regression model utilizing the adjusted age and sex were shown to be significantly different ($P < 0.0001$). However, these variations were not statistically significant in an unadjusted regression model. Yet, lifestyle factors such as age, sex, ethnicity, and biochemical parameters as albumin, creatinine, and alkaline phosphatase may be associated with vitamin B6 status and, is important to take these factors into proper consideration (Schorgg et al., 2021).

2.2.2 Symptoms and Signs of Vitamin B6 Deficiency.

Critically vitamin B6-deficient adults may suffer seizures and commonly present with rashes, normocytic anemia, cheilitis with scaly lip skin, cracks in the corner of the mouth, glossitis (swelling of the tongue), and mental status changes such as depression. Existing studies evaluating the function of vitamin B6 deficiency in cancer, heart disease, and health conditions associated with cognition, refute that supplementation of vitamin B6 beyond the normal dietary intake have further effect. Nonetheless, certain studies have shown a decrease of symptoms in premenstrual syndrome with vitamin B6 supplementation, especially a reduction in irritability, moodiness, and inattention. In addition, vitamin B6 supplementation of 1.9 mg/day is recommended for hyperemesis

gravidarum (severe nausea and vomiting throughout pregnancy) by The American College of Obstetrics and Gynecology (Rollón et al., 2014).

The findings of asthenia, paresthesia, additional sensory or dermatological symptoms may support the diagnosis. Still, symptoms and clinical findings linked with vitamin B6 are extensive. Certain conditions with equivalent symptoms include beriberi (thiamine deficiency), porphyria, normocytic anemia, folic acid deficiency, depression, isoniazid toxicity, and neonatal seizures. It can be hard to test in real-time for vitamin B6 status in various clinical scenarios. In order to examine B6 status, the measurement of key biomarkers, including vitamers in plasma, serum, erythrocyte, and urine is applied. Active serum PLP measurement is available during few clinical settings however, it is not widely accessible or timely.

In developed countries like the United States and Canada, the likelihood of acquiring vitamin B6 deficiency is less probable compared to developing countries, but it is still likely in the presence of a chronic condition or a specific disease state. Despite this observation, it is important to note that patients under isoniazid therapy and other drugs such as cycloserine, and penicillamine, that form a complex with PLP must be educated about the side effects of these medications and the significance of vitamin B6 supplementation and an adequate intake of food rich in B6 given the fact that those drugs act as B6 antagonists.

2.3 Impact of Vitamin B6 Deficiency.

2.3.1 Transsulfuration Pathway and Vitamin B6.

It is evident that a low status of vitamin B6 caused by a nutritional inadequacy or an inflammatory response can result in a tissue-specific or systemic absence of cellular PLP (Paul et al., 2013). Through an experiment inducing vitamin B6 deficiency, some modifications in metabolite patterns proved the ability for variations in PLP-dependent pathways (including transsulfuration). These factors could lead to pathogenesis (Silva et al., 2013). Moreover, a study examining the impact of altering dietary vitamin B6 intake on decreasing PLP levels in the liver. While levels of cysteine were only moderately modified by vitamin B6 deficiency, cystathionine in liver (the transsulfuration intermediate) increased by a factor of ~4. These observations are crucial since it indicates that a metabolic blockage occurs at the level of cystathionine y-lyase (CSE) in the transsulfuration pathway in the presence of significant vitamin B6 deficiency, but that there is enough production to inhibit a significant decrease of cysteine levels in liver with a minor vitamin B6 deficiency (Lima et al., 2006).

Furthermore, a study observed that when several inflammatory diseases exist concurrently, there is increased demand for B6, leading to very low plasma PLP levels in the human body. In a case-control study conducted in 2,686 adults from the United States, an inverse relation between vitamin B6 and inflammatory responses was shown, and this relationship did not include other B-vitamins. These authors concluded that there is a relationship between the level of protection against inflammation with a higher intake of vitamin B6 and the maximum protection against vitamin B6 deficiency boosted in the presence compared to the absence of inflammation. However, the mechanisms underlying

these associations between PLP in plasma and the inflammatory markers remain to be identified (Morris et al., 2010). Nevertheless, the nutritional status may be the key factor to understand the past associations.

A study conducted by Mayengbam et al. (2015a) studied the effect of a moderate B6 deficiency along with an anti-pyridoxine factor on plasma B6 vitamers, SAA, and CBS and CGL enzyme activity in male rats. The study observed that both enzyme activities were reduced in the moderate B6 group (0.7 mg/kg-diet pyridoxine HCI) compared to the B6 adequate (7.0 mg/kg-diet pyridoxine HCI) with a $P < 0.001$. These findings support the argument that a moderate B6 deficiency was able to independently inhibit activities of these two enzymes who play a vital role within the transsulfuration pathway and are PLP-dependent. Additionally, PLP in plasma were significantly different between both moderate and adequate diet groups exhibiting 63.4 nmoles/L vs 731.1 nmoles/L respectively, suggesting a dose-dependent association between B6 intake and plasma. In summary, this study highlights how a moderate B6 deficiency can impact various parameters within the transsulfuration pathway, which are essential for the formation of crucial substrates and amino acids such as GSH, cysteine, etc.

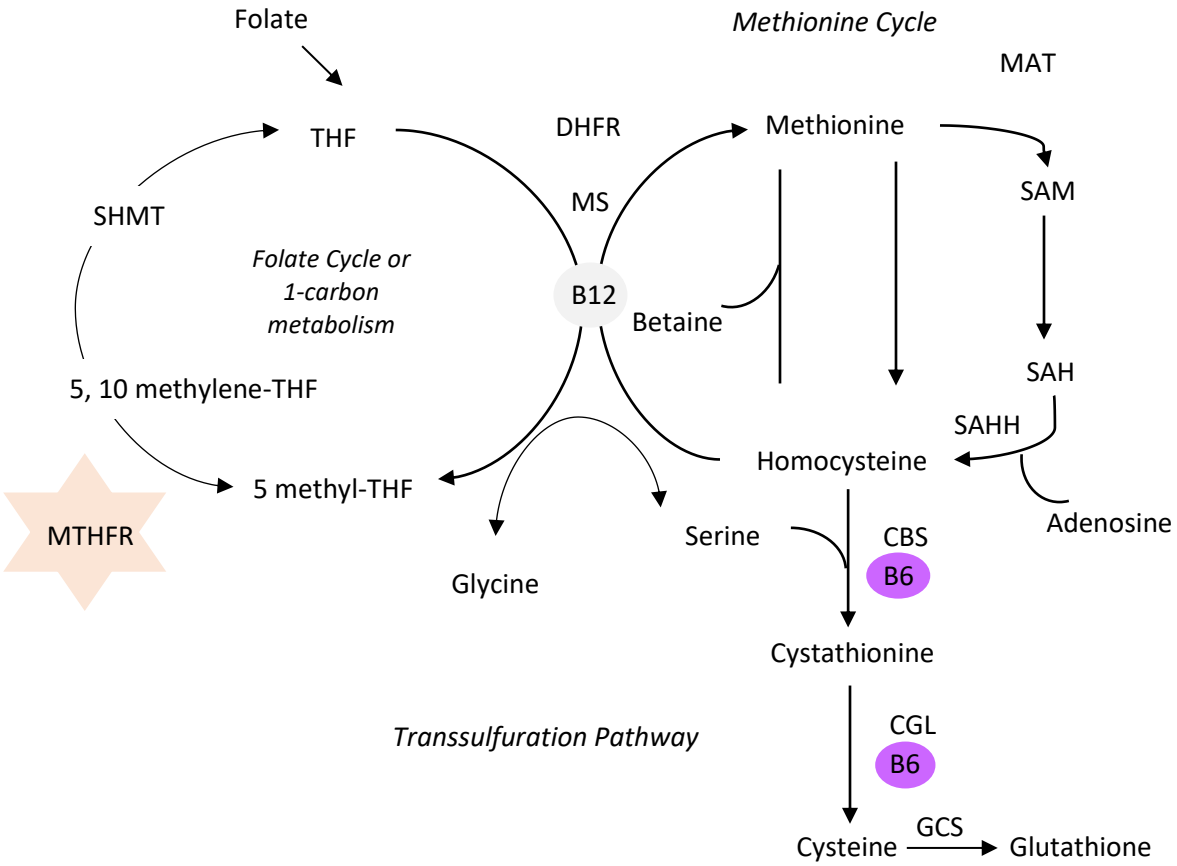
2.4 Sulfur Amino Acid Nutrition and Metabolism.

2.4.1 The Methionine Role in Supplying Cysteine.

Methionine is an aliphatic, sulfurized amino acid; for practical purposes it is a nutritionally essential (indispensable) sulfur amino acid (SAA). The other sulfur amino acid is cysteine, and it is considered to be semi-essential or conditionally indispensable because of the capacity of the body to generate it from methionine. The metabolic pathway leading

from methionine to cysteine includes homocysteine as an intermediate (Figure 1.) (Davis et al., 2005). The notable uncertainty, thus, is how changes in tissue availability of SAA and the end products of their metabolism impact the immune system and related metabolic activities. An adequate metabolic amount of SAA from the diet and tissue protein breakdown is vital for proteins synthesis and peptides implicated in the typical immune responses (Grimble, 2006a). Several studies (Medina et al., 2020) have explored the impacts of excessive methionine and cysteine consumption on growth and mortality in rodents, primarily rats. However, little research has been performed in rats studying the influence of the ratio of dietary SAA and their intake on the inflammatory process.

Figure 1. Folate and Methionine Cycle, and The Transsulfuration Pathway



MAT: Methionine adenosyltransferase.

SAM: S-Adenosylmethionine.

SAH: S-Adenosylhomocysteine.

SAHH: S-Adenosylhomocysteine hydrolase.

SHMT: Serine hydroxymethyltransferase

MTHFR: Methylenetetrahydrofolate reductase

MS: Methionine synthase.

CBS: Cystathionine β-synthase.

CGL: Cystathionine y-lyase.

GCS: y-Glutamylcysteine Synthetase.

DHFR: Dihydrofolate reductase

THF: Tetrahydrofolic acid

2.4.2 Sulfur Amino Acid Metabolism in Response to Intake Changes and its linkage to Health.

Since the metabolism of sulfur has significant implications in the synthesis of crucial metabolic intermediates, a limited intake of SAA can alter numerous metabolic pathways. For instance, it alters the natural capacity of inception and development of specific pathways leading to inconsistent responses, such as causing inflammation and deterioration of changes related to normal aging that may cause several pathologies (Nimni et al., 2007). A study exposed rats to a poor SAA intake to examine variation in the metabolism of 3'-Phosphoadenosine-5'-phosphosulfate (PAPS). The authors observed relative stability of acetaminophen sulfation after a lack intake of PAPS. (Gregus et al., 1994). Remethylation of the metabolic product is preferred over transsulfuration at minimal methionine intracellular concentrations, where methionine is preserved. Furthermore, when methionine intake increases, the transsulfuration pathway is enhanced to supply a substrate for glutathione synthesis. However, it appears that elevated dietary consumption of methionine (5-6 g/day) increases plasma levels of homocysteine, regardless of a sufficient intake of vitamin B6 (Ditscheid et al., 2005). Besides, at greater dietary protein consumption, SAA requirements are most likely met. In essence, methionine included in the diet after needs are met is oxidized through the transsulfuration pathway and few amino acids are discharged through the urine under normal physiological conditions. Hence, adult humans require 13 mg/kg per day of methionine plus cystine based on highest estimate of requirement to achieve nitrogen balance, this according by the (FAO/WHO, 1985). Nevertheless, certain researchers (Nimni et al., 2007) have suggested doubling this amount due to the limitations of the studies used to establish the recommended intake; human

studies are challenging to conduct, given their complexity, cost, and susceptibility to numerous variables.

2.4.3 Homocysteine and Cysteine Status Influence in Health.

When homocysteine circulating levels are high, it is known as hyperhomocysteinemia, and this has been historically identified as a coronary, cerebral, and peripheral atherosclerosis risk factor (McCully, 2011). A flaw in the metabolism of methionine can increase homocysteine levels, and this can be caused by anomalies in the coding genes for homocysteine metabolism enzymes or deficiencies of key vitamin cofactors, including B6, folate, B12 and riboflavin (Rothenbacher et al., 2002).

Some studies have determined that total-homocysteine levels are a solid predictor of mortality in patients with angiographically confirmed coronary artery disease (Lotto et al., 2011); (Ebbing et al., 2010). Additionally, high serum homocysteine concentrations have been associated with sudden death in patients with diabetes (Burke et al., 2002). There is further evidence that higher homocysteine levels damage the artery tissue and cells and it tends to induce cytokines and cyclins liberation, along with other mediators of inflammation and cell division (McCully & Wilson, 1975).

As for cysteine, deficient levels are related to several conditions such as cardiovascular disease (CVD), ischemic stroke, diabetes, lung cancer, renal malfunction, and vitiligo (Bin et al., 2017). This SAA is grouped as semi-essential since it can be obtained from the diet or produced by methionine. It acts as a building block for approximately 2% of the proteins and performs a crucial role in biological processes (Sameem et al., 2019). Furthermore, promising studies have shown that a L-cysteine-rich

diet is helpful in numerous pathological affections including neurodegenerative diseases and type-2 diabetes (Yin et al., 2016). A supplementation with L-cysteine in patients with type-2 diabetes has shown to decrease oxidative stress, which is considered a risk factor in vascular inflammation development in diabetes (Manna & Jain, 2013). Cysteine also assumes a fundamental role in iron-sulfur and lipid biosynthesis and is, thus critical for skeletal muscle integrity (Bak et al., 2019).

Both cysteine and homocysteine have a variety of essential roles in the body, however homocysteine is considered toxic at higher concentrations (Table 3). Hyperhomocysteinemia is related to several medical challenges because of alterations in metabolic pathways and a deficiency of vitamin B6. There is evidence that both homocysteine and cysteine can be potential biomarkers of a large number of diseases such as cancer, diabetes mellitus, CVD, neurological disorder, and so forth due to the high homocysteine concentrations and low cysteine levels observed in these diseases. Whereas the cysteine role in CVD is unclear, additional investigation is needed so future usage of cysteine and homocysteine can be applied clinically (Rehman et al., 2020).

Table 3. Homocysteine low, normal, and high blood levels in humans.

Homocysteine	Levels (micromoles/liter)
Low	<5
Normal	5-15
Hyperhomocysteinemia	>15

Based on the National Center for Biotechnology Information (Son & Lewis, 2021)

2.5 The Role of Vitamin B6 in Sulfur Amino Acid Metabolism.

2.5.1 Vitamin B6 Involvement in the Methionine Transsulfuration Pathway and the Role of Deficiency.

The transsulfuration pathway contributes to cysteine synthesis and homocysteine regulation, and involves reactions that are catalyzed by cystathionine-*y*-lyase (CGL) and CBS (Zhang et al., 2012). This pathway is susceptible to vitamin B6 status since both CBS and CGL need PLP as a coenzyme. A study examining the effect of moderate vitamin B6 deficiency and flaxseed as an anti-pyridoxine factor examined SAA, CBS and CGL enzyme activity and PLP (Mayengbam, 2015b) discovered that flaxseed extract or synthetic 1 amino-d-proline administration resulted in a significant reduction ($P= 0.001$) in hepatic CGL enzyme activity by 26% when compared to the corresponding controls. However, CBS enzyme activity was not significantly different ($P= 0.122$) between the control group and the moderate deficient diet; severe deficient rats exhibited significantly lower CBS activity by more than half when compared to the control group. Nonetheless, when rats administered synthetic 1 amino-d-proline compared to those fed flaxseed extract in the moderate B6-deficient group, the decline in hepatic CBS enzyme activity was greater. However, in the adequate B6 group compared to the moderate B6 deficient, hepatic CBS activity was similar.

Thus, a deficient vitamin B6 status (<20 nmoles/L) might lead to impaired regulation of cellular and plasma homocysteine concentrations and a decrease in cysteine synthesis (Finkelstein & Chalmers, 1970). A study including selenium and vitamin B6 supplementation discovered that pigs supplemented with both selenium and vitamin B6 had a higher gene expression of GPX1 and selenocysteine lyase, which indicates that the

transsulfuration pathway was triggered (Dalto & Matte, 2017). The importance of dietary vitamin B6 in enhancing the efficacy of selenium on tissue levels of selenium and glutathione peroxidase has been studied; researchers observed higher selenium levels in the liver of rats deficient in vitamin B6 (Yin et al., 1991). Selenium, an essential micronutrient, is closely intertwined with the methionine cycle and transsulfuration pathway in metabolism. A study (Wischhusen et al., 2020) revealed that parental selenium nutrition influenced the methionine cycle, resulting in lower levels of free methionine and SAM, alongside higher levels of methionine synthase in both selenium-supplemented treatments. In addition, it has been observed that when methionine intake increased, the transsulfuration pathway that supplies cysteine as a substrate for glutathione was enhanced. Nevertheless, it was seen that elevated dietary consumption of methionine (5-6 g/day) increases plasma levels of homocysteine, regardless of a sufficient intake of vitamin B6 (Ditscheid et al., 2005).

2.5.2 Vitamin B6 Status Influence on Cysteine Production.

Previous research has investigated one-carbon metabolism and transsulfuration reactions in rats under two conditions: those under adequate intake of vitamin B6 and those with a critical deficiency of this vitamin. Enzyme activity was assessed through tests following a protocol involving bolus injection of methionine and serine tracers. The results indicated that a shortage of vitamin B6 led to a minimal reduction of 7.4% in hepatic CBS activity. However, the plasma ratios of cysteine and cystathionine reflected a significant change in the transsulfuration pathway, particularly indicating a phase known as CGL. It was observed that in rats with sufficient vitamin B6, the flux through this pathway was only

8.8%. This implies that CGL, determined by the conversion of cystathionine to cysteine, was notably affected in the deficient state. (Martinez et al., 2000).

A study developing a rodent model of moderate vitamin B6 deficiency determined that, when comparing the control diet and the moderately deficient diet groups, plasma cysteine did not alter substantially across both treatments (175.9 vs 167.0 respectively) (Mayengbam et al., 2015c).

Furthermore, past research (Zhang et al., 2009) examining cysteine levels in presence of depleted vitamin B6 (2.25 mg/kg) in piglets determined that plasma cysteine concentrations compared before and after deficiency were 198.7 vs 80.3 μmol/l, respectively. These results deliver solid evidence that plasma cysteine is altered by more drastic levels of low vitamin B6 and that these modifications are mediated by changes in enzyme's key activity within their metabolism.

2.5.3 Vitamin B6 Status and Influence on Methionine's Bioavailability within the Transsulfuration Pathway.

SAM and CBS are essential to preserving the balance between methionine via transmethylation. Under a limitation of methionine, the transsulfuration pathway is coordinately regulated to assures sufficient methionine supply. Anomalies during redox homeostasis and methylation are frequent to numerous diseases that include liver pathologies, in hepatocellular carcinoma and alcoholic liver disease, a growth in markers of oxidative stress is reflected (Mckillop & Schrum, 2005). Thus, it is likely that when methionine levels are minimal, flux via the transsulfuration pathway has to be

downregulated to maintain the amino acid pillar in the methionine cycle (Martinov et al., 2000).

In addition, a study (Ubbink et al., 1996) performed oral methionine tests on 22 human patients that experienced B6-deficiency induced by an antagonistic drug vs a control group with normal B6 status for 6 weeks. The study revealed that individuals with a deficiency in vitamin B6 experienced notably higher increases in circulating total homocysteine ($P < 0.01$) and cysteine ($P < 0.05$) levels following methionine loading. However, after six weeks of supplementation with vitamin B6 (20 mg/day), the deficient subjects showed a decrease in post-methionine load levels of homocysteine and cysteine, however this reduction was not statistically significant in the control group. Moreover, both groups exhibited a significant decrease in circulating cystathionine concentrations after B6 supplementation following methionine loading. In conclusion, a lack of vitamin B6 might lead to disrupted transsulfuration and an irregular methionine load test, which has links to premature vascular disease.

2.6 Exploring Sex Differences

2.6.1 Sex Differences in Homocysteine and Cysteine Plasma Levels

In a cross-sectional study (Cohen et al., 2019), it was found that plasma homocysteine concentrations were higher in males compared to females. The study conducted a subgroup analysis, comparing individuals younger than 55 years old with those older, as homocysteine levels may be influenced by estrogen contributing to sex differences (Morris et al., 2000). In fact, concentrations were higher in women above the age of 55 compared to the younger group from 20 years old to <55 years old. At the time of the study,

participants were not hospitalized. A medical history was obtained, and a physical examination, including blood, urine, and X-rays, was conducted. In addition, homocystinuria is an uncommon but likely fatal inherited condition. It indicates that the body is unable to adequately metabolize methionine. This results in a dangerous buildup of homocysteine in the blood and urine (Sacharow et al., 2017). The disease occurs more frequently in males than in females, and it is congenital in nature (Balaghova, 2022). Previous research in humans and mice has linked homocystinuria to significantly lower plasma total cysteine levels. This reduced cysteine level has usually been attributed to a block in endogenous synthesis of this amino acid caused by CBS inactivating mutations (Jiang et al., 2015a).

In addition, a study (Jiang et al., 2015b) involving female and male Cgl null mice reported that females had a significantly higher plasma total homocysteine and methionine adenosyltransferase 1, alpha expression than males. This result was linked with a sex-specific 70% reduced methionine synthase expression, which was followed by significantly lower plasma methionine. Likewise, in female Cgl null mice a reduced plasma cysteine levels were linked to female-specific cysteine dioxygenase expression. Data also showed that the normal cysteine levels in male Cgl null mice may be due, at least in part, to repression of hepatic dioxygenase expression serving on preserving tissue levels of this compound. In this regard, the finding that female Cgl null mice have significantly lower plasma cysteine levels than male Cgl null mice is intriguing because both sexes are similarly blocked in endogenous cysteine biosynthesis due to deactivating alterations in CBS, as mentioned before (Gupta et al., 2014).

The latter represents one of the few reported studies documenting significant sex differences in homocysteine and cysteine metabolism and emphasizes the need to consider sex effects in nutrition studies. As a result, there is currently little data on metabolite levels among males and females in this regard (Kraus et al., 2009).

2.6.2 Sex Differences in Vitamin B6 Plasma Levels

A recent study was conducted (Mayengbam & Chleilat, 2020), including female and male Sprague-Dawley rats 3 weeks old, to explore the impact of B6 status on gut microbiota. Rats were randomly assigned to one of three dietary groups: Low vitamin B6 (0.07 mg/kg), high vitamin B6 (70 mg/kg), and a control group (7 mg/kg). Results showed that the low B6 group significantly reduced body weight in both sexes. Similarly, feed intake was lower in this dietary group. Additionally, they found a significant sex effect in serum metabolites in low B6 when compared to control and high B6 groups. However, this study did not examine plasma vitamin B6 and metabolite levels. Nonetheless, it was previously demonstrated that inducing vitamin B6 deficiency in female rats via the feeding of a diet devoid of B6 feeding during 2 weeks, reduced vitamin B6 blood plasma levels by 97% while increasing homocysteine levels by 89% after 14 days (Weingaertner et al., 2005).

A recent study (Kim et al., 2021a), including healthy female and male adults, aimed to investigate whether sex differences associated with vitamin B6 intake and plasma PLP were significant. A total of 864 participants between the ages of 20-59 were included, pregnant women were excluded because hormonal changes may reduce plasma PLP levels during pregnancy and lactation (Ueland et al., 2015). A 24-hour dietary recall method was

used to assess habitual B6 intake and supplement usage. Data provided evidence that, of those participants reporting, supplement usage was common. Nutrient consumption was assessed concerning the average overall energy intake and blood plasma samples were taken to assess vitamin B6 levels. Results showed significant interactions between sex and PLP, however, no interaction between sex and B6 intake was shown. This study determined that sex differences in plasma PLP were identified, resulting in significant immediate correlations in men solely among young and middle-aged adults. Furthermore, while both men and women consumed the same amount of vitamin B6, the mean plasma PLP level in women was less than that in men (33.5 nmol/L for women versus 51.1 nmol/L for men). The variations in plasma PLP levels might be due, in part, to the prevalence of vitamin B6 deficiency (plasma PLP 20 nmol/L) in women, which was roughly three times higher than in men (29.0% for women, 10.4% for men) (Kim et al., 2021b). However, more studies involving sex differences within a moderate B6 deficiency are still needed.

2.6.3 Sex Differences in Hormone Plasma Levels (Estrogen and Progesterone)

Literature supporting the understanding of gender and sex differences in cardiac health and pathology is sorely lacking. Regardless of the fact that both male and female sex hormones influencing cardiac function, a review study concentrated on the genetic effects of estrogen as the primary mediator of sex differences (Blenck et al., 2016). The role of sex hormones in rodents, especially estrogen, is notably similar to humans regarding the cardiac gene expression (Isensee et al., 2008). The findings point to estrogen playing a sexually dimorphic role in the adjustment of signalling activity in cardiac myocytes.

There is an important absence of knowledge about the role of progesterone in males. The majority of what it is known about progesterone function comes from female studies. Even though progesterone has always been thought to be a "female hormone," adult male rats have circulating levels of progesterone that range from 1.5 to 2 ng/ml, whereas females have levels that range from 3 to 35 ng/ml during the rat estrus cycle (Priest & Pfaff, 1995).

A study (Bychowski & Auger, 2012) included 4-month-old male rats that were administered either progesterone hormone via subcutaneous injection or oil as a control group. The objective was to study the specificity of progesterone-induced impairment of social recognition. Data displayed impaired a trend for social discrimination within the progesterone-injected group although not statistically significant. The above highlights the lack of studies that include both sexes in rats with the objective of identifying sex differences in the hormone plasma levels overall in many topics, with vitamin B6 one of particular interest in this research where data is limiting.

2.7 Vitamin B6 and the role in the 1-Carbon Metabolism

Vitamin B6 is a recognized cofactor in over 100 metabolic reactions, including 1-carbon metabolism, and has been linked to oxidative stress, immune system regulation, and carcinogenesis. Vitamin B6 is a cofactor for serine hydroxymethyltransferase (SHMT), CBS, and CGL in 1-carbon metabolism. DNA synthesis, repair, methylation, defense against oxidative stress, and detoxification all rely on 1-carbon metabolism (Wit, 2011). As a result, sufficient levels of vitamin B6 must exist to keep these processes running. In vitro and animal studies have shown that vitamin B6 deficiency inhibits the development of lymphoid organs, lymphocyte proliferation, cytotoxicity, delayed-type hypersensitivity

reaction, antibody production, and the production of interleukins involved in a T-helper 1 response. The impacts of vitamin B6 status on 1-carbon metabolism are the main hypothesised mechanism of vitamin B6 deficiency's abnormality of immune system regulation. Vitamin B6 has been shown to protect against colorectal cancer (Zhang et al., 2006). Several mechanisms have been proposed, including 1-carbon metabolism disruption, cell proliferation gene expression, detoxifying carcinogenic substances, protection against oxidative stress and angiogenesis, oxidative stress, inflammation, and nitric oxide synthesis. Theoretically, DNA methylation is reduced in vitamin B6 deficiency because the methyl group donor is less available. Hypomethylation of SAM DNA results in boosted gene transcription (Zhang et al., 2006). Gene hypomethylation results in increased gene transcription. Furthermore, vitamin B6 deficiency may inhibit the transsulfuration pathway of one-carbon metabolism, and thus GSH formation. GSH is essential for protecting against oxidative stress and detoxifying carcinogenic compounds. DNA may be weakened against oxidative stress and toxins in vitamin B6 deficiency, contributing to carcinogenesis (Larsson et al., 2005).

Chapter 3 Research Gap

3. Research Gap.

The effect of a moderate vitamin B6 deficiency within the transsulfuration pathway, specifically on methionine levels to be converted into cysteine, remains ill-defined. The unknown cysteine and homocysteine availability within the body during this nutritional state needs to be studied. Furthermore, previous studies of vitamin B6 deficiency have primarily used and examined the impacts in male rats. It is critical that effects in both sexes are studied to have a better knowledge of the variations that exist due to sex. Additionally, studies involving hormone plasma levels of progesterone and estrogen are lacking to better understand the role that hormones play in sex differences. The analysis of plasma metabolites of 1-carbon metabolism is less evident in the literature, and the use of high throughput, semi-quantitative analysis provides an opportunity to gain further insights into the interplay between B6 status and sex on markers of sulfur amino acid and 1-carbon metabolism.

Chapter 4 Hypothesis and Objectives

4.1 Hypothesis

A moderate vitamin B6 deficient diet in rats (0.7 mg/day) will affect B6, sulfur amino acids, and 1-carbon metabolite levels in plasma compared with the adequate vitamin B6 group (7.0 mg/day). Sex effects on the above parameters will also be evident.

4.2 Objectives

To determine:

1) The effect a moderately B6 deficient diet has in male and female seven-week-old Sprague-Dawley rats on sulfur amino acids, B6 vitamers, estrogen, progesterone, and metabolites of 1-carbon levels in plasma, and CBS and CGL enzyme expression in the liver.

2) The effect that sex has on markers of B6 metabolism, including sulfur amino acid concentrations, enzyme expression of CBS and CGL, and metabolites of 1-carbon metabolism under a moderate B6-deficient diet.

Chapter 5 Materials and Methods

5.1 Animals and Diets.

Forty-six female (n=23) and male (n=23) Sprague-Dawley rats, aged seven-weeks-old, were allowed to acclimate for six days before starting the experiment. Studies have shown that rats become sexually mature at around seven weeks with an average weight of 300 g and adulthood begins after the eight-week of post-natal life (Sengupta, 2013). There is evidence showing that metabolism depend on sex steroids, immunological responses to exogenous and self-antigens differ between males and females as do innate and adaptive immune responses (Klein & Flanagan, 2016). In conclusion, adults are distinguished by significant variances in sex hormone concentrations that impact their immune and inflammatory responses (Casimir et al., 2010).

The animals were weighed and randomly assigned to one of the two dietary groups (n= 23/group) (Figure 2). Base diets formulations are listed in Table 4 and are based on the AIN-93G diet (Reeves et al., 1993a). A randomized complete block design was proposed since groups were divided into six blocks (n= 8 rats; block 1-5, n=6 rats; block 6) to fulfill the experiment. Groups consisted in two levels of vitamin B6 [moderate deficiency (MD): 0.7 mg/kg vs. adequate (AD): 7.0 mg/kg pyridoxine-HCl/kg diet] (Nfational Research Council, 1995a). For the purposes of this study, MD refers to dietary B6 levels that, when consumed, yields low plasma B6 but with no changes in homocysteine levels (Mayengbam et al., 2015) observed. An equal number of animals per sex were considered for randomization. Pre-mixed vitamins (Dyets INC from Pennsylvania, U.S.A) were formulated to meet micronutrient laboratory rat needs (National Research Council, 1995b) but, vitamin B6 was eliminated so that it could be added in prescribed amounts depending on the diet group adequacy (Table 4). Animals received diets and unrestricted water access

to allow *ad libitum* feeding for five weeks. Likewise, a 12:12 hour light cycle with exposure to a minimal level during the 12-hour dark phase (with 0.01 $\mu W/cm^2$) at night was preferred. A previous study experimenting with rats at different luminosity levels showed that light intensity of (0.02 $\mu W/cm^2$) or constant brightness exposure during the dark phase alters circadian patterns. Mostly, glucose, lactic acid, plasma melatonin, and corticosterone. In addition, minimal light contamination interrupts normal circadian rhythms and can affect the investigation outcome (Dauchy et al., 2010).

A record of food consumption was conducted daily by weighing feed residue on the next day and weight was recorded every week; weight was measured after the acclimatization stage and then until termination. Feed efficiency was calculated as: Feed intake (g)/ Body weight (g).

During the experimentation, enforcement of protocols for animal use from the University of Manitoba Animal Care Committee in agreement with the Canadian Council of Animal Care regulations were applied (Canadian Council on Animal Care, 2020).

Figure 2. Flow Design of The Moderate Vitamin B6 Deficiency

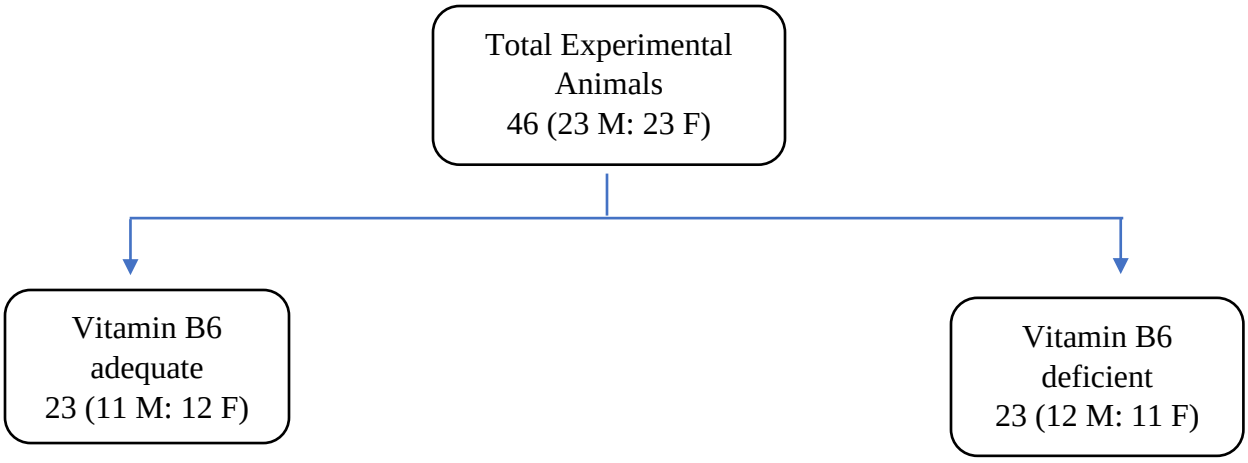


Table 4. AIN-93G Base Mix Diet for Rats (g/kg)

Ingredients	Diet Inclusion (g/kg)
Cornstarch	397
Casein-Vitamin Free	200.0
Dextrose	132
Sucrose	100.0
Powdered Cellulose	50
L-Cystine	3.0
AIN-93G Mineral Mix	35
AIN-93 Vitamin Mix- No B6	0.93
Soybean Oil	70.0
Choline Bitartrate	2.5
T-Butylhydroquinone	0.014
Pyridoxine (g) for adequate and deficient diets:	
Pyridoxine- adequate	0.007
Pyridoxine- deficient	0.0007

Diets based on AIN-93G (Reeves et al., 1993b).

5.2 Plasma and Tissue Collection.

After five weeks of the dietary trial, animals were sedated using isoflurane at 5% and provided with a supply of oxygen at 0.8-1.0 L/min. Next, rats were euthanized via a cardiac puncture, blood samples (3 mL) were collected at the time as quickly as possible into heparin tubes 4 mL tubes. Right after collection, blood was quickly centrifuged at 10,000 x g for 15 min. Plasma was taken and preserved at -80°C for subsequent analysis (Medina et al., 2020a). At the end of the blood collection procedures, liver tissue samples were collected and instantly frozen and stored at -80°C for subsequent analyses.

5.3 Determination of Total Plasma Cysteine and Homocysteine.

Total plasma cysteine and homocysteine levels were determined by treating plasma with 20 μL 10% tris 2-carboxyethyl phosphine (TCEP) (Mayengbam et al., 2015d) from Pierce Chemical Co, USA, and samples were incubated for 30 minutes at room temperature. Next, samples were deproteinized with 125 μL of 0.06 M perchloric acid, and centrifuged at 5,000 x g for 10 min. Then, 50 μL of the supernatant were added to 100 μL of potassium borate buffer (pH 10.5), covering 5 mmol/L ethylenediaminetetraacetic acid (EDTA) and 50 μL of 1 mg/mL ammonium 7-fluorobenzo-2-oxa-1, 3-diazole-4-sulfonate (SBD-F) from Wako Chemicals, VA, USA. For 60 min the solution was incubated at 60°C and then chilled to 4°C. For evaluating cysteine and homocysteine plasma concentrations, reverse phase-HPLC (Shimadzu Inc, Nakagyo-82ku, Kyoto, Japan) with fluorescence detection (λEx= 385 nm, λEm= 515 nm) was used (Medina et al., 2020b).

5.4 Determination of Plasma Metabolites of 1-Carbon Metabolism

Plasma samples were sent to TMIC laboratory located in Edmonton Alberta for the analysis of plasma metabolites of 1-carbon metabolism. A TMIC internal standard solution containing isotope-labeled glutathione, methionine, glutamic acid, cysteine, glycine, serine, nicotinamide, B6 and SAH was made in 50% methanol. A stock solution of the targeted metabolites was prepared in water. This solution was serially diluted with the same solvent to make 10-point standard solutions, in a concentration rang of 0.00001 to 10 nM for each compound. Next, 20 μL of each plasma sample or each standard solution was mixed with 20 μL of the isotope solution and 60 μL of acetonitrile. The mixtures were vortexed for 2 min and then placed on ice for 30 min before centrifugation at 21,000 g for 10 min. The clear supernatants of the solutions were diluted 6-fold with water. Furthermore, 5-μL aliquots were then injected to run UPLC-fMRM/MS on an Agilent 1290 UHPLC system coupled to an Agilent 6495C triple-quadrupole mass spectrometer with positive ion detection. A polar C18 column (2.1*150 mm, 1.6 μm) was used for chromatographic separation. A mobile phase composed of an ammonium formate buffer (A) and methanol (B) was used for binary solvent gradient elution (0% to 20% B in 15 min) at 45 ºC and 0.30 mL/min (The Metabolomics Innovation Centre, 2023).

The detected analyte concentrations in the preceding LC-MRM/MS runs were calculated using their internal standard calibration. This methodology should be viewed as semi-quantitative, yielding relative differences between treatment combinations, as the methods are not specifically optimized for all analyses. For instance, within the laboratory total homocysteine and cysteine are optimally determined following a reducing step (TCEP) and will thus yield higher numbers. However, this method has the potential to offer insights into the interplay between nutrition and sex via the sheer number of

metabolites under study. These data look to serve as a basis upon which new studies can be generated.

5.5 Determination of Enzyme Expression of CBS and CGL in Liver

Total RNA was extracted from liver samples (12 mg) utilizing the RNeasy Mini Kit according to the manufacturer's protocol, which included on-column DNA digestion with the DNase Set kit (Qiagen Canada Inc.). Total RNA concentration was determined using the Nanodrop UV-Vis spectrophotometer 2000 (Thermo Fisher Scientific Inc.) at an optical density of 260 nm, and RNA quality was measured by evaluating the ratio of optical density of 260-280 nm. The Superscript® VILOTM cDNA synthesis kit (Invitrogen; Thermo Fisher) was used to reverse-transcribe 1 g of total RNA, and the resulting cDNA was preserved at 20°C. Based on previous laboratory research, PCR primers were chosen. Ct was calculated using the comparative Ct method, and β -actin was used to normalize target gene expression (Li et al., 2023).

qRT-PCR analysis was carried out on the Step OneTM Real-Time PCR System using the Fast SYBR® Green Master Mix kit (Applied Biosystems; Thermo Fisher). The following were the amplified conditions: 95°C for 20 seconds, followed by 40 cycles of denaturation at 95°C for 3 seconds and combined annealing and extension at 61°C for 30 seconds, followed by a 3-segment cycle of the melting curve (95°C/15 seconds, 60°C/1 min, 95°C/15 seconds). The samples were run in duplicates, and the mean was used for further analysis. The comparative Ct method (ΔΔCt) (Livak & Schmittgen, 2001) was used to determine relative mRNA expression levels, and β-actin was used to normalize target gene expression.

5.6 Determination of Plasma Hormone Levels (Estrogen and Progesterone)

Plasma estrogen was analyzed by an ELISA kit (Competitive EIA) with an Inter-Assay CV <10% and a detection range of 15.6-1000 pg/ml, purchased from LSBio Inc. Samples, replicates (n=1), and standards (n=5) went through a microplate reader with 450nm wavelength filter. Room temperature samples were transferred onto a well plate and then 50μL of 1x Biotin-detection antibody was added and further incubated for 45 minutes at 37°C. Wells were aspirated and then washed 3 times with a buffer. Subsequently, 100μL of 1x HRP-Streptavidin Conjugate was added to every sample well and left to incubate for 30 minutes at 37°C. Afterwards, wells were washed 5 times and 90μL of TMB substrate solution was added and plate was incubated for 20 minutes at 37°C. Immediately after, 50μL of stop solution was added to every sample well and plate was read at 450nm.

Plasma progesterone was also analyzed by an ELISA kit (Competitive EIA) with Inter-Assay CV <12% and a detection range of 1.23–100 ng/ml, purchased from LSBio Inc. Samples, replicates (n=1), and standards (n=5). For progesterone a correction wavelength set at 600- 630nm was used. Room temperature samples were transferred onto a well plate and then 50μL of HRP-conjugate and antibody were added to each well and further incubated for 1 hour at 37°C. Wells were aspirated and washed 3 times with a buffer. Next, 50μL of Substrate A and Substrate B were added to each well and incubated in the dark for 15 minutes at 37°C. Immediately after, 50μL of stop solution was added, finally plate was read at 450nm.

5.7 Statistics.

The study consisted of forty-six animals, with four treatments (Tx): B6 adequate, B6 moderate deficient, both sexes (male rats and female rats). The study was a completely randomized block design, having six blocks (n= 8 rats; block 1-5, n=6 rats; block 6). Each block was performed every day during six days. Hence, a two-way ANOVA was used. The IBM SPSS Statistics software was used to perform the statistics of the data collected. To evaluate equality of error variances a Levene's test was used. Furthermore, a test of between-subjects-effects was applied to define interactions and effects among both dietary groups (B6-MD and B6-AD) and the sex factor under a vitamin B6 moderate deficiency vs an adequate vitamin B6 intake. To measure mean differences and interactions a Bonferroni test with the P value <0.05 threshold was utilized for all tests (Medina et al., 2020e).

Chapter 6 Results

6.1 Performance Data

Rats in the moderate B6-deficient diet showed significant differences (P <.001) in the initial and final body weight, the weight gain, total feed intake and total feed efficiency compared to the B6 adequate diet. However, no significant differences in relative liver (P= 0.848) weights were found when compared to the adequate B6 diet based on diet as observed in Table 5. Yet, rats displayed significant sex differences (P<.001) in the initial body weight, weight gain, total feed efficiency, total feed intake, and relative liver weight (P= .008). In addition, a sex-by-diet interaction was shown in the initial body weight (P= 0.010), weight gain (P= 0.026).

Table 5. Performance and weight of tissues derived from male and female rats that had consumed vitamin B6 adequate or moderate B6-deficient diets.

	Diet			Sex			Interaction				
	AD n=23	**MD n=23**	**P value**	**F n=23**	**M n=23**	**P value**	**AD-F n=12**	**AD-M n=11**	**MD-F n=11**	**MD-M n=12**	**P value**
Total feed efficiency (g/g)	0.0719 ± 0.001	0.0616 ± 0.001	**<.001**	0.072 ± 0.001	0.062 ± 0.001	**<.001**	0.74± 0.04	0.64± 0.04	0.69± 0.04	0.60± 0.04	0.76
Total feed intake (g)	21.44 ± 0.6	30.27 ± 0.6	**<.001**	21.40 ± 0.4	30.28 ± 0.4	**<.001**	22.17± 0.61	30.70± 0.63	20.63± 0.63	29.86± 0.61	0.580
Initial body weight (g)	281.48 ± 4.4	453.77 ± 4.4	**<.001**	351.78 ± 3.1	382.72 ± 3.1	**<.001**	274.7^{c} ± 6.0	288.8^{c}± 6.3	428.7^{b} ± 6.3	$476.6a^{a}$ ± 6.0	**0.010**
Final body weight (g)	322.55 ± 6.8	561.19 ± 6.8	**<.001**	444.23± 4.8	439.28± 4.8	0.262	327.2± 10.7	317.4± 11.2	561.2± 11.2	561.1± 10.7	0.619
Weight gain (g)	41.07 ± 3.6	108.46 ± 3.6	**<.001**	92.45 ± 2.6	56.56 ± 2.6	**<.001**	52.4^{d} ± 5.1	28.6^{c} ± 5.3	132.4^{a} ± 5.3	84.4^{b} ± 5.1	**0.026**
Relative liver weight (g/kg BW)	3.56± 0.4	3.58± 0.4	0.848	3.47 ± 0.5	3.68 ± 0.5	**0.008**	3.44± 0.74	3.69± 0.77	3.49± 0.77	3.67± 0.74	0.265

Values are given as mean ± standard error, means with different superscripts (a, b, c, d) are significantly different within row and section and are based on $p < 0.05$. Total feed efficiency= Feed intake/Body weight gain, AD: adequate diet, MD: moderate deficient, BW: body weight, F: female, M: male.

6.2 Determination of Total Cysteine and Homocysteine Levels in Plasma

Rats in the moderate B6-deficient diet showed no significant differences in total homocysteine (P= 0.867) or cysteine (P= 0.446) plasma levels compared to the B6 adequate diet as observed in Table 6. However, a significant difference (P= <.001) in sex was observed in total cysteine in which females had higher plasma levels than males. Normal cysteine plasma levels in rats are between 80-200 μM/L (Stipanuk et al., 2006). Hence, levels are within normality.

Table 6. Total homocysteine and cysteine plasma levels derived from male and female rats that had consumed vitamin B6 adequate or moderate B6-deficient diets.

	Diet			Sex			Interaction				
	AD n=23	**MD n=23**	**P value**	**F n=23**	**M n=23**	**P value**	**AD-F n=12**	**AD-M n=11**	**MD-F n=11**	**MD-M n=12**	**P value**
Total Hcy (μM)	9.08± 1.3	8.78± 1.3	0.867	10.15± 1.2	7.71± 1.2	.191	10.89± 1.7	7.28± 1.8	9.41± 1.8	8.15± 1.7	0.526
Total Cys (μM)	126.87± 2.8	129.93± 2.8	0.446	136.46 ± 2.8	120.46 ± 2.8	**<.001**	135.23± 3.8	118.51± 4.0	137.69± 4.0	122.17± 3.8	0.881

Values are given as mean ± standard error; means with significant difference are based on $p < 0.05$. Hcy: Homocysteine, Cys: Cysteine, AD: adequate diet, MD: moderate deficient, F: female, M: male.

6.3 Determination of Plasma Metabolites of 1-Carbon Metabolism

Rats in the moderate B6-deficient diet showed a significant difference compared to the B6 adequate diet in the following metabolites: 5-MTFH (P value= 0.046), glutathione (P value= 0.004), cystathionine (P value <.001), glycine (P value = 0.028), and cystine (P value= 0.025). Although pyridoxine did not show a significant difference between diets there is a trend towards a significant difference with P value= 0.054. In addition, data showed a significant difference between sexes in the following parameters with a P value <.001: 5-MTFH, methionine, acetylcholine, betaine, cystathionine, glutamic acid, glycine, and GSSG. Additional sex differences were shown in DHFR (P value= 0.009), vitamin B12 (P value= 0.034), vitamin B2 (P value= 0.005), 5-MTHF (P < .001), acetylcholine (P < .001), choline (P value= 0.041), SAM (P value= 0.019), betaine (P < .001), glutamic acid (P < .001), glycine (P < .001), GSSG (P < .001), serine (P= 0.011). cystathionine (P < .001), and serine (P value= 0.011). Finally, a sex-by-diet interaction was shown in glycine (P= 0. 041) as observed in Table 7.

Table 7. Relative plasma metabolites of 1-Carbon metabolism levels derived from male and female rats that had consumed vitamin B6 adequate or moderate B6-deficient diets.

	Diet			Sex			Interaction				
	AD n=23	**MD n=23**	**P value**	**F n=23**	**M n=23**	**P value**	**AD-F n=12**	**AD-M n=11**	**MD-F n=11**	**MD-M n=12**	**P value**
5-MTFH (nM)	112.1 ± 2.1	120.9 ± 2.1	**0.046**	125.2 ± 3.01	107.8 ± 3.01	**<.001**	103.5 ± 4.1	120.8 ± 4.3	112.2 ± 4.3	129.7 ± 4.1	0.982
Pyridoxine (μM)	3.2 ± 0.082	2.9 ± 0.082	0.054	3.14 ± 0.117	3.08 ± 0.115	0.701	3.30 ± 0.1	3.30 ± 0.1	3.30 ± 0.1	3.30 ± 0.1	0.932
Pyridoxamine (μM)	9.8 ± 1.3	7.7 ± 1.3	0.257	8.6 ± 1.3	8.8 ± 1.3	0.909	9.61 ± 1.8	10.11 ± 1.9	7.75 ± 1.9	7.67 ± 1.8	0.878
Methionine (μM)	132469.04 ± 3926.1	123636.37 ± 3926.1	0.119	110602.1 ± 3926.1	145503.2 ± 3926.1	**<.001**	116851.0 ± 5430.3	148087.0 ± 5671.7	104353.2 ± 5671.7	142919.4 ± 5430.3	0.513
DHFR (nM)	0.695 ± 0.3	0.703 ± 0.3	0.900	0.78 ± 0.04	0.61 ± 0.04	**0.009**	0.62 ± 0.60	0.762 ± 0.63	0.602 ± 0.063	0.804 ± 0.60	0.582
Glutathione (μM)	2735.7 ± 89.5	2190.1 ± 89.5	**0.004**	2477.3 ± 126.6	2448.5 ± 126.6	0.873	2693 ± 175.1	2261 ± 182.9	2778 ± 182.9	2119 ± 175.1	0.530
Folic Acid (nM)	1.22 ± 0.04	1.18 ± 0.04	0.547	1.23 ± 0.04	1.17 ± 0.04	0.272	1.25 ± 0.056	1.18 ± 0.059	1.21 ± 0.059	1.16 ± 0.056	0.905
Vitamin B12 (nM)	0.43 ± 0.003	0.41 ± 0.003	0.525	0.046 ± 0.002	0.038 ± 0.002	**0.034**	0.040 ± 0.03	0.046 ± 0.03	0.037 ± 0.03	0.045 ± 0.03	0.822
Vitamin B2 (nM)	46.2 ± 1.5	47.8 ± 1.5	0.469	43.7 ± 1.5	50.3 ± 1.5	**0.005**	43.43 ± 2.1	49.04 ± 2.2	44.09 ± 2.2	51.57 ± 2.1	0.672

Table 7. Relative plasma metabolites of 1-Carbon metabolism levels derived from male and female rats that had consumed vitamin B6 adequate or moderate B6-deficient diet (*continued*).

	Diet			Sex			Interaction				
	AD n=23	**MD n=23**	**P value**	**F n=23**	**M n=23**	**P value**	**AD-F n=12**	**AD-M n=11**	**MD-F n=11**	**MD-M n=12**	**P value**
Acetylcholine (nM)	188.5 ± 19.4	180.1 ± 19.4	0.763	242.0 ± 19.4	126.6 ± 19.4	**<.001**	130.27 ± 26.9	246.73 ± 28.1	122.98 ± 28.1	237.29 ± 26.9	0.969
Betaine (nM)	94787.6 ±4545.8	100105.3 ±4545.8	0.413	1198080.1 ± 4545.8	75812.9 ± 4545.8	**<.001**	74529.3 ± 628	115046.1 ± 656	77096.6 ± 656	123114.0 ± 628	0.671
Choline (nM)	6006.4 ± 292.5	5885.9 ± 292.5	0.772	6382.5 ± 292.5	5509.8 ± 292.5	**0.041**	5701 ± 404.6	6311 ± 422.6	5318 ± 422.6	6453 ± 404.6	0.530
Cystathionine (nM)	155.3 ± 11.1	286.3 ± 11.1	**<.001**	284.6 ± 15.7	156.9 ± 15.7	**<.001**	113.4 ± 21.72	197.2 ± 22.68	200.5 ± 22.68	372.1 ± 21.72	0.054
Glutamic Acid (nM)	91067.1± 3967.4	85.94.1 ± 3967.4	0.293	98964.4 ±3967.4	77196.8 ±3967.4	**<.001**	82722.1 ± 5487.5	99412.2 ± 5731.5	71671.5 ± 5731.5	98516.6 ± 5487.5	0.371
SAM (μM)	175.4 ± 12.1	180.7 ± 12.1	0.755	199.0 ± 12.1	157.1 ± 12.1	**0.019**	156. 6 ± 16.7	194.1 ± 17.5	157.6 ± 17.5	203.9 ± 16.7	0.800
SAH (μM)	27.23 ± 3.5	27.05 ± 3.5	0.972	24.89 ± 3.5	29.40 ± 3.5	0.378	25.03 ± 4.9	29.43 ± 5.1	24.74 ± 5.1	29.36 ± 4.9	0.983

Table 7. Relative plasma metabolites of 1-Carbon metabolism levels derived from male and female rats that had consumed vitamin B6 adequate or moderate B6-deficient diet (*continued*).

	Diet			Sex			Interaction				
	AD n=23	**MD n=23**	**P value**	**F n=23**	**M n=23**	**P value**	**AD-F n=12**	**AD-M n=11**	**MD-F n=11**	**MD-M n=12**	**P value**
Glycine (nM)	65740.3 ±2838.07	78645.7 ±2838.07	**0.028**	89742.1 ±4013.6	54643.9 ±4013.6	**<.001**	54187.4^{c} ± 555	77293.2^{b} ± 579	55100.4^{bc} ± 579	102191.0^{a} ± 555	**0.041**
Serine (μM)	258716.6 ±7610.3	245978.5 ±7610.3	0.243	266740.7 ±7610.3	237954.4 ±7610.3	**0.011**	245132.5 ± 10526.1	272300.7 ± 10994.1	230776.3 ± 10994.1	261180.8 ±10526.1	0.881
Taurine (nM)	160598.6 ± 9429.5	147627.6 ± 9429.5	0.336	149263.2 ± 9429.5	158962.9 ± 9429.5	0.471	165342.4 ± 13042.2	155854.7 ± 13622.2	133184.07 ± 13622.2	162071.1 ± 13042.2	0.158
GSSG (nM)	42.8 ± 1.2	42.7 ± 1.2	0.989	46.8 ± 1.2	38.7 ± 1.2	**<.001**	38.58 ± 1.67	47.01 ± 1.75	38.97 ± 1.75	46.58 ± 1.67	0.814
Cystine (nM)	20537.2 ± 607.7	23361.4 ± 607.7	**0.025**	22029.2 ± 859.5	21869.4 ± 859.5	0.896	21246.1 ± 1188.8	19828.2 ± 1241.6	22812.3 ± 1241.6	23910.0 ± 1188.8	0.307
Vitamin B3 (nM)	2439.7 ± 237.4	2392.4 ± 237.4	0.889	2742.7 ± 237.4	2089.4 ± 237.4	0.058	2798.8 ± 328.3	2080.7 ± 342.9	2686.6 ± 342.9	2098.2 ± 328.3	0.848

Values are given as mean ± standard error and means with different superscripts (a, b, c, d) are significantly different within row and section and are based on $p < 0.05$. AD: adequate diet, MD: moderate deficient, F: female, M: male. * P= 0.054 shows a trend towards a significant difference between B6 diets. DHFR: Dihydrofolate reductase, SAM: S-adenosylmethionine, SAH: S-adenosylhomocysteine, GSSG: Glutathione disulfide.

6.4 Determination of Enzyme Expression of CBS and CGL Levels in Liver

Rats in the moderate B6-deficient diet showed no significant difference in CBS nor CGL enzyme expressions compared to the B6 adequate diet. Yet, data showed a significant difference between sexes observed on the CBS enzyme expression (P= 0.025). Additionally, a sex-by-diet interaction was found in CBS (P= 0.011) as observed in Table 8. In conclusion, females had significantly higher CBS expression levels in the liver compared to male groups, females within the moderate vitamin B6-deficient diet showed higher values than those observed in the B6 adequate diet. Conversely, males within the B6 adequate diet showed higher values compared to the moderate B6-deficient diet. However, there were no significant differences found on CGL enzyme expression levels.

Table 8. mRNA enzyme expression of CBS and CGL in liver derived from male and female rats that had consumed vitamin B6 adequate or moderate B6-deficient diets

	Diet			Sex			Interaction				
	AD n=23	**MD n=23**	**P value**	**F n=23**	**M n=23**	**P value**	**AD-F n=12**	**AD-M n=11**	**MD-F n=11**	**MD-M n=12**	**P value**
mRNA CBS expression	1.003 ±0.6	1.051 ±06	0.592	1.13 ±0.6	0.922 ±0.6	**0.025**	0.98^{ab} ± 0.08	1.02^{ab} ± 0.08	1.27^{a} ± 0.08	0.82^{b} ± 0.08	**0.011**
mRNA CGL expression	1.06 ±0.10	1.25 ±0.10	0.213	1.28 ±0.1	1.03 ±0.1	0.111	1.08± 0.14	1.03± 0.14	1.47± 0.14	1.03± 0.14	0.080

Values are given as mean ± standard error and means with different superscripts (a, b,) are significantly different within row and section and are based on $p < 0.05$). AD: adequate diet, MD: moderate deficient, F: female, M: male, CBS: Cystathionine β-lyase, CGL: Cystathionine-γ-lyase.

6.5 Determination of Plasma Estrogen and Progesterone Hormone Levels

Rats within the moderate B6-deficient diet showed no significant differences compared to the B6 adequate diet. Yet, significant differences were seen among sexes (P= 0.025) in estrogen plasma levels in which, females had higher means than those of males Furthermore, there were no significant differences in progesterone plasma levels as observed in Table 9.

Table 9. Estrogen and Progesterone plasma levels derived from male and female rats that had consumed vitamin B6 adequate or moderate B6-deficient diets.

	Diet			Sex			Interaction				
	AD n=23	**MD n=23**	**P value**	**F n=23**	**M n=23**	**P value**	**AD-F n=12**	**AD-M n=11**	**MD-F n=11**	**MD-M n=12**	**P value**
Estrogen (pg/ml)	1554.14 ± 78.02	1526.27 ± 78.02	0.802	1668.56 ± 55.1	1411.84 ± 55.1	**0.025**	174.9± 107.9	136.3± 112.7	159.1± 112.7	145.3± 107.9	0.287
Progesterone (pg/ml)	53.20 ± 4.13	53.75 ± 4.13	0.926	58.40 ± 2.9	48.55 ± 2.9	0.099	62.8± 5.7	43.5± 5.9	53.9± 5.9	53.5± 5.7	0.115

As required, data were log-transformed to homogeneity of variance before analysis. Values are given as mean ± standard error; means with significant differences are based on $p < 0.05$. AD: adequate diet, MD: moderate deficient, F: female, M: male.

Chapter 7 Discussion

7.1 Performance Data

The feed efficiency indicates that for every unit (g) of feed consumed, there is a weight gain, and it is determined by three major factors including the quality of raw materials, diet formulation, and the absorption and utilization of nutrients by the animal. For instance, the cumulative efficiency with which a rat uses dietary nutrients for preservation, lean gain, and lipid accretion is represented by feed efficiency. It is closely related to energy metabolism because all metabolic processes are driven by the oxidation of carbon-containing elements in the feed (Patience et al., 2015). It has been investigated (Sauberlich, 1961) that rats experienced improved growth and efficiency of gains when the diets were enriched with higher levels of either pyridoxine or the specific amino acid being investigated, or a combination of both. When the diet contained an optimal amount of vitamin B6 (7.0 mg/kg/day) D-methionine, methionine hydroxy analog demonstrated growth-promoting capabilities equivalent to L-methionine. It was observed that dietary pyridoxine levels influenced the plasma free-amino acid pattern. Hence, the significantly lower total feed efficiency observed in rats fed the moderately B6 deficient diet compared to those on the B6 adequate diet aligns with existing literature. A moderately deficient B6 diet leads to reduced nutrient availability for efficient utilization by the animal's body, as defined by feed efficiency (Patience et al., 2015). Furthermore, considering both the rat's feed intake and body weight gain adheres with the accurate method of calculating feed efficiency, ensuring the precision of these results.

Additionally, the initial and final body weight, and weight gain were significantly different between diets showing higher values in rats within the moderate B6-deficient diets compared to the B6 adequate diets. These results differ from past research done within the

laboratory following a similar dietary trial in male rats which showed no significant differences between diets. However, as a study including both sexes, the initial body weights would not be similar due to sex differences. It is expected that the initial body weight of male rats significantly differs from that of females because males tend to be bigger than females. Furthermore, according to the study conducted by (Mayengbam et al., 2015) who followed a moderately B6 deficient diet in male rats, total feed intake and feed efficiency were not significantly different between diets after 5 weeks of the trial. This outcome differs from the current study which displays a significant difference between diets; higher means are found in the B6 adequate diet in total feed efficiency compared to the moderately deficient one. In addition, the feed intake appeared higher within the moderately deficient compared to the B6 adequate diet. Furthermore, differences in total feed intake and feed efficiency were significantly different among sexes, with females having higher means in total feed efficiency and males having higher means in the total feed intake values. Now, the relative liver weight differed significantly between sexes, with males having significantly higher liver weights than those in females, however these differences were minor and may be a function of body composition when scaled to body weight.

7.1.1 Amino Acids

Cysteine is formed from methionine utilizing homocysteine and cystathionine as intermediates. Cysteine acts as a direct precursor for the biosynthesis of glutathione. These reactions are catalyzed by CBS and CGL through the condensation of serine and homocysteine to water and cystathionine, which is then hydrolyzed to cysteine. In this study cysteine plasma levels were higher in female groups. However, the higher means did not overpass the normal cysteine plasma levels in rats shown in the literature review (80-200 μM/L) (Stipanuk et al., 2006). Cysteine results in this study differ from those shown in a study conducted by Mayengbam et al. 2015, which showed higher means for homocysteine plasma levels in the moderate B6 deficient diet in rat males taking an oral anti-pyridoxine factor; interestingly no significant effect was shown in cysteine as seen in the current study. It can be suggested that males could have used cysteine for muscle formation, since body weight appeared to be significantly higher in males than females. Moreover, it is worth noting that rats utilized in Mayengbam's study were three weeks old, whereas those in the current study were seven weeks old. These differences, along with the inclusion of both female and male rats, may contribute to the disparities observed even when comparing males from both studies. Particularly, regarding sex maturity and variations in hormone proportions may have affected plasma cysteine differently. Homocysteine plasma levels are considered to be within the normal range from 5-15 micromoles/liter (Son & Lewis, 2021) which would not represent risk for cardiovascular disease. It can be suggested that the moderately B6 deficient diet (0.7 mg/kg of pyridoxine hydrochloride) feeding for 5 weeks did not yield a negative impact nor effect on plasma homocysteine levels in this study and rat age utilized. Then, methionine plasma levels were

higher in males regardless of the group diet showing a sex effect. However, methionine ranges from 13 to 45 μmol/L (Kovalska et al., 2021), which was not exceeded and it showed to be lower than that with 1.48 μM for the B6 adequate group and 1.42 μM for the B6 moderately deficient. Nonetheless, this parameter was analyzed in a foreign laboratory under a different method which was not entirely comparable to the literature or cysteine and homocysteine data results obtained. A unit conversion was required to compare where these results stood towards the normal ranges. A similar method of deproteination is needed to be comparable to the literature and results obtained in the current study. However, it would be interesting to study this under a longer period of vitamin B6 moderately deficient diet to see the possible different outcomes in homocysteine and methionine concentrations following the same analysis methods in order to be comparable with one another.

7.1.2 Enzyme Expression

The transsulfuration pathway contributes to cysteine synthesis and homocysteine regulation, and involves reactions that are catalyzed by CBS and CGL (Zhang et al., 2012). This pathway is susceptible to vitamin B6 status since both CBS and CGL need PLP as a coenzyme. Thus, inadequate vitamin B6 status might lead to a decrease in cellular SAM concentrations resulting in a diminished in vivo activity of CBS, consequently reducing flux through the transsulfuration pathway (Lima et al., 2006). This study's data showed a significant sex-by-diet interaction on the CBS enzyme expression, with the male group having significantly higher means compared to females in the B6 adequate diet. The contrary was found in the vitamin B6 moderate deficient diet, where females had significantly higher means than those found in males. It has been shown that the activity of

CGL is affected by stimuli such as oxidative stress, and nutrient deprivation, such as vitamin B6, whereas CBS is affected at a less variable rate (Werge et al., 2021). Although enzyme activity and expression are not entirely similar, it can be implied that what occurs in the activity may similarly translate to the expression level. It is interesting to see that CBS expression was significantly affected in the current study versus past research conducted by (Mayengbam et al., 2015) which did not show a significant difference between the control group and the moderate B6 deficient diet in the CBS enzyme activity. Interestingly, CGL showed a decreased activity of 26% in the moderate B6 deficient diet, when no significant effect on CGL expression was found in the current study. However, activity and expression are different and can not be one hundred percent comparable; enzymes are proteins that aid in the speeding up of biochemical reactions, and how much of an enzyme is produced by a cell is referred to as its expression (Robinson, 2015). This is significant because it can provide information about a cell's metabolism.

While a deficiency in vitamin B6 was documented to cause a notable decrease in hepatic CBS activity in rats, some studies have indicated minimal reductions in CBS activity in conditions of potentially less severe deficiencies. A previous study (Sturman et al., 1969) conducted a comparison of CGL and CBS activities in rats that were given a high-pyridoxine diet (50 mg PN/kg diet) and those that were deficient in dietary pyridoxine exhibited reduced liver CBS activity but caused a much greater reduction in CGL activity. Furthermore, a study conducted by (Lima et al., 2006) including rats that consumed diets lacking in pyridoxine at doses of 0 and 0.1 mg/kg showed only 30% of the CGL activity compared to those rats that were fed diets containing 2 mg/kg of pyridoxine. Liver CBS activity remained unaffected by both the type of diet (AIN-76A or AIN-93G) and the

concentration of dietary pyridoxine, regardless of whether SAM was added for allosteric activation or not. The basal and PLP-stimulated CGL activities showed a significant and positive correlation with dietary pyridoxine concentration (P= 0.001) both without and with PLP, but these activities were not influenced by the type of diet. Thus, the extent to which CBS and CGL enzymes are affected by a moderate B6 vitamin diet indicates that the enzyme expression may not be consistent with previous studies. It can be suggested that the higher expression of CBS appears to be such a response to compensate for the inadequate vitamin B6 status. Furthermore, sex differences are involved in such outcomes as seen in the moderate B6 deficient female group. Estrogens can impact the liver both directly, through the actions of hepatic endoplasmic reticulum, and indirectly, by interacting with endocrine, metabolic, and sex-differentiated functions of growth hormone. Substantial evidence supports the idea that estrogens play a role in influencing growth hormone-regulated endocrine and metabolic functions in the human liver, particularly through their involvement in the GHR-STAT5 signaling pathway (Udy et al., 1997). Consequently, the biology of growth hormone can have a significant effect on liver physiology during both mammalian development and adulthood. In humans, the sexually dimorphic pattern of hormone secretion exists, although it is not as pronounced as observed in rats (Ohlsson et al., 2009). Furthermore, various hepatic genes have been discovered to undergo up or down-regulation in response to distinct patterns of growth hormones or sex-steroid exposure. These transcripts, influenced by both hormones and sex-dependent factors, encompass genes related to plasma proteins, enzymes, transcription factors, and receptors. They play roles in the metabolism of proteins, carbohydrates, lipids, and signaling regulation (Flores-Morales et al., 2001). There is an understanding that the primary contributor to sexual dimorphism in the liver is the response to sex-specific patterns of

hormones, for instance estrogens (Flores-Morales et al., 2001). Nevertheless, it is probable that factors beyond the sexually dimorphic estrogen secretion pattern contribute to certain sex differences observed in the rat liver. Gaining insight into the intricate interplay of these factors in physiological, biological, and pathological states could enhance comprehension of sex-related distinctions in liver characteristics, enzyme activities, and their expression in the liver.

7.1.3 Hormones

It is important to note that the role of sex hormones in rodents, especially estrogen, is notably similar to humans regarding the cardiac gene expression (Isensee et al., 2008). The findings point to estrogen playing a sexually dimorphic role in the adjustment of signalling activity in cardiac myocytes. However, there is a lack of studies related to vitamin B6 deficiency and the role hormones play in sex differences. The current study shows a significant sex effect on estrogen plasma levels in which, females had higher estrogen than that of males. Thus, the varied effects noted could be attributed to differences in initial hormonal proportions, sex, and the phase within the estrous cycle in which rats were. The menstrual cycle as it is known in humans would be equivalent to the estrous cycle in rats; during the proestrus phase, akin to the human follicular stage, there is an increase in circulating estrogen levels and a minimal surge in prolactin (Heape, 1990). It can be suggested that rats were in this stage when euthanized, as higher estrogen levels were observed in females compared to males.

In addition, numerous studies have proposed diverse methods for assessing the estrous cycle by monitoring changes in the animal's physiology and anatomy. Such as, a

visual inspection, vaginal cytology, and histological analysis of reproductive organs (McLean et al., 2012). However, none of these approaches were employed to assess the estrous cycle due to time and resource limitations.

Yet, there is a dearth of studies examining the proportions of estrogen and progesterone in both sexes in rats, as well as the effects of moderate vitamin B6 deficiency on these hormones. Further research is necessary to gain a clearer understanding of the biological responses and the influence that hormones like progesterone and estrogen may exert on plasma levels in both sexes in rats.

7.1.4 Metabolic Data

1-carbon metabolism is essential for DNA synthesis and repair, DNA methylation, detoxification, and oxidative protection. As a result, vitamin B6 deficiency can disrupt these processes, promoting carcinogenesis (Larsson et al., 2005). The majority of studies done about the 1-carbon metabolism and vitamin B6 are human studies on the relationship of vitamin B6 with carcinogenesis revolving around colorectal cancer, diabetes, cardiovascular disease, among others. The majority of them looked at the relationship between 1-carbon metabolism nutrients like vitamin B6, vitamin B12, folate, methionine, homocysteine, cysteine, also factors such as alcohol and cancer risk (Wit, 2011). However, studies in both female and male rats are lacking in the literature as it relates to sex effects in relation to 1-carbon metabolism, including vitamin B6 deficiency. The parameters showing a significant difference between diets and sex groups are shown above in Table 7. - 7.1.

A study (Lamers et al., 2009) conducted in humans gave subjects either B6 adequate or moderately deficient diets. Blood results showed that vitamin B6 restriction

decreased plasma PLP concentration from 55.4 nmol/L to 23.1 nmol/L, consistent with moderate deficiency, while increasing plasma glycine concentration (P= 0.01). Results align with the current study in which glycine was significantly higher in the moderate B6-deficient diet (P= 0.028) compared to the B6 adequate. Additionally, they determined that the in vivo GCS and SHMT reactions were relatively resistant to the effects of moderate vitamin B6 deficiency, most likely due to a compensatory effect of increasing substrate concentration. A way to better understand this, is knowing that PLP as the active form of vitamin B6 is required as a coenzyme for glycine decarboxylation via the GCS and glycine-serine transformation via SHMT. One being dependent to the other suggests that in a B6 moderate deficient status glycine will be affected as explained above.

Although B6 vitamers showed no significance; pyridoxine showed a trend towards a diet difference with a P value= 0.054. This states that even when pyridoxine is not the primary active form of vitamin B6 it can be observed a peculiarity in this parameter to be affected by the moderately B6 deficient diet. Furthermore, the concentration of cystathionine in plasma usually serves as a highly sensitive biomarker for vitamin B6 insufficiency, surrounding levels from slightly below optimal to outright deficient states of vitamin B6 status (Lima et al., 2006). Hence, when cystathionine plasma levels are higher than those of the B6 adequate group, there is most likely a vitamin B6 deficiency status as seen in the cystathionine plasma levels in the current study. Higher means were found in the moderate diet. Furthermore, a lack of vitamin B6 has been observed to result in increased levels of glutathione in the liver of rats and in the plasma of humans. A study (Davis et al., 2006) revealed a 38% higher concentration of glutathione in plasma when vitamin B6 was restricted in the diet compared to the baseline. Hence, regardless of the lack

of PLP analysis, cystathionine, pyridoxine, and glutathione serve to determine B6 status. In this case, glutathione was found to be significantly different between diets; means appeared higher in the moderately B6 deficient diet compared to the B6 adequate diet. It can be suggested that based on these plasma levels, there is not a severe deficiency but more of a moderate B6 deficiency. Moreover, sex differences have been found to influence these findings. Further research is needed to gain a better understanding of how hormones influence metabolic processes.

Additionally, it has been shown that CBS has a higher affinity for serine, and CGL has a higher affinity for cystathionine compared to cysteine. If serine levels are elevated, the production of cystathionine will increase, and if cystathionine is elevated, the production of cysteine will increase (Werge et al., 2021). In the end, all parameters in the transsulfuration pathway are connected and work in conjunction. It may be bold to suggest that it is expected that when several parameters have been altered, then other parameters will most likely be too. Plasma cystathionine was higher in the moderately B6 deficient diet compared to the B6 adequate diet, and males showed higher values than females. There have been studies that mentioned that plasma cystathionine concentrations increase in B6 deficiency mainly due to the bottleneck caused by reduced CGL activity which is observed in these data results. It has been found that because of the increase in cystathionine, the canonical production of cysteine is largely maintained even during vitamin B6 deficiency (Gregory et al., 2016). However, it's interesting to see how this process works differently depending on the sex. This study shows the importance of including both sexes in research, to provide a better understanding of the biological and physiological mechanisms. Furthermore, alterations in glycine metabolism have been observed in rats at different

levels of vitamin B6 deficiency, with hepatic glycine levels inversely proportional to B6 intake. It can be hypothesized that the increase in glycine plasma levels observed during moderate vitamin B6 deficiency is due to a decrease in the activity of the B6-dependent GCS, which is a major source of cellular 5-MTFH production, as observed in the Lamers study (Lamers et al., 2009). The presence of a loss of function can result in an accumulation of glycine, the current study observed higher plasma levels in the moderate B6-deficient diet compared to the B6 adequate diet, and male groups showed higher mean values compared to females.

Because GCS is B6 dependent and is a major source of cellular 5-MTHF production, it can be deducted that the plasma levels on 5-MTHF would be impaired under conditions of moderate B6 deficiency. As seen before, higher plasma levels are found in the moderate B6-deficient diet for the 5-MTHF parameter. In addition, the literature has shown that 5-MTHF is the main form of dietary folate. Organs like the testis and ovaries are encased in a capsule that acts as a partial, regulated barrier to circulating blood, and they have an increased need for folates in a depleted environment. Although the synthetic form of folate as folic acid was analyzed this may explain why higher 5-MTHF plasma levels were found in the male groups that had lower estrogen levels compared to females (Menezo et al., 2022). There is significant sex variation in this feature, and estrogens are triggers and controllers of methylation resetting, according to the literature that could explain why there are sex differences in plasma levels across several parameters.

As for betaine, it has been shown that tissue betaine serves as both an intracellular osmolyte and a reservoir for methyl groups. Despite its crucial role, there is limited data in its content. A study (Slow et al., 2009) measured betaine levels in plasma and various

tissues of male and female rats to assess gender-specific differences and the relationship between tissue and plasma levels. Betaine was most abundant in the liver and kidney (1.6 to 9.5 mmol/l and 2.0 to 5.4 mmol/l, respectively). Plasma betaine concentrations were generally lower compared to tissues; lung, liver, kidney, spleen, and intestine exhibited significant accumulations related to both plasma and independent of plasma levels. Betaine content in the skin, liver, and kidney did not differ significantly between males and females, but it was notably higher in males in plasma and all tissues analyzed ($P < 0.01$). This information aligns similarly to the current study, which showed higher plasma levels on male rats compared to females. The differences in reported betaine content across studies may stem from various factors, such as genetic variability among animals and variations in pre-study animal treatment. Two crucial factors are likely to be significant: the betaine levels in the diet and the hydration status of the animals, which were not assessed in the current study.

In conclusion, this methodology should be viewed as semi-quantitative, yielding relative differences between treatment combinations, as the methods are not specifically optimized for all analyses. For instance, within the laboratory total homocysteine and cysteine are optimally determined following a reducing step (TCEP) and will thus yield higher numbers. However, this method has the potential to offer insights into the interplay between nutrition and sex via the sheer number of metabolites under study. These data look to serve as a basis upon which new studies can be generated.

7.2 Strengths and Limitations

The current study utilized established research methodologies and standards but also quite novel methods of analysis for 1-carbon metabolism and the analysis of enzyme expression in the liver of CBS and CGL. These methods were efficiently carried out by employing an ultra-performance liquid chromatography-mass-spectrometry and qPCR methods, which resulted in robust data results in the methodology. Additionally, the inclusion of hormone determination in plasma allowed us to gather data that looks to provide a better understanding and knowledge of sex differences and the importance of including both sexes in research. There are significant sex effect results in these data, ones that are expected to help direct attention to this factor in science for the upcoming studies in nutrition. Furthermore, as explained in the introduction to this paper, moderate vitamin B6 deficiency is well defined in humans as plasma levels < 20-30 nmol/L. In animals, however, this may not be the case; the development of a rat model of moderate B6 deficiency was well used to replicate a subset of the human population of interest and provided an advantage when performing in vivo studies. The diets followed a well-known diet base that fulfilled the animal requirements and was modified to achieve research objectives. As far as it is known, this is the first study to investigate the effects of moderate B6 deficiency on hepatic CBS and CGL enzyme expression, estrogen and progesterone hormone plasma levels, and 1-carbon metabolism in plasma. As a result, data obtained may promote future research with the inclusion of both sexes. It hopes to serve as one of many crucial components of future pre-clinical studies aimed at enhancing risk assessment and treatment approaches tailored to the patient's sex. Furthermore, the metabolomics analysis of 1-carbon metabolism broadens the scope of the research project.

On the other hand, the time and resources limitations present in the study impacted the flow treatment design and the inclusion of the analysis of the primary form of vitamin B6 (PLP). The current study could not show the plasma levels in the blood for this parameter, limiting the objective and the need to have a more specific result and comparison with the existing literature review. Furthermore, because a rat model was employed to represent moderate B6 deficiency in humans, the potential effects of this diet must be evaluated in human clinical trials. Nonetheless, the current short-term study provided data on the potential effects of a moderate B6 deficiency on SAA such as cysteine, as well as parameters including hepatic CBS enzyme expression, estrogen in plasma, 5-MTFH, cystathionine, serine, glutathione, and many other 1-carbon metabolism parameters (Table 10).

7.3 Future Directions

As the majority of population in Canada consumes animal protein, most of them may be getting plenty of vitamin B6 through their diet. Still, 20.8% of Canadians have a lower B6 intake than the EAR of 1.1 mg/day. This can be better explained indicating that the population observes a moderate deficiency in B6 rather that a severe one. Hence, this study may only reflect those that are under a moderate B6 deficiency. In addition, factors such as the dated vitamin B6 intake survey conducted in Canada, the consumption of drugs like metformin for type 2 diabetes and the growing trend of veganism, as discussed in the paper's introduction, suggest a potential increase in vitamin deficiency in the coming years. Furthermore, the current study has shown the significance of studying both sexes due to the importance that hormones play in metabolism and how sex-specific differences result in variations. Nevertheless, the short-term dietary trial provides not more than a basic knowledge of the effects of a moderately vitamin B6 deficient diet and the impact it has on SAA that takes place in the transsulfuration pathway and their metabolism. A long-term study that uses the same model in addition to an inflammatory challenge might be required to strengthen data before moving on to clinical trials. Further research on the potential impacts of a moderate vitamin B6 deficiency under an inflammatory challenge is a promising step to see the effect on other metabolic pathways. Ultimately, a better understanding of the importance of adequate vitamin B6 under more harsh circumstances will open a broader scenario that will look to provide enough data to help the treatment and prevention of this vitamin deficiency in patients with inflammatory-related diseases.

7.4 Conclusion

Vitamin B6, in its active form as PLP, is an essential cofactor in numerous enzymes involved in metabolism and amino acid synthesis. Inadequate vitamin B6 intake impairs the effective functioning of those PLP-dependent enzymes, which is linked to the occurrence of numerous prevalent conditions such as diabetes, obesity, and cardiovascular and metabolic defects (Leklem, 1990). However, a meaningful proportion of the population suffers from moderate vitamin B6 deficiency in North America and Europe (Haller et al., 1991). Sulfur amino acids and vitamin B6 are both part of two major pathways, transsulfuration and 1-carbon metabolism, which includes methionine and its conversion to cysteine via homocysteine, the formation of folate, DNA repair, methylation of DNA, protein, and lipids, among others (Larsson et al., 2005). Vitamin B6 acts as a coenzyme in PLP form to important enzymes such as CBS and CGL which are critical to the formation of cysteine and glutathione which plays an important role to combat oxidative stress, free radicals, and tissue repair just to mention a few (Diotallevi et al., 2017). This study shows the importance of including both sexes in research, given that many results had a significant sex effect and recognizes that hormone levels are crucial as they may impact metabolism and responses in different ways. For instance, cysteine, methionine, CBS enzyme expression, and SAM were affected most likely by estrogen levels and sex, these parameters play important roles in the metabolism of sulfur amino acids and 1-carbon cycle, and the transsulfuration pathway. It also highlights the significance of an adequate vitamin B6 intake and its role in maintaining a well-working transsulfuration pathway and SAA metabolism. Although, the hypothesis could not be proven in its entirety since PLP in plasma was not analyzed, the trend observed in pyridoxine is headed in the right direction

towards a significant diet effect. In addition, SAA levels such as cysteine, and methionine were affected by sex differences as predicted. The objectives were accomplished by analyzing the hormones, sulfur amino acids, the CBS and CGL enzymes, metabolites of 1-carbon, and three B6 vitamers by obtaining a diverse of significant effects including sex and diet. However, further research including the analysis of the primary active form of vitamin B6 in plasma (PLP) in a long-term study is needed for a more precise result. The addition of an inflammatory challenge would give a better insight into how rats would respond to it and the impact of a vitamin B6 deficiency under this scenario.

Definitively, a moderate deficient intake of certain micronutrients can have impacts on body functions like the ones shown in the vitamin B6 moderate deficient state. Hence, it is important to keep studying the role of this nutrient because a well-functioning body is vital to endure viruses and diseases.

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