Assessing the Relationship Between Cobalamin Deficiency and Methylation Capacity in a Vegetarian Population

by

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ABSTRACT

According to a 2016 census, eight million adults conform to a vegetarian diet within the United States, and about 50% of these adults follow a vegan diet. The census determined that plant-based diets are quickly growing in popularity particularly in young adults between the ages of 18 to 34 years. Many Americans are aware of the health benefits of a plant-based diet, however, the dietary risks associated with these diets are not well emphasized. Health concerns such as vitamin deficiencies and altered metabolism are heightened in vegetarian populations.

One Particular nutrient that is commonly lacking in the vegetarian diet is vitamin B12. Vitamin B12 is found mainly in animal-derived food sources such as meat, poultry, fish, dairy, and eggs. Although some vegetarians, called lacto-ovo vegetarians, consume dairy and eggs, vegans do not consume any animal products at all. Vitamin B12 deficiency can have devastating consequences on the human body due to its role as a methylation cofactor. Metabolism, DNA replication, and cancer formation all involve methylation processes.

This cross-sectional, differential study aimed to further understand the relationship between vegetarianism, vitamin B12 status, and methylation capacity in healthy adults. A group of 34 healthy adults (18 vegetarians and 16 omnivores) was recruited to analyze serum B12, homocysteine, methylmalonic acid, serum total folate, and transcobalamin II status. It was hypothesized that (1) vegetarians would have a lower vitamin B12 status, and thus, a lower methylation capacity than omnivores and that (2) low vitamin B12 status would be correlated with low methylation capacity.

The data show that vegetarians did not have significantly lower vitamin B12 r methylation capacity status than omnivores. Nor was vitamin B12 status correlated with methylation capacity. However, the data revealed that diet quality had a positive influence on folate status. There was also a statistical trend ($p=0.08$) for homocysteine reduction in participants consuming high-quality diets. The data herein suggest that methylation capacity may be impacted by the quality of diet rather than the type of diet.

DEDICATION

This thesis is dedicated to my parents, Cynthia Ugarte and Robert Ugarte, Jr. Your endless outpouring of love, patience, and encouragement are appreciated more than you know. I love you.

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CHAPTER 1

INTRODUCTION

According to a census conducted in 2016, eight million adults conform to a vegetarian diet within the U.S. Of these eight million vegetarians, about four million adults follow a vegan diet. The census determined that plant-based diets are quickly growing in popularity within the U.S., particularly in young adults within the ages of 18 to 34 years.[1] Most American's have been made aware of the health benefits of a plant-based diet,[2] however, the dietary risks may not have been thoroughly understood. Possible concerns such as vitamin deficiencies and altered metabolism are always looming within the vegetarian population.

There are several nutrient deficiencies that may impact individuals who conform to a vegetarian diet. Cobalamin (B12), niacin (B2), vitamin D, calcium, selenium, and iron are all nutrients that are typically deficient in vegetarians.[3] Nutrient deficiencies can prove detrimental because they directly impact certain metabolic pathways that are essential for life. A critical nutrient that is commonly lacking in vegetarian diets is cobalamin. Cobalamin acts as a methylation cofactor in various biological reactions.[4,5,6] This cofactor role impacts many aspects of normal physiology, including food metabolism, neurological functions, and oxygen perfusion via the circulatory system.[4,7,8]

Numerous studies have assessed serum cobalamin status among the vegetarian population.[6,9,10,11,12,13] In recent years, researchers have explored the relationship between serum cobalamin status and plasma homocysteine levels within vegetarian individuals. One particular study determined that hyperhomocysteinemia is a consequence of low cobalamin status, and that vegetarians are more likely to experience

hyperhomocysteinemia due to their significantly lower cobalamin status compared to omnivores.[14] Another study concluded that vegetarians were more likely to experience B-vitamin deficiencies, and that cobalamin deficiency, specifically, impacted homocysteine levels in vegetarians.[3] These studies have been critical in linking cobalamin deficiency with hyperhomocysteinemia within the vegetarian diet. Elevated homocysteine levels within the blood is a risk factor for developing cardiovascular disease, neurodegenerative conditions, osteoporosis due to homocysteine's oxidative nature.[15] However, homocysteine is not the only biomarker for inhibited methylation. Methylmalonic acid and serum total folate are two additional biomarkers can indicate inhibited methylation capacity.

To date, the correlation between cobalamin deficiency and methylation capacity in the vegetarian population has yet to be explored. Homocysteine seems to be most researched, but other methylation biomarkers, such as methylmalonic acid and serum total folate, have been neglected in the literature. It is possible that the widespread cobalamin deficiency observed within the vegetarian population can lead to an overall decreased methylation capacity, which could have serious physiological consequences.

Purpose of the Study

This cross-sectional study examined the relationship between vitamin B12 status and methylation capacity in vegetarians and omnivores in Phoenix, Arizona. Serum homocysteine, methylmalonic acid, and serum total folate were used to assess total methylation capacity in both diet groups.

Hypotheses

H1: Vegetarians will have significantly lower serum cobalamin levels and lower methylation capacity compared to omnivores.

H2: There is a direct correlation between low serum cobalamin levels and low methylation capacity.

Definition of Terms

- Vegetarian – a plant-based, alternative diet that excludes flesh foods. Vegetarianism is a broad term that encompasses a wide spectrum of plant-based alternative diets including, but not limited to, lacto-ovo vegetarianism and veganism.
    - Lacto-Ovo Vegetarian – a plant-based, alternative diet that excludes flesh foods, but includes milk and egg products.
    - Vegan – a plant-based, alternative diet that excludes flesh foods, as well as all other animal bi-products.
- Omnivore – a common Western diet that includes flesh foods, plant products, and animal bi-products on most days.
- Dietary Supplement – a substance taken by mouth to increase dietary intake of vitamins, minerals, amino acids, or botanicals.
- Methylation – a biochemical reaction that involves the donation of a methyl group from one molecule to another. The molecule receiving the methyl group is referred to as the methylated molecule. The molecule donating the methyl group is referred to as the methyl donor.

- Methylation Capacity (MC) – in this study, methylation capacity was measured by determining the serum concentrations of three methylation biomarkers: homocysteine, methylmalonic acid and folate.
- Homocysteine (Hcy) – the unmethylated derivative of S-adenosylhomocysteine in the Methionine Cycle
- Methylmalonic Acid (MMA) – a methylated derivative of malonate. It is a key output of the Tricarboxylic Acid Cycle (TCA)
- Serum Total Folate – the measure of biologically active folate forms within the blood. This includes 5-methyltetrahydrofolate, unmetabolized folic acid, tetrahydrofolate, 5-formyltetrahydrofolate, 5,10-methenyltetrahydrofolate.
- Transcobalamin II – the active transport protein for cobalamin.
- Hyperhomocysteinemia – elevated homocysteine levels within the blood; defined as equal to or greater than 15 µmol/L.

Limitations

- Using a convenience sample
- Cross-sectional study – hence causality cannot be examined
- Self-reported frequency of consumption of B12 and folic acid fortified foods
- Self-reported frequency, brand, and/or dose of dietary supplements
- Small sample size
- Healthy college student population

Delimitations

- Lacto-ovo vegetarians, vegans, and omnivores for at least 6 months

- Aged 18 – 65 years
- Non-smokers
- No known medications that inhibit methylation processes
- No known metabolism polymorphisms
- Phoenix residents

CHAPTER 2

REVIEW OF LITERATURE

Defining the Vegetarian Diet

Vegetarianism is a broad term used to describe various diets that exclude animal products. The level of exclusion varies between the different subsets of vegetarianism. The subsets are most commonly broken into five categories: vegan, lacto-vegetarian, lacto-ovo vegetarian, pesco-vegetarian, and semi-vegetarian (sometimes referred to as flexitarian).

Definitions of each category will vary because there are several assessment methods. Adherence to a vegetarian diet category is most often assessed through standardized food frequency questionnaires. A commonly utilized questionnaire is the food frequency questionnaire that was used in the Adventist Health Study-2 (AHS-2).[16] Although the questionnaire excluded lacto-vegetarians, and allowed for some consumption of flesh foods, it imposed a 12-month time limit on the frequency of food consumed. The AHS-2 questionnaire defined vegans as individuals who did not consume any animal products and reported eating red meat, poultry, fish, eggs, milk, or dairy for less than once per month in the previous 12-month period. Lacto-ovo vegetarians were defined as individuals who consumed diary and eggs but reported eating meat, poultry, or fish less than once per month in the previous 12-month period. Pesco-vegetarians were defined as individuals who consumed dairy, eggs, and fish more than once per month but reported eating meat and poultry less than once per month in the previous 12-month period. Semi-vegetarians were defined as individuals who consumed dairy, eggs, fish, meat, and poultry more than once per month, but never more than once per week. The

AHS-2 food frequency questionnaire defined omnivores as individuals who consumed dairy, eggs, fish, meat, and poultry more than once per week.[16]

Another example of a vegetarian food frequency questionnaire is the questionnaire utilized within the National Family Health Survey conducted in India (NFHS-3).[17] The NFHS-3 is a government-ran health census that has generated reliable data about India's population. Unlike the AHS-2 food frequency questionnaire, the NFHS-3 food frequency questionnaire included all categories of vegetarianism. The questionnaire provided a standardized list of foods: milk/curd, pulses/beans, dark green leafy vegetables, fruits, eggs, fish, chicken/eggs, and meat. Individuals were asked to indicate how often they consumed each of these foods: daily, weekly, occasionally, or never. Based on the data gathered, individuals were placed into different diet categories. Vegans were defined as individuals who never consumed animal products. Lacto-vegetarians were defined as individuals who did not consume fish, chicken/eggs, nor meat. Lacto-ovo vegetarians were defined as individuals who did not consume fish, chicken, nor meat. Pesco-vegetarians were defined as individuals who did not consume chicken nor meat. Semi-vegetarians were defined as individuals who consumed fish and poultry less than one time per week. The NFHS-3 food frequency questionnaire defined non-vegetarians (omnivores) as individuals who consumed all foods listed.[17]

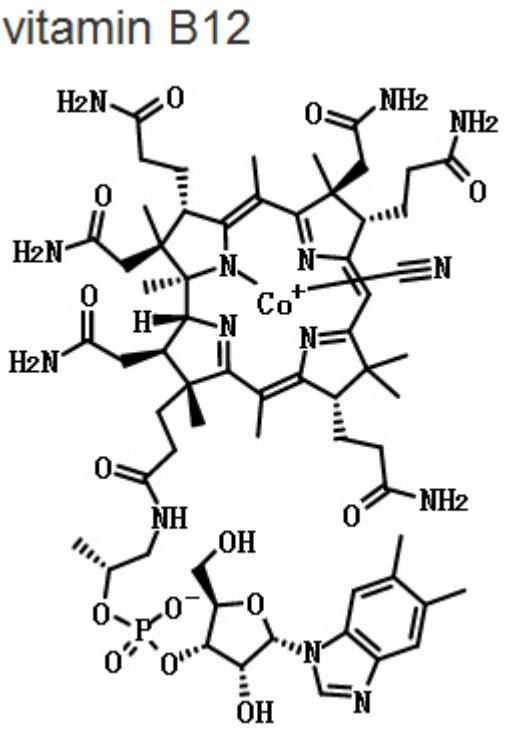
Although definitions of each vegetarian category are usually similar, some definitions have not yet been agreed upon. Semi-vegetarian, or flexitarian, is a category that most often has various definitions between popular vegetarian food frequency questionnaires. Because no standard vegetarian food frequency questionnaire has been published, studies that analyze and compare the various vegetarian categories are faced

with the responsibility to utilize a food frequency questionnaire that has been validated and tested.

Vegetarianism and Cobalamin Status

Cobalamin, most frequently known as vitamin B12, is a water-soluble B vitamin that is a part of the corrinoid compound group. Its chemical structure is comprised of one corrin macrocycle and various organic configurations (see Figure 1). The specific configuration determines what type of cobalamin it is. 5'-Deoxyadenosylcobalamin, methylcobalamin, hydroxocobalamin, and cyanocobalamin are all known configurations of cobalamin. Each configuration plays a unique enzymatic role for a unique enzyme.[10]

Figure 1. The chemical structure of cobalamin.



Cobalamin is essential to the human body. The American Dietary Reference Intakes (DRI) states that healthy adult individuals should consume 2.4 micrograms of cobalamin per day.[18] The bioavailability within meat, poultry, fish, and dairy are above 50%.[9] This means that the DRI is often met by the majority of Americans due to the traditional Western diet. However, eggs and plant-based foods have extremely poor bioavailability. For example, the cobalamin in an egg has less than 9% bioavailability. According to a review article published in 2014, dried purple laver (nori, algae), is the best source of plant-based cobalamin. The review also investigates the amount of cobalamin in other plant-based foods (see Table 1). Ultimately, the data show that plant foods do not provide enough cobalamin to meet the DRI in a normal diet.[10] This is because the presence of cobalamin on plant foods is often related to bacterial contamination. Bacteria which produce cobalamin can be found on plant surfaces, as well as within vegetation in some occasions. For this reason, plant-derived cobalamin levels are highly inconsistent and should not be seen as a reliable source.[19]

Table 1. Plant based foods and their cobalamin content (µg/100 g).[10]

Soybean	Undetectable
Tempeh (fermented soybean)	0.7 – 0.8
Tea leaves	0.1 – 1.2
Mushroom (black trumpet, golden chanterelle)	1.09 – 2.65
Algae (dried purple laver)	32.3

Because animal-derived cobalamin is the most reliable food source, populations that exclude animal foods from their diets face an increased risk of cobalamin deficiency. A review of cobalamin status and the vegetarian population was published recently. In this review, the researchers analyzed several aspects of this topic from eight different countries: Austria, Italy, USA, India, Turkey, Germany, The Netherlands, and Slovakia. The data show that vegetarians from each of these countries expressed mild to severe cobalamin deficiency. The researchers also assessed the validity of several cobalamin markers. It was determined that holotranscobalamin II (HTCII), methylmalonic acid (MMA), and homocysteine (Hcy) levels were acceptable methods to assess cobalamin status in humans. However, it was noted that different cut-off levels for each test can be found in different countries (see Table 2).[6] The lack of a universal definition of cobalamin deficiency is primarily due to the lack of declaration by government agencies and national surveys. The European Food Safety Authority is one example of an entity who has clearly defined what constitutes as a cobalamin deficiency: a serum cobalamin level of less than 140 pmol/L, a MMA serum level more than 750 nmol/L, and a Hcy serum level more than 15 µmol/L.[6]

Table 2. Lab value criteria for cobalamin deficiency by country.[6]

Country	B12 (pmol/L)	Hcy (µmol/L)	MMA (nmol/L)
USA	<150	Not reported	>376
India	<150	>15	>260
The Netherlands	<156	>12	>271
Germany	<250	>15	>271

Austria	<110	>12	Not reported
Turkey	<150	>15	Not reported

This table lists some reference ranges that have been defined in studies conducted in various countries.

Many studies have been conducted to analyze the relationship between the vegetarian diet and cobalamin status. In 2003, a team of researchers gathered 174 healthy individuals from Germany and The Netherlands who identified as lacto-vegetarian or lacto-ovo vegetarian (n=66), vegan (n=29), and omnivorous (n=79). Fasting blood was extracted from each participant, and several biological tests were performed. Among them, the researchers analyzed HTCII, MMA, and Hcy. The data showed that 73% of the vegetarians and 90% of the vegans expressed low HTCII, compared to 11% of the omnivores. Sixty-one percent of the lacto-vegetarians and lacto-ovo vegetarians and 86% of the vegans expressed high MMA, compared to 5% of the omnivores. Thirty-three percent of the lacto-vegetarians and lacto-ovo vegetarians and 55% of the vegans expressed high Hcy, compared to 16% of the omnivores.[11] These data demonstrate a significant association between vegetarianism and cobalamin deficiency. However, this is not the only correlational study that determined this association. Krajcovicova-Kudlackova et al. determined that there was a significant association between elevated Hcy levels and vegetarians when compared to omnivores from Slovakia.[14] Majchrzak, et al. also analyzed the relationship between Hcy levels among vegetarians compared to omnivores from Austria. The data from this study showed that vegans had significantly lower levels of cobalamin when compared to omnivores. Lacto-ovo vegetarians demonstrated lower cobalamin levels when compared to omnivores, although not

significantly lower.[3] The absence of significance in this particular study can be attributed to the consumption of fortified plant-based foods such as soy milk and wheat products. Although these particular studies did not analyze HTCII nor MMA, Hcy has been recognized as a reliable and valid method to estimate cobalamin status in humans. In fact, many studies that have assessed the relationship between cobalamin status and vegetarianism have used Hcy status as the sole indicator of cobalamin status.[20]

These correlational studies have provided enough data to generate interest in conducting scientific interventions. Unfortunately, interventions to improve cobalamin status in the vegetarian population are not plentiful within the scientific literature. There are two main approaches that are used within these intervention studies: supplementation or the addition of animal-derived foods. Because most individuals are reluctant to alter their plant-based diets, supplementation interventions are most common within the literature. One randomized, double-blind, cross-over trial tested the effects of cyanocobalamin supplementation (500 µg/day) on 50 healthy, vegetarian individuals. The data showed that the supplementation intervention significantly increased serum cobalamin levels and significantly decreased Hcy levels. In addition to this, supplementation also significantly increased flow mediated dilation of the brachial artery.[13] There are also intervention studies that target diet modification (the addition of animal-derived foods into diets) that have yielded positive results. Naik, et al. assessed the effects of increased milk consumption in 51 vegetarians who consumed less than 2 cups of cow's milk per day. The participants were divided into two groups based on their cobalamin status: normal versus deficient. The deficient participants were instructed to consume 200 ml of milk twice per day. At the end of the intervention, the data showed

that increased milk consumption resulted in a significant increase in serum cobalamin status.[12]

Cobalamin status in the vegetarian population has been the subject of many studies. To date, there is a general consensus that vegetarianism is associated with cobalamin deficiency. This deficiency is due, mainly, to the exclusion of animal foods and the lack of supplementation within the population.[6] Despite the acquired knowledge of this problem, very little interventional studies have been conducted to correct this phenomenon.

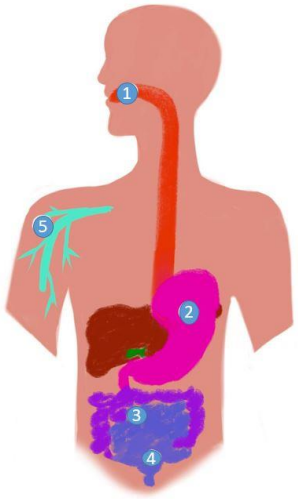
Molecular Roles of Cobalamin

Cobalamin is most frequently tightly bound to proteins within our food. The process of absorption first begins within the mouth (see Figure 2). When food is consumed, the oral cavity secretes copious amounts of saliva. The saliva contains specialized proteins that form additional bonds to the cobalamin-protein complex in order to protect the vitamin from the acidic environment within the stomach. Meanwhile, parietal cells within the stomach secrete hydrochloric acid. The secretion of hydrochloric acid within the stomach triggers the production of intrinsic factor (inactive). When the bolus is swallowed, the proteins bound to cobalamin denature and break away slowly. The freed cobalamin binds to a protein called transcobalamin I in order to avoid damage from the hydrochloric acid. As gastric emptying occurs, intrinsic factor and the cobalamin-transcobalamin I complex (holotranscobalamin I or HTCI) enter the duodenum. Several critical events occur within the duodenum. The first is that transcobalamin I gets denatured by pancreatic peptidases, which frees cobalamin. The second is that the higher pH level within the duodenum activates intrinsic factor, and it

binds to the freed cobalamin. In the ilium, the cobalamin-intrinsic factor complex gets absorbed into the cells through a specific receptor complex called cubilin-amnionless receptor.[21] Intrinsic factor is then broken down and the freed cobalamin enters the bloodstream. In the blood stream, 70% of cobalamin binds to transcobalamin I and is transported to the liver. The holotranscobalamin I complex is considered to be the inactive form of cobalamin because it is unable to enter the vast majority of cells, except hepatocytes, while bound to transcobalamin I.[4] Although the majority of circulating cobalamin is bound to the inactive transcobalamin I, the biological function of holotranscobalamin I is unclear.[22] Thirty percent of cobalamin binds to transcobalamin II to form holotranscobalamin II, the active form of cobalamin, and is transported into various cells within the body.[4]

The process of holotranscobalamin II absorption has been a topic of much scrutiny. The active complex is transported into cells via calcium-dependent receptor-mediated endocytosis. The specific receptor that recognizes holotranscobalamin II is a low-density lipoprotein receptor called CD320. This receptor has been found to have an unusually high specificity and affinity for the holotranscobalamin II complex. In fact, the receptor is so specific, it does not recognize holotranscobalamin I. This lack of receptor recognition is the reason holotranscobalamin I has been deemed the inactive form.[23]

Figure 2. Cobalamin absorption.



(1) Oral cavity: salivary proteins bind to cobalamin (Cbl)-protein complex in oral cavity. **(2) Stomach:** Cbl binds to transcobalamin I (TCI) and intrinsic factor (IF) is produced. **(3) Duodenum:** TCI is denatured; IF is activated and binds to Cbl. **(4) Ilium**: Cbl-IF complex is absorbed into cells, where IF is destroyed. **(5) Bloodstream**: 70% of Cbl binds to TCI; 30% binds to TCII. Original illustration by Robert Ugarte.

Once transported into the cells, cobalamin is chemically converted into different subsets. The two most common forms of cobalamin within the cells are methylcobalamin and 5'-Deoxyadenosylcobalamin. These two forms play critical cofactor roles within the human body. Methylcobalamin aids in the methylation of homocysteine to methionine and the demethylation of S-adenosylmethionine to S-adenosylhomocysteine in the methionine cycle. 5'-Deoxyadenosylcobalamin aids in the methylation of L-methylmalonyl coenzyme A to succinyl coenzyme A in the TCA cycle.[4]

These specialized coenzyme roles play a critical role in several bodily systems, most notably, the nervous and circulatory system. There is emerging evidence to suggest that deficiency of cobalamin can negatively impact the nervous system, including cognition.[24,25] A review of cobalamin deficiency and the neurological effects was recently

published. In this review, it is stated that the most common side effect of cobalamin deficiency within humans is neurological degeneration. This can manifest in several ways; however, the most common manifestation is through myelopathy. Myelopathy is a chronic disease related to the spinal cord which often does not express signs or symptoms. Optic neuropathy, paresthesia, and neuropsychiatric disorders may also occur in individuals deficient in cobalamin. Unfortunately, research in neuropsychiatric disorders related to cobalamin deficiency are scarce. Some neuropsychiatric disorders that are thought to manifest during chronic cobalamin deficiency include memory loss, psychosis, and in extreme cases, delirium and coma.[7]

Cobalamin deficiency can also lead to complications within the circulatory system, including problems specific to red blood cell production. Because cobalamin is an essential cofactor for DNA production and methylation, red blood cells are directly impacted by this vitamin deficiency.[4,8]

Vegetarianism and Folate Status

Folate is another essential B vitamin that plays various critical roles within the human body. Much like cobalamin, there are several molecular forms of folate that allow the vitamin to carry out numerous functions. The active forms are 5-methyltetrahydrofolate, unmetabolized folic acid, tetrahydrofolate, 5-formyltetrahydrofolate, and 5,10-methenyltetrahydrofolate. There is also the inactive form 5-methyltetrahydrofolate oxidation product, sometimes abbreviated as MeFox.[26] It should be noted that the in vivo occurrence of MeFox is highly debated, and it is still unknown if MeFox formation only occurs after blood sample draws, in vitro.[26] Thus,

MeFox is often not included in serum total folate assessment. Only the established active forms are typically counted in this laboratory assessment.

All forms of folate have the same chemical base structure comprised of a pteridine ring, p-aminobenzoic acid, and one or more glutamate residues. These components, when bound together, are referred to as tetrahydrofolate. There are also various carbon units that can be attached to either the N5, N10, or both positions.[27] Thus, folate molecules are named based on where the carbon group is attached. For example, 5,10-methenyltetrahydrofolate denotes that the carbon unit (methenyl) is attached to the base structure at the N5 and N10 positions. The glutamine residue in the base structure is what differentiates folate from folic acid. Folate contains polyglutamate, which must be hydrolyzed prior to being absorbed by the human body. Folic acid contains monoglutamate, which can be readily absorbed.[27]

Folate intake guidelines have been established in various countries around the world. The United States has set the Recommended Dietary Allowance (RDA) for folate at 300 µg per day for male adults and women past childbearing age. The RDA for women of childbearing age is 400 µg per day.[28] Unfortunately, gauging how much folate is consumed may be a difficult task. Bioavailability of folic acid is estimated to be about 50%. However, bioavailability of folate can range from 30-98%.[28] This high variability has lead the intake of folate and folic acid to be quantified in dietary folate equivalents (DFEs).[28] DFEs were introduced as the standard measure for the RDA in the United States in 1998. One µg DFE is equivalent to 1 µg of folate, 0.6 µg of food fortified folic acid, or 0.5 µg supplemental folic acid taken without food.[29] This helps standardize the variable bioavailability between folate and folic acid for better estimation of intake.

Folate can be found naturally occurring in food sources. Fruits, specifically citrus varieties, vegetables, specifically green leafy varieties, legumes, pulses, whole grains, and liver have been established as foods rich in naturally occurring folate.[28,30,31] Folic acid is mainly found in fortified food sources, although it can be naturally occurring in trace amounts.[27] In 1997, the United States government made it mandatory for cereal grains to be enriched with folic acid in order to fight birth defects associated with low total serum folate levels in women of childbearing age. One hundred and forty μg of folic acid must be fortified per 100 grams of grains.[32] According to the Food Fortification Initiative Network, the United States has since deemed folic acid fortification mandatory for wheat, maize, and rice products.[33]

The 2011-2012 National Health and Nutrition Examination Survey (NHANES) was one of the first national surveys to investigate the serum total folate status in a representative sample of the United States population. The data show that the serum total folate in the country ranged from 3.26 to 375 nmol/L. The researchers noted that these levels did not greatly vary from previous NHANES, though the sample of these previous publications were not representative of the whole national population.[26] This wide range indicates that folate status in the United States, despite fortification efforts, is still highly variable.

Unfortunately, there are not many studies that examine the relationship between serum total folate status and vegetarianism. One Swedish cross-sectional study assessed folate status in a nationally representative sample of 1,657 adults. Sweden is one of the few countries that does not have government mandated folate fortification, thus folate status in participants was almost completely dependent of natural food source

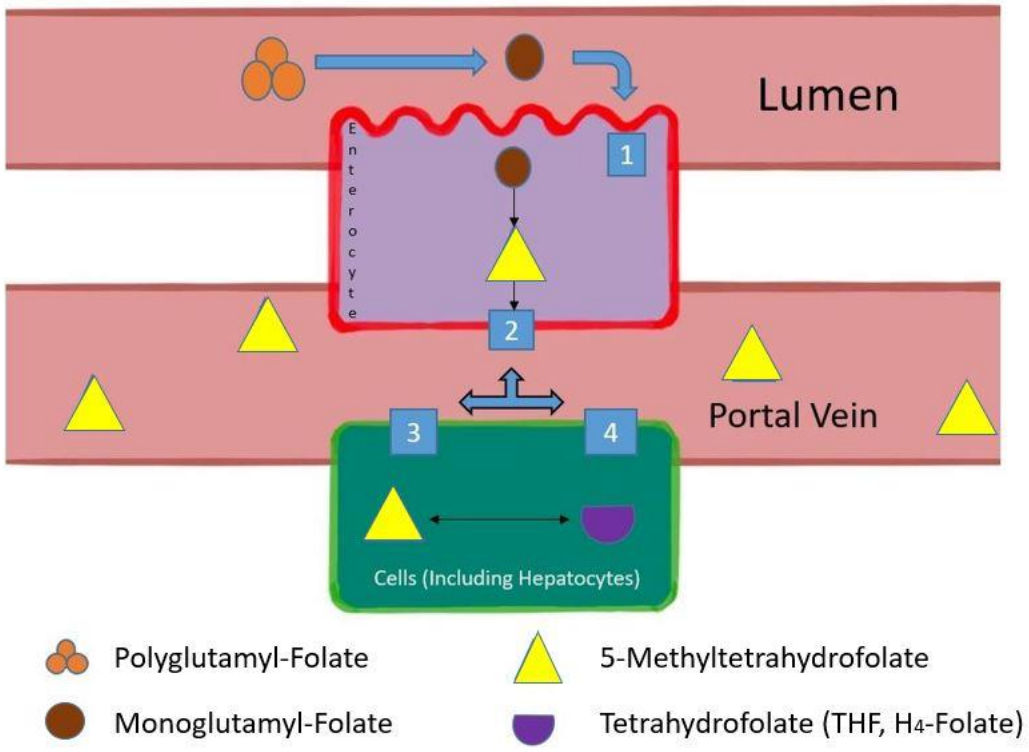
consumption. One of the lifestyle factors assessed within the study was the vegetarian diet. The data show that the vegetarian diet positively correlated with elevated folate status. Other factors that were found to be positively correlated with higher folate status were older age, male gender, and higher education level.[31] These data are not surprising when the food sources of folate are considered. As stated above, fruits, vegetables, legumes and pulses are all high in naturally occurring folate. Unlike vitamin B12, which is found mainly in animal products, folate-rich foods are not excluded in vegetarian diets. However, more research is needed in the literature base examining the relationship between folate status and the vegetarian diet.

Molecular Roles of Folate

Folate absorption is a well-explored process (see Figure 3). As previously described, folate is chemically comprised of a polyglutamate residue.[27] In the brush border of the jejunum, the polyglutamate must first be hydrolyzed by glutamate carboxypeptidase II in order to be small enough to absorb. The newly hydrolyzed monoglutamyl-folate is then transported into the jejunal enterocyte via a proton-coupled folate transporter. Within the enterocyte, the monoglutamyl-folate is methylated and converted into 5-methyltetrahydrofolate, the most dominant form of folate within the human body.[26,27] 5-Methyltetrahydrofolate is then transported into the portal vein and subsequently the hepatocytes. The liver contains about half of the absorbed folate.[28] However, 5-methyltetrahydrofolate is also disbursed into other cells within the body. Once in the cell, 5-methyltetrahydrofolate can undergo polyglutamylation, and be converted into tetrahydrofolate-polyglutamate (H_4-Folate, otherwise known as tetrahydrofolate). This pathway can be reversed to reproduce 5-methyltetrahydrofolate

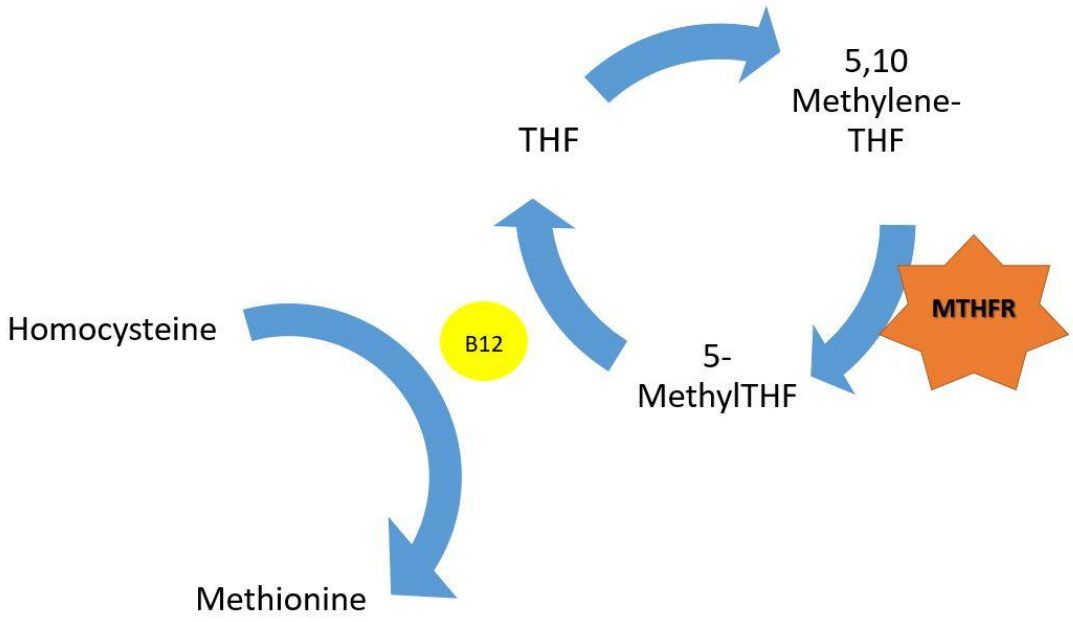
via the remethylation pathway (see Figure 4).[34] In this particular pathway, tetrahydrofolate is converted into 5,10-methylenetetrahydrofolate and then remethylated via the methylenetetrahydrofolate reductase (MTHFR) enzyme to form 5-methyltetrahydrofolate.[34] This interconversion process between the methylated 5-methyltetrahydrofoalte and demethylated tetrahydrofolate is what fuels folate's methylation cofactor abilities.

Figure 3. Folate absorption and transport proteins.



(1) Protein-coupled folate transporter. **(2)** Multi-drug resistance-associated protein. **(3)** Reduced folate carrier. **(4)** Folate receptors. Original illustration by Robert Ugarte.

Figure 4. Folate remethylation pathway.



5-Methyltetrahydrofolate demonstrates these essential cofactor roles within the body's cells. This substance is an essential cofactor required specifically in the methylation cycle and nucleotide synthesis.[28] In the methylation cycle, 5-methyltetrahydrofolate donates its methyl group to cobalamin to create methylcobalamin. Methylcobalamin in turn donates its methyl group to homocysteine to produce methionine.[28,35,36] The byproducts of this chain reaction are demethylated tetrahydrofolate and demethylated cobalamin, which can both be utilized to create nucleotides and repair DNA.[36]

The interdependent relationship and similar molecular roles between cobalamin and folate can cause a series of inter-related complications: the folate trap, alternate folic acid metabolism, and B12 masking. The folate trap is a metabolic roadblock that occurs

when a person experiences cobalamin deficiency.[36] In the methionine cycle, 5-methyltetrahydrofolate is unable to donate its methyl group to cobalamin because there are insufficient amounts of cobalamin within the cells. This ultimately results in a deficiency of demethylated tetrahydrofolate production. Not only does this trap lead to an elevation of homocysteine, it also halts the nucleotide synthesis and DNA repair that would be performed by demethylated tetrahydrofolate and demethylated cobalamin.[36,37] The chronic absence of nucleotide synthesis often leads to megaloblastic anemia because red blood cells cannot be properly synthesized.[37] However, folic acid obtained from fortified foods or supplements is able to convert itself into tetrahydrofolate without donating a methyl group to cobalamin. This alternate folic acid pathway allows for the production of tetrahydrofolate and subsequent nucleotide synthesis which ultimately remedies megaloblastic anemia.[36] Although this may seem advantageous, this alternate metabolic pathway can simply mask a cobalamin deficiency by resolving the anemia.[36,37] Thus, it is general good practice to identify the correct cause of megaloblastic anemia (folate versus cobalamin deficiency) in order for the correct supplement to be prescribed.[37] Hyperhomocysteinemia also requires proper identification of folate versus cobalamin deficiency due to their highly interdependent role within the methionine cycle.

Aside from its importance in the development of megaloblastic anemia, folate plays its greatest role in rapid cell division. Methylation is an essential process needed for DNA synthesis to occur. The ability to facilitate DNA synthesis is what makes folate essential for proliferating cells.[38] One of the most demanding times for cell proliferation is during pregnancy. It has long been known that inadequate folate status is tied to increased rates of neural tube defects such as anencephaly and spina bifida.[32] Research

shows that these birth defects are a direct consequence of inadequate cell proliferation due to insufficient DNA synthesis.[38] In short, the methylation function of folate is needed for the production of DNA, which in turn allows for quicker cell production.

Methylation Mechanisms

There are two main methylation pathways that are dependent on cobalamin as a cofactor: the methionine cycle and the tricarboxylic acid (TCA) cycle. The methionine cycle is a pathway that occurs within the cytosol of various cells within our body (see Figure 5a). The products of the methionine cycle are critical for the production of methionine, polyamine synthesis, and methyl-group transfers essential for DNA methylation.[39] There are two keys steps where cobalamin is required in the methionine cycle. Methylcobalamin, along with 5-methyltetrahydrofolate, are the cofactors for the enzyme methionine synthase. This enzyme is responsible for adding a methyl group to homocysteine in order to create methionine. The production of methionine is essential for the production of cysteine, an amino acid, and DNA methylation. The second place where methylcobalamin is required is during the production of S-adenosylhomocysteine. S-adenosylmethionine is a methylated structure that undergoes demethylation via several S-adenosylmethionine-dependent methyltransferases to create S-adenosylhomocysteine. These two metabolites act as regulators for the methionine cycle.[39] When methylcobalamin or 5-methyltetrahydrofolate are deficient, the methionine cycle is halted at several points. This pathway dysfunction can ultimately impact protein synthesis and DNA methylation throughout the body.[40]

The second pathway that is dependent on cobalamin is the TCA cycle, usually referred to as the TCA cycle (see Figure 5b). 5'-Deoxyadenosylcobalamin is the key

cofactor that aids methylmalonyl coenzyme A mutase in the methylation of L-methylmalonyl coenzyme A into succinyl coenzyme A. The reverse reaction also occurs with the help of 5'-deoxyadenosylcobalamin and methylmalonyl coenzyme A mutase. The production of methylmalonic acid (MMA) is due to two main reasons: (1) the genetic mutation of methylmalonyl coenzyme A mutase or (2) the absence of 5'-deoxyadenosylcobalamin.[41] When either of these occur, the pathway cannot continue and L-methylmalonyl coenzyme A is converted into MMA. Since the TCA cycle is a critical step in the production of ATP, along with other metabolites, interruption of this cycle can negatively impact metabolism as a whole.

Figure 5a. The methionine cycle.

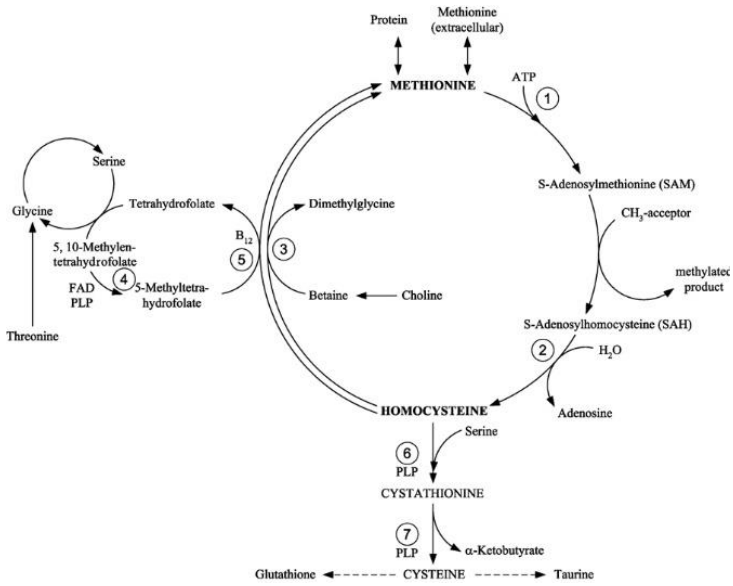
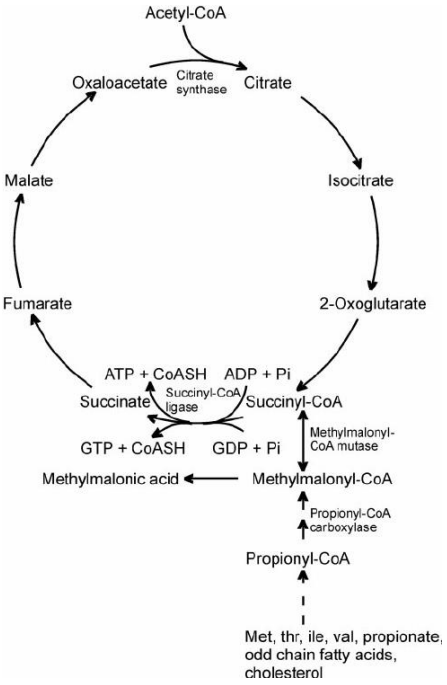


Figure 5b. The tricarboxylic acid cycle.



The Importance of Methylation for Health

Because the methionine and TCA cycles are critical in the regulation of numerous biological functions, interruption of either of these cycles can have detrimental effects on human health. Cancer, cardiovascular disease, and altered cognition are common health complications that develop when methylation capacity diminishes.[42,43,44]

It is well established within the literature that hypomethylation plays a critical role in oncogenesis.[42] Because folate also plays a role in the methionine cycle, some studies have analyzed the relationship between folate deficiency, hypomethylation, and the development of cancerous cells. Although these studies do not focus on cobalamin

deficiency, the data still show that hypomethylation, a result shared between folate and cobalamin deficiencies, causes an imbalance between S-adenosylmethionine and S-adenosylhomocysteine, which is associated with cancer development.[45] This is not to say that data on cobalamin deficiency and oncogenesis have not been analyzed. Piyathilake, et al. performed an in vitro investigation that analyzed the effects of various methylation treatments on blood samples of 75 HPV16 positive women who expressed abnormal cervical cells. The data showed that increased methylation resulted in a 79% lower chance of being diagnosed with cervical intraepithelial neoplasia.[46] Several years later, Piyathilake, et al. analyzed the relationship between folate and cobalamin status and the development of cervical intraepithelial neoplasia. The team recruited 315 HPV 16 positive women who had abnormal cervical cells and determined plasma concentrations of folate and cobalamin in each participant. The data showed that higher folate and cobalamin status were associated with higher methylation and lower risk of developing cervical intraepithelial neoplasia.[47] Although the majority of studies analyze the relationship between hypomethylation and oncogenesis, it should be noted that hypermethylation is also associated with oncogenesis.[48]

The relationship between hypomethylation and cardiovascular disease is well explored within the literature. DNA methylation is speculated to play a critical role in the development of cardiovascular disease within humans.[43] DNA methylation is a method used in vivo to mark certain genes within the genome and signal whether the gene should be activated or silenced. Because S-adenosylmethionine donates its methyl group to DNA, alterations within the methionine cycle due to cobalamin deficiency can indirectly impact gene expression. Altered DNA expression may alter vascular smooth muscle

cells, inflammation pathways, and lipid metabolism. All of these factors may lead to the development of cardiovascular disease.[49] Hyperhomocysteinemia, elevated serum homocysteine (Hcy) levels, is also linked with a high incidence of atherosclerosis. As discussed previously, cobalamin deficiency results in the buildup of homocysteine. Elevated levels of homocysteine within the blood can damage the smooth muscles of blood vessels over time. As homocysteine circulates, it begins to bind to lipoproteins. These complexes stick to the vessel wall and begin to form plaques. Over time, these fatty plaques damage the smooth muscle cells within the blood vessels, which results in the development of atherosclerosis.[50] Atherosclerosis is also known to increase stroke risk.[50] In 2016, a cross-sectional study was conducted to assess the relationships between metabolic syndrome, atherosclerosis, and serum homocysteine levels. The study recruited 76 overweight and obese individuals. These individuals were divided into two groups: a metabolic syndrome group, and a group without metabolic syndrome. In addition to homocysteine analysis, the researchers gathered data on blood pressure, cholesterol, triglycerides, apoproteins, fasting insulin, hemoglobin A1c, c-reactive protein, microalbuminuria, and uric acid. The data showed that individuals in the metabolic syndrome group had significantly higher serum homocysteine levels than the group without metabolic syndrome. The data also showed that individuals with elevated serum homocysteine expressed increased risk of developing atherosclerosis.[51] Despite the findings of this study and other similar findings, recent census data show that hyperhomocysteinemia has a weak correlation with dietary patterns and the development of carotid intima-media thickness, which is a parameter associated with atherosclerosis.[52]

The mixed results within the literature suggest that more research is needed to better understand the relationship between hyperhomocysteinemia and atherosclerosis.

Another area of study that appears to need more research involves the relationship between hypomethylation and impaired cognition. The Barbados Nutrition Study (BNS) was a cross-sectional study that analyzed the relationship between protein energy malnutrition in children, DNA methylation, and their cognitive development 48 years later. The data showed that malnutrition that occurs during the early developmental stages of life has a significant impact on DNA methylation. Hypomethylation and hypermethylation were both detected within gene promoters, the gene body, and intergenic regions. The data also showed that incidences of attention deficits increased in children who experienced protein energy malnutrition in early stages of life.[44] These findings suggest that protein energy malnutrition may lead to alterations in DNA methylation processes, which may directly impact cognitive development within humans. This cross-sectional study provides very interesting information, unfortunately, similar studies are lacking within the scientific literature.

Mortality in the Vegetarian Population

It is commonly stated that vegetarianism is an alternative diet that can lead to numerous health benefits. A plethora of studies that have been conducted on the health implications of vegetarianism show that there are many positive health outcomes associated with vegetarianism. Vegetarians have lower rates of obesity, diabetes, ischemic heart disease, and diverticular disease. However, atherosclerosis, stroke, bone fracture risk, and cancer risk do not have lower incident rates in the vegetarian population.[53]

These finding suggest that, although vegetarianism can elicit many health benefits, there may also be health concerns associated with this alternative diet. A recent review article analyzed mortality incidence in vegetarians and non-vegetarians. The data collected were corrected for smoking, alcohol consumption, physical activity, marital status, nutritional supplementation, and BMI. The data were then adjusted so that omnivores expressed a hazard ratio (HR) of 1.0 for each cause of mortality (see Table 3). It was determined that colorectal disease, lung disease, female breast cancer, mental and behavioral disease, circulatory disease, and cerebrovascular disease all had higher mortality incidence in vegetarians compared to omnivores.[54]

Table 3. Mortality causes and rates in omnivores compared to vegetarians.[54]

Cause of Mortality	Omnivore HR	Vegetarian HR
Colorectum diseases	1.00	1.11
Lung disease	1.00	1.07
Female breast cancer	1.00	1.12
Mental and behavioral diseases	1.00	1.12
Circulatory diseases	1.00	1.13
Cerebrovascular diseases	1.00	1.19

Based on the mechanisms discussed previously, one can speculate that the lower cobalamin status in the vegetarian population has negatively impacted the methionine and TCA cycle, which has altered DNA methylation. Many studies have already established

that hypomethylation is associated with oncogenesis, cardiovascular diseases, and cognition – many of the same diseases that have been shown to cause increased mortality in the vegetarian population.

The Effects of Dietary Supplementation on Methylation

As discussed above, both cobalamin and folate can be obtained through dietary means. However, for individuals who either do not consume enough animal products to obtain sufficient cobalamin, or do not consume enough produce to obtain sufficient folate, dietary supplementation may be necessary. Dietary supplements are defined as substances taken by mouth to increase dietary intake of vitamins, minerals, amino acids, or botanicals.[55] Often, these supplements can be manufactured to contain multiple vitamins or minerals, termed multivitamin or multimineral supplements.

The most common form of cobalamin found in supplements is cyanocobalamin. This inactive form is both cost effective and easy to produce, but it must be converted into methylcobalamin in order to benefit the consumer.[6] The simple decyanation and reduction process that converts cyanocobalamin to methylcobalamin occurs intracellularly in most mitochondrial-containing cells with NADPH, including hepatocytes, via the methylmalonic aciduria and homocystinuria type C protein (MMACHC).[56] As of yet, there is limited research on whether vegetarianism affects this conversion process. This is not to say that cobalamin supplementation cannot prove beneficial to vegetarian populations. In fact, there have been clinical trials showing the effectiveness of cobalamin supplementation recently. A randomized placebo-controlled study assessed the effects of cobalamin supplementation (500 µg methylcobalamin) on serum cobalamin and homocysteine levels in non-pregnant, vegetarian women in India.

The women that received supplements (n=20) for the duration of the 6-week study were found to have elevated serum cobalamin levels and reduced serum homocysteine levels.[57] These findings suggest that supplementation is an effective method to correct low serum cobalamin levels within the vegetarian population. However, it is important to remember that dosage plays a critical role in supplement effectiveness. In a recent randomized controlled trial, researchers found that several small doses of cyanocobalamin (50 µg/day) was more effective in correcting marginal cobalamin deficiency in vegetarians and vegans than large bolus doses (2000 µg/week).[58] Ultimately, the use of cobalamin supplements, regardless of the form, has been shown to be an effective means to correct suboptimal serum levels. For this reason, the supplementation of cobalamin by vegetarians is a lifestyle behavior that may affect overall methylation capacity.

Because folate plays an integral role in the methionine cycle, folic acid supplementation should also be considered when assessing overall methylation capacity. Current research is inconclusive on whether or not folate supplementation is an effective intervention for lowering serum homocysteine levels.[59] One notable randomized, double-blind clinical trial assessed the effectiveness of folic acid supplementation (200 µg/day), 5-methyltetrahydrofolate supplementation (200 µg/day), folate-rich dietary intake (200 µg/day), placebo supplements on lowering moderately elevated serum homocysteine levels in 149 participants. The data showed that all three interventions significantly lowered serum homocysteine levels and that there was no significant difference in effectiveness between them.[60] On the other hand, an intervention trial conducted in China found that folic acid supplementation (5 mg/d for 3 months) was only 56.41% effective in lowering serum homocysteine levels in 484 participants with hyperhomocysteinemia.[59]

These findings suggest that, although folate has been established as an essential cofactor in the methionine cycle, the effectiveness of folate supplementation on lowering homocysteine levels may still need more research to be better understood.

A factor that may play a role in assessing the efficacy of supplemental treatment is the fickle regulation of supplement manufacturers. Regulation of dietary supplements has been a long, arduous task for the United States government. Currently, the Dietary Supplement Health and Education Act of 1994 ensures that the Food and Drug Administration (FDA) take investigative action against supplements accused of being unsafe or adulterated.[55] Although this act ensures that potentially harmful supplements will be taken off the market, it does not enforce the FDA to ensure the quality of supplements before sale. This means that, though supplements may not be harmful, they may exhibit inconsistencies between dosages, bioavailability, and labeling accuracy. These inconsistencies make it difficult to compare the results of scientific supplementation studies, as well as accurately collect supplementation information from subjects.[61] Researchers assessing the metabolic effects of supplementation should be wary of this shortfall.

CHAPTER 3

METHODOLOGY

Participants and Study Design

Participants were accepted into the study if they were healthy, adhered to a vegetarian or omnivore diet for at least more than six months, were between the ages of 18 and 65 years of age, non-smokers, were not taking medications that altered methylation processes, had stable medication adherence for three or more months, and had no known methylation polymorphisms. Participants who did not adhere to these criteria or were pregnant, planning to be pregnant, and/or lactating were not selected to take part in the study. Preliminary studies examining homocysteine concentrations in vegetarians and omnivores provided data for sample size calculations (see Appendix A).[3,14,20] Target sample size for this study was estimated at 70 participants (35 vegetarian, 35 omnivore). The actual sample size achieved was 34 participants (18 vegetarian, 16 omnivore).

All participants were recruited by list serves and fliers. List serves were obtained from Arizona State University. Recruitment fliers were strategically placed in Arizona State University locations. Individuals who were interested in participating in the study were directed to an online survey tool to assess general health and adherence to the inclusion criteria. Individuals who met the inclusion criteria were then recruited as participants of the study. IRB approval and written consent were obtained (see Appendix B).

The study utilized a cross-sectional, differential research design that spanned one week (see Appendix C). After recruitment, selected participants were asked to participate

in Visit 1. This visit lasted 60 minutes. During this initial visit, the participants underwent verbal and written consent and completed a general health history questionnaire that included a MET survey, modified REAP-S survey, and 24-hour dietary recall (see Appendix D, E, F, respectively). Height, weight, and waist circumference were measured using a stadiometer, electronic weighing scale, and Gulick tape measure, respectively. Participants were then asked to participate in Visit 2. This visit lasted 30 minutes. The subjects were asked to attend Visit 2 in a fasted state. The researcher ensured that the participants had fasted for 12 hours by verbal confirmation. If participants were fasting at Visit 1, the blood sample was collected at that time, eliminating the need for Visit 2. Five milliliters of venous blood were extracted from each participant. Both visits were scheduled in the morning to accommodate fasting considerations.

Independent Variable

Adherence to a vegetarian or omnivore diet was the independent variable within the study. The vegetarian diet was defined as following a lacto-ovo vegetarian or vegan diet for at least six months and the exclusion of flesh foods for at least six months. The omnivore diet was defined as following a diet that consisted of both plant-based and animal-based foods, including flesh foods daily, for at least six months. Pesco-vegetarians were considered to be omnivores for the purpose of this study. Adherence to the diet was established through verbal confirmation and the 24-hour dietary recall.

Dependent Variables and Blood Analysis

This study examined two dependent variables: serum B12 status and methylation capacity. Methylation capacity was measured with homocysteine, methylmalonic acid and serum total folate. Transcobalamin II was also measured to determine B12 deficiency

as there are B12 status criteria for both total serum B12 and TCII (see Table 2). These metabolites were tested using fasting venous blood samples drawn during Visit 2 or Visit 1, depending if the participant was in a fasted state at the first visit.

Laboratory Analysis

Five milliliters of venous blood were extracted from each participant into a 7-milliliter lavender cap vacutainer containing the anticoagulant Ethylenediaminetetraacetic acid *(EDTA)*. The blood samples were then spun in a refrigerated 4 degrees Celsius centrifuge (2,800 RPM x 10 min.) and promptly frozen at -80 degrees Celsius for 15 minutes. Samples were then thawed and centrifuged a second time (3,000 RPM x 15 min.) to achieve deproteination. Vitamin B12 was examined through a SNB test kit (MPBio SimulTRAC SNB kit, item number 06B264806). Homocysteine was measured through an ELISA test kit (MyBiosource ELISA kit, item number MBS7252797). Methylmalonic acid was measured through an ELSA test kit (MyBiosource Methylmalonic Acid ELISA Kit, item number 288266). Serum total folate and transcobalamin II were measured through an RIA kit (SimulTRAC-SNB Vitamin B12/Folate RIA Kit, item number 06B264806). After the blood tests were performed, the blood samples were frozen at -80 degrees Celsius and stored. All test procedures were performed according to the specific kit directions. No modifications to the published procedures were done.

Assessing Diet Quality

Diet quality was assessed using a modified Rapid Eating Assessment for Participants – shortened version (REAP-S). The REAP-S is a shortened version of the original REAP survey which assesses parameters such as fat, fiber, sugar, fruit, and

vegetable intake. Validation of the REAP-S has been published in the literature.[62,63] In the traditional REAP-S, possible scores ranged from 13-39 (scores directly indicate diet quality).[63] The REAP-S used in this study was modified to include vegetarian eating styles. Possible scores in the modified REAP-S ranged from 15-45 (scores directly indicate diet quality).

Assessing Leisure-Time Physical Activity

Physical activity was assessed using the Godin-Shephard Leisure-Time Physical Activity Questionnaire (GSLTPAQ). In this measure, a leisure score is calculated by multiplying the number of times mild, moderate, and strenuous leisure-time physical activity is performed for at least 15 minutes in a typical week (frequency score) by a metabolic equivalent of task, or MET, score (3, 5, 9 for mild, moderate, and strenuous, respectively), and then summed. In the traditional GSLTPAQ, a score ≤ 23 indicate an insufficiently active lifestyle.[64]

Statistical Analysis

Preliminary studies examining methylation biomarkers in vegetarians and omnivores provided data for sample size calculations. The alpha error level for the outcomes of this study was set at 0.05 and the beta error level at 0.20 (a power of 80% that a difference will be seen in methylation biomarkers due to the intervention). A 15% drop out rate was anticipated. The estimated sample size was 35 participants per group (35 vegetarians, 35 omnivores); a total sample size of 70 participants was required. The assumption was made that homocysteine would be approximately 13.30±3.25 µmol/L (mean±SD) in vegetarians, a difference of ~3 µmol/L versus omnivores.

Data were represented as a mean plus and minus a standard deviation (mean±SD). For comparison of methylation capacity outcome variables (serum homocysteine, methylmalonic acid, serum total folate) between diet type and diet quality, an independent sample T-test was conducted for normally distributed variables. Serum B12 and transcobalamin II were non-normally distributed and were assessed using the Mann-Whitney test. For assessment of correlation between serum cobalamin and methylation capacity outcome variables (serum homocysteine, methylmalonic acid, serum folate), a Spearman Rho correlation test was performed. Serum B12 deficiency status was compared between diet type groups using the Pearson Chi Square test. Data were analyzed on BMI SPSS software, version 24.

CHAPTER 4

RESULTS

Subject Characteristics

A total of 146 individuals (including 44 vegetarians) completed the online screening measure. Eighteen of the vegetarians met the eligibility criteria and were matched to an omnivore respondent. Thirty-four individuals (18 vegetarians: 3 men/14 women; 16 omnivores: 3 men/13 women) agreed to meet with investigators and were enrolled in the study. Physical activity was quantified in metabolic equivalents (METs), which is a ratio of the rate of energy expenditure during physical activity to the rate of energy expenditure during rest. Diet quality was quantified with the Rapid Eating and Activity Assessment for Participants short version (REAP-S) modified for vegetarians. Of the descriptive variables (Table 4), there were no significant differences between the two groups. One vegetarian participant was morbidly obese, but removing her from the data analysis did not change the significance for any of the variables.

Table 4. Subject characteristics. A comparison of baseline data between diet types. Data presented as mean ± SD.

Variable	Omnivore (n= 16)	Vegetarian (n= 18)	P-value
Age (years)	29.4±9.1	27.7±9.0	0.59
Weight (kg)	64.8±9.4	68.0±26.4	0.67
Height (cm)	166.9±6.4	167.6±7.8	0.80
BMI (kg/m^2)	23.2±2.8	24.5±11.3	0.66
Waist Circumference (cm)	77.6±7.7	79.6±19.9	0.71
METs ($kcal \cdot kg^{-1} \cdot wk^{-1}$)	50.5±30.7	51.4±26.1	0.93
REAP-S score	37.7±3.1	37.5±3.0	0.86

Data are the mean ± SD; the p-value is for independent sample T-tests. METs <23 kcal/kg/wk indicate inactive adults.[64] REAP-S scoring range: 15-45 with higher scores indicating greater diet quality.

Outcome Variables by Diet Type

Table 5 shows the outcome variables for the sample and by diet type. Blood samples were analyzed for plasma B12, transcobalamin II, folate, homocysteine, and methylmalonic acid. There were no significant differences between the diet groups. Interquartile ranges for plasma B12 and transcobalamin II are 254/369 and 156/337, respectively for the omnivores/vegetarian. Reference ranges from a recent CDC report are provided alongside participant data.[65] The national average for transcobalamin II status is currently unknown.[66]

Table 5. Outcome variables by diet type. Data presented as mean ± SD.

Variable	Reference Values	Total sample (n=34)	Omnivore (n=16)	Vegetarian (n=18)	P-value
Plasma B12 (pmol/L)	360.9 – 377.1	439.7±216.3	381.9±150.6	491.1±254.7	0.31
Transcobalamin II (pmol/L)	Unknown*	346.9±171.9	297.5±117.2	390.7±202.4	0.21
Folate (nmol/L)	27.2 – 28.6	32.8±12.5	31.7±10.6	33.8±14.1	0.63
Homocysteine (umol/L)	8.1 – 8.4	8.1±1.8	7.9±1.3	8.3±2.2	0.46
Methylmalonic Acid (ng/mL)	18**	28.8±3.6	29.1±4.0	28.6±3.2	0.70

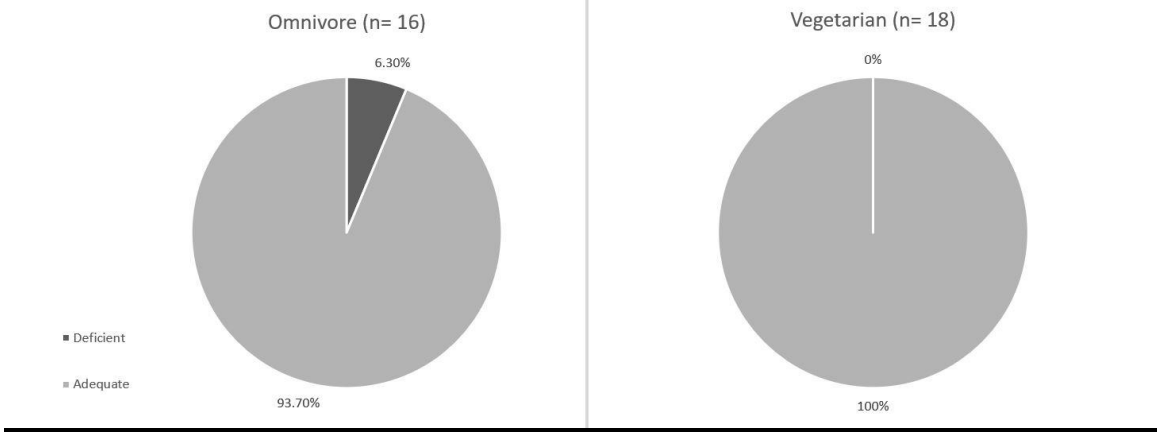
Data are mean ± SD; the p-value is for independent sample T-test for diet comparisons with the exception of B12 and TCII which represent the Mann-Whitney test. National average range based on recent CDC report.[65]
* National status of transcobalamin II is unknown.[66]
**MMA: National Institute of Standards and Technology.[67]

Cobalamin Status Between Diet Types

Cobalamin status was assessed as one of the outcome variables. Figure 6a shows the percent of participants who were B12 deficient between the two groups (6.3 and 0% for omnivores and vegetarians respectively, p=0.95). Figure 6b shows the percent of

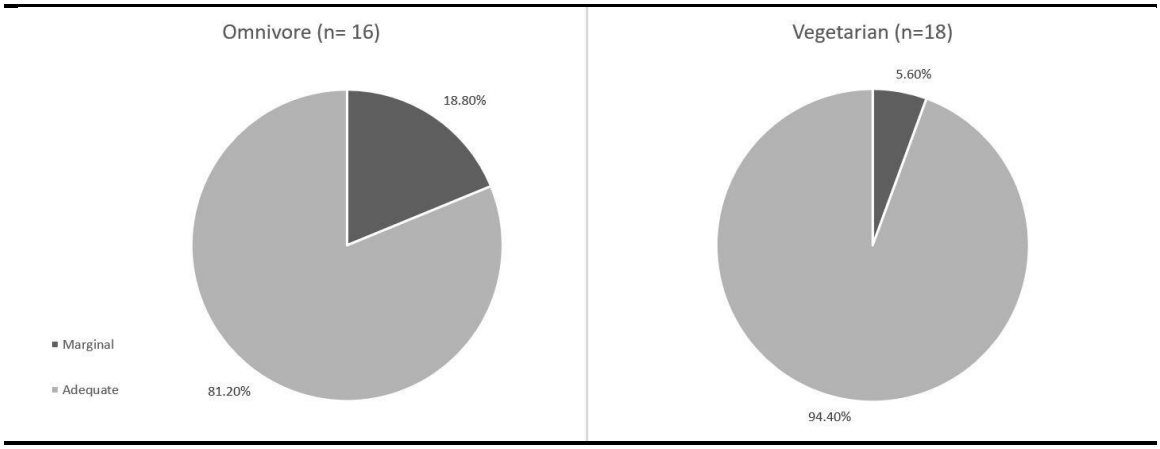
participants who were B12 marginally-deficient between the two groups (18.8 and 5.6% for omnivores and vegetarians respectively, p=0.51).

Figure 6a. B12 status deficient between diet groups.



Data were analyzed with Pearson Chi Square Test with continuity correction: p=0.95

Figure 6b. B12 status marginal between diet groups.



Data were analyzed with Pearson Chi Square Test with continuity correction: p=0.51

Supplements Taken by Diet Type

Information about the subject's supplemental regimen was gathered subjectively. Pertinent supplements to this particular study were defined as vitamin B12, multivitamins, folate, B-complex, and prenatal. Four omnivores and 12 vegetarians

reported supplementing at least one pertinent supplement on a regularly scheduled basis (see Figure 7a). Of the four omnivores who reported taking a pertinent supplement, three reported taking a multivitamin and one reported taking a prenatal vitamin (see Figure 7b). Of the 12 vegetarians who reported taking a pertinent supplement, 8 reported taking a B12 supplement, 8 reported taking a multivitamin, and one reported taking a B-complex vitamin (see Figure 7b). One omnivore reported supplementing B-complex only when sick. This subject was not included in the figures below. None of the subjects reported taking a folate supplement.

Figure 7a. Supplement behavior between diet groups. Graphs show the percent of individuals who supplemented pertinent supplement(s) on a regular basis between omnivore and vegetarian groups.

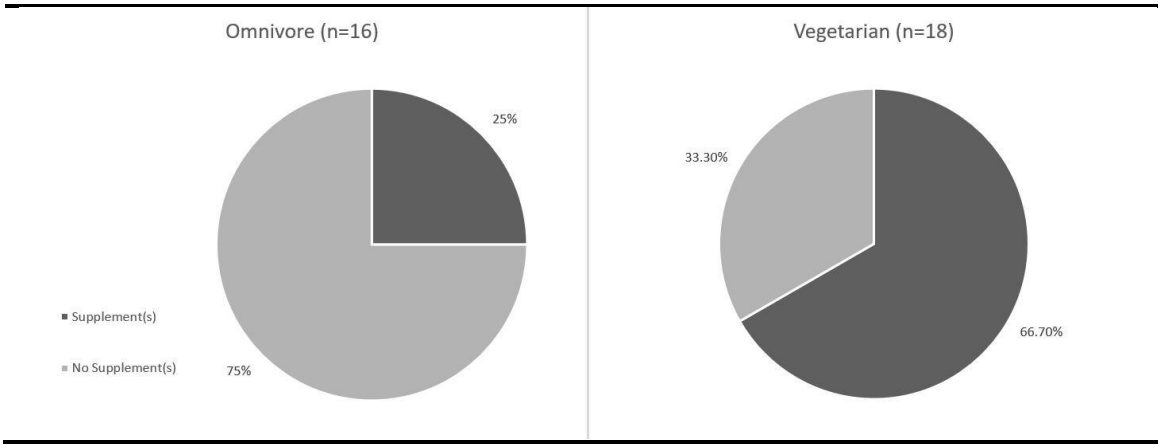
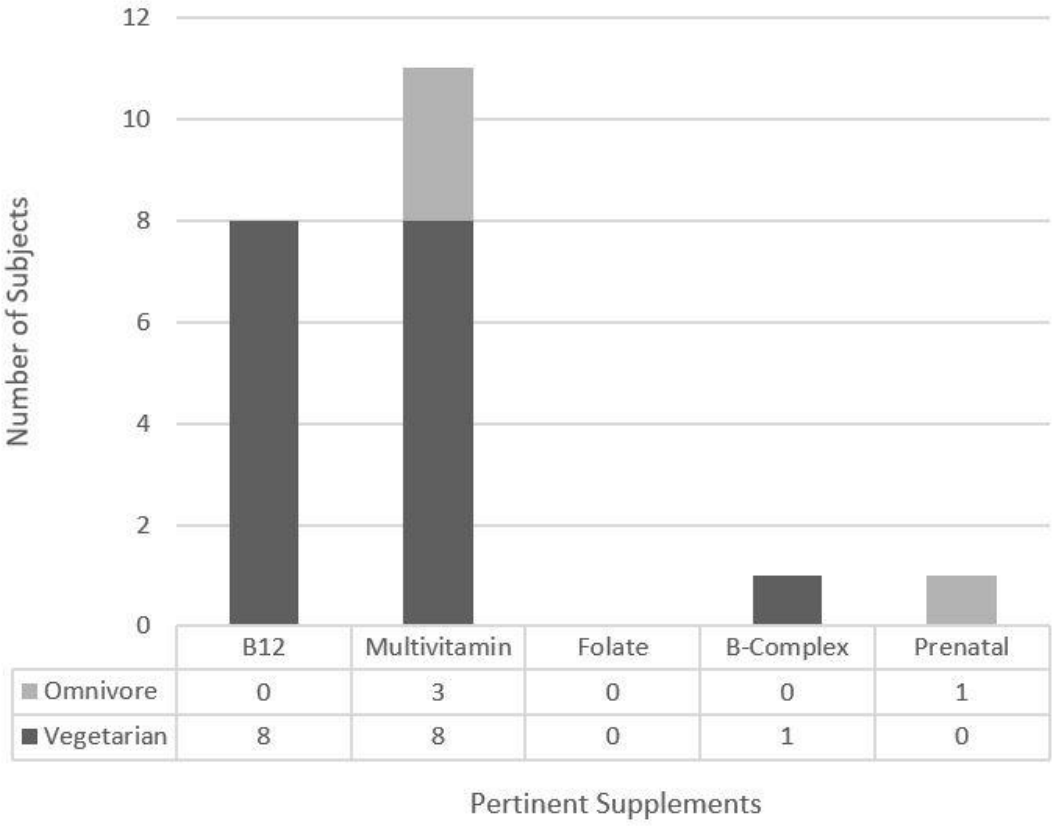


Figure 7b. Types of pertinent supplements taken between omnivore and vegetarian groups.



Correlations Between Outcome Variables

Correlations between plasma B12 levels and outcome variables for methylation capacity were assessed using the Spearman Rho correlation test. Table 6 shows the significance and correlation coefficients between each outcome variable. The data show there is a high correlation between plasma B12 and transcobalamin II levels ($r=0.974$; $p<0.001$). There is a weak direct correlation between folate and methylmalonic acid concentrations ($r=0.287$; $p=0.099$). All other variables were not significantly correlated.

Table 6. Correlations Between outcome variables. (n=34)

Variable	Plasma B12	Trans-cobalamin II	Folate	Homocysteine	Methylmalonic acid
Plasma B12	--	**.000**	.203	.900	.529
	[1.00]	**[.974]**	[.224]	[.022]	[.112]
Transcobalamin II		--	.358	.896	.567
		[1.00]	[.163]	[.023]	[.102]
Folate			--	.351	.099
			[1.00]	[-.165]	[.287]
Homocysteine				--	.239
				[1.00]	[.208]
Methylmalonic Acid					--
					[1.00]

P-value and correlation coefficient (in brackets); the p-value is for Spearman's Rho correlation test.

Outcome Variables by Diet Quality

Table 7 shows the outcome variables for the sample when regrouped based on diet quality. Diet quality was quantified with the REAP-S survey (REAPS scoring range: 15-45 with higher scores indicating greater diet quality). The data show that there is a significant difference between diet quality groups for folate status. There is a statistical trend (p=0.08) for homocysteine reduction in the group with the higher diet quality. Interquartile ranges for plasma B12 and transcobalamin II are 162/269 and 125/179, respectively for low/high diet quality. National average ranges from a recent CDC report are provided alongside participant data.[65] The national average for transcobalamin II status is currently unknown.[66]

Table 7. Outcome variables by diet quality. Data presented as mean ± SD.

Variable	Reference Values	Low Diet Quality (≤37; n=16)	High Diet Quality (>37; n=18)	P-value
Plasma B12 (pmol/L)	360.9 – 377.1	491.0±234.7	394.1±193.7	0.16
Transcobalamin II (pmol/L)	Unknown*	385.5±180.4	312.5±161.5	0.11
Folate (nmol/L)	27.2 – 28.6	27.0±10.6	38.0±11.9	0.01
Homocysteine (umol/L)	8.1 – 8.4	8.7±2.0	7.6±1.5	0.08
Methylmalonic Acid (ng/mL)	18**	29.2±3.9	28.5±3.4	0.58

Data are the mean ± SD; the p-value is for independent sample T-test for diet comparison with the exception of B12 and TCII which represent the Mann-Whitney test. National average range based on recent CDC report.[65]

* National status of transcobalamin II is unknown.[66]

** MMA: National Institute of Standards and Technology.[67]

CHAPTER 5

DISCUSSION

These data indicate that our population of healthy vegetarians did not have significantly lower serum cobalamin levels nor lower methylation capacity compared to omnivores. Furthermore, there were no direct correlations between low serum cobalamin levels and low methylation capacity. Methylation capacity was measured with three outcome variables: folate, homocysteine, and methylmalonic acid. Data were compared against diet type (omnivore versus vegetarian) and there were no significant differences between the two diet groups. Furthermore, diet quality based on the modified REAP-S measure did not differ significantly between diet groups.

One limitation of the study was the participant population. Recruitment was done through fliers posted around the Arizona State University downtown campus. The population demographic is mainly comprised of female-dominated, college-aged students. This demographic is clearly mirrored in the participant characteristics (mean age=28.6 years, 82.4% female). The campus is also home to Arizona State University's College of Health Solutions. This may have impacted the physical activity scores (mean METs=50.9 kcal/kg/week reference value for sedentary American adults, 36 kcal/kg/week[64,68]) and diet quality (mean REAP-S=37.6; scoring 15 (low) to 45 (high)) of the participants.

Although there was no significant difference in serum cobalamin levels and methylation capacity between the diet types, there was a notable difference in serum cobalamin deficiency and marginal deficiency status. The data show 6.3% of omnivores and 0.0% of vegetarians were deficient in serum cobalamin; 18.8% of omnivores and

5.6% of vegetarians were marginally deficient in serum cobalamin. One variable that could explain these data is supplementation behavior. Participants were asked to subjectively report regularly scheduled supplements they consumed. Pertinent supplements examined in this study were vitamin B12, multivitamins, folate, B-complex, and prenatal vitamins due to their known B12 and folate content.[61] The subjective data show that 25.0% and 66.7% of omnivores and vegetarians, respectively, supplemented either one or a combination of pertinent supplements. Vegetarians were the only participants who supplemented B12 on a regularly scheduled basis. None of the participants took a folate supplement. There is a considerable lack of research on vegetarian knowledge of supplementation. However, the data collected herein suggests that vegetarian knowledge of dietary vitamin disparities was adequate in this population. It is evident that the vegetarians that participated in this study were aware of the importance of vitamin B12 supplementation. Unfortunately, consistent collection of dietary supplementations is a difficult task due to the inconsistent regulatory definitions, varying bioavailability, and dosage amounts between brands.[61] Supplementation data in this study were reported from memory and thus not optimally documented. Future studies should collect supplementation data in a more structured manner. Exact B12 and folate doses should be recorded, along with the supplement brand name. These measures may help better estimate the amount of B12 and folate obtained through supplementation versus diet.

The data show a significant direct correlation between serum cobalamin and transcobalamin II levels ($r=0.974$; $p<0.001$). This correlation is to be expected due to the bound nature of cobalamin.[69] The transport process of cobalamin has been well

established in the literature. There are several proteins that bind to cobalamin throughout the digestion, absorption, and metabolic processes. Transcobalamin I (TC; also referred to as haptocorrin) first binds to cobalamin in the mouth and remains bound until it reaches the higher pH of the upper small intestine. Intrinsic factor then binds to cobalamin and allows it to enter the enterocytes in the terminal ileum. Finally, transcobalamin II (TCII) binds to cobalamin and allows it to enter cells throughout the body. Transcobalamin II is the active-bound form of cobalamin due to its ability to allow cobalamin into cells where it can be utilized. Cobalamin bound to TCI and TCIII are considered analogues because they cannot be taken into or utilized by the cells. However, total serum cobalamin tests measure both analogue and active forms of cobalamin.[21] It is estimated that up to 70-90% of bound cobalamin are analogues and only 10-30% of cobalamin are active forms bound to TCII.[21] For this reason, it is imperative to ensure cobalamin deficiency is not due to insufficient TCII production and polymorphisms. The direct correlation between total serum cobalamin and transcobalamin II levels suggests that the participants had adequate transcobalamin II production and that subsequent deficiencies in total serum cobalamin were not related to transcobalamin II polymorphism.

The correlational data also show a weak direct correlation between folate and methylmalonic acid concentrations ($r=0.287$; $p=0.099$). It has been well established that folate, cobalamin, homocysteine and methylmalonic acid are inter-related. High levels of serum folate can lead to vitamin B12 masking and complicate the diagnosis of B12 deficiency.[37] Homocysteine and methylmalonic acid measurement have been established as reliable tests for cobalamin status for nearly a decade.[37] Homocysteine and

methylmalonic acid both increase in the presence of cobalamin deficiency. However, methylmalonic acid has not been established as a reliable indicator of folate status. The data herein suggest that, although methylmalonic acid can be used as a marker of cobalamin status, it may also be affected by folate status. Theoretically, excessive folate may mask a B12 deficiency which would increase methylmalonic acid. However, although the mean folate concentration in the present study was above average for American adults (27.2 – 28.6 nmol/L), methylmalonic acid ranges were near normal (15 – 17 ng/ml) according to a most recent CDC report.[65] More research is needed to better understand the mechanism behind the weak direct correlation between methylmalonic acid and folate.

To further examine diet adequacy of the sample, participants were regrouped based on diet quality. Participants were reconfigured into a low diet quality group or high diet quality group based on their modified REAP-S score (the median value, 37, was the cut-off used to reconfigure the sample). The reconfigured data show that the high diet quality group had a significantly higher folate status ($p=0.01$). Folate is a naturally occurring vitamin that can be found in fruits and vegetables. Pulses, citrus fruits and leafy vegetables contain the highest levels of naturally occurring folate.[28] Processed foods can be enriched with the synthetic form of folate called folic acid. This synthetic form must be converted into folate by the enzyme methylenetetrahydrofolate reductase (MTHFR). Unfortunately, the MTHFR conversion process can be compromised by a common polymorphism. Consequently, obtaining folate from naturally occurring sources is a much more effective way to ensure proper folate status.[34] The participants in the high diet quality group reported consuming more fruits and vegetables than the participants in the

low diet quality group. Thus, it can be expected that the high diet quality group had a significantly higher serum folate status. In fact, serum folate is used as an indicator of diet quality. A retrospective cohort study assessed the relationship between serum folate status and fruit and vegetable consumption in 5,536 adults. The researchers concluded that serum folate was a reliable biomarker to assess fruit and vegetable intake in adults.[30] These data are consistent with the folate-diet quality findings in this study.

The data also show a statistical trend for homocysteine reduction in the high diet quality group ($p=0.08$). Much like cobalamin, folate is an essential cofactor in the methylation cycle. Specifically, THF is required to provide a methyl group to cobalamin in order to convert homocysteine to methionine. As stated above, the high diet quality group had a significantly higher folate status. This finding suggests that higher folate status may be associated with lower homocysteine levels due to the vitamin's essential cofactor role.

There were several limitations within the study that should be addressed. The first limitation involves the study's convenience sample and cross-sectional design. These factors prevent causality from being examined. The sample population was also a limitation due to the health-oriented college demographic. These students may have had a higher knowledge base on supplementation and the importance of cobalamin due to the health-related majors on the campus. It should be noted that this is only speculation because specific information about participant college majors were not recorded. Frequency, brand, and dose of dietary supplementation was self-reported from memory. This made it hard to understand the relationship between supplementation behavior and serum cobalamin levels. In addition to these limitations, the study utilized a smaller

sample size than what was calculated (calculated n=70, actual n=34). All participants were lacto-ovo vegetarians, vegans, or omnivores for at least six months, between the ages of 18 to 65 years old, non-smokers, and Phoenix residents. The participants confirmed that they did not take any known medications that inhibit methylation and had no known methylation polymorphisms.

This healthy vegetarian sample had similar methylation capacity as a matched omnivore sample. However, future trials should examine methylation capacity in vegetarian populations that are less likely to supplement. Additionally, the diet quality data give future studies a new direction to explore: the effects of diet quality on methylation capacity. Reorganizing the participants into diet quality groups raises the question on whether or not vegetarian diets are truly healthier than omnivore diets since similar numbers of vegetarians and omnivores (9 and 9, respectively) were placed into the high diet quality group. In sum, these data suggest that individuals can have a healthy diet regardless of diet type but vitamin/mineral supplementation may be a determining factor for healthy vegetarian diets.

CHAPTER 6

CONCLUSION

In summary, serum cobalamin levels and methylation capacity were not significantly lower in vegetarians than omnivores. Additionally, there was no correlational relationship between serum cobalamin and methylation capacity. However, when the subjects were regrouped by diet quality scores, high diet quality was positively correlated with serum total folate status. There were also lower homocysteine levels in the high diet quality group, though not significant. These findings suggest that diet quality may actually have a greater impact on methylation capacity than diet type. Future studies should focus on gathering a larger, representative subject pool of vegetarians residing in the U.S. Researchers should also gather supplementation information and fortified food intake more rigorously to better assess how these behaviors may impact methylation capacity.

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APPENDIX A

SAMPLE SIZE CALCULATIONS

Author	Year	Hcy in Vegetarians (Mean±SD, µmol/L)	n Per Group	Calculated n	Age Range	Subject State	Test
Krajcovicova-Kudlackova, et al.	2000	13.18±2.05	51	9	19-77	Healthy	HPLC method with fluorescence detection and SBD-F as derivative agent
Obersby, et al	2013	13.91±3.15	13	20	27-59	Healthy	Majority – HPLC method with fluorescence detection
Majchrzak, et al	2006	12.80±4.56	39	81	19-65	Healthy	HPLC method with fluorescence detection
AVERAGE	-	**13.30±3.25**	**34**	**35**	**20-65**	-	-

APPENDIX B

PARTICIPANT CONSENT FORM WITH IRB APPROVAL

Diet and Metabolism Study

INTRODUCTON
The purposes of this form are (1) to provide you with information that may affect your decision as to whether or not to participate in this research study, and (2) to record your consent if you choose to be involved in this study.

RESEARCHERS
Dr. Carol Johnston, a Nutrition professor, and Noel Ugarte, a graduate student, at Arizona State University Downtown, Phoenix, have requested your participation in a research study.

STUDY PURPOSE
The purpose of this research study is to compare the methylation capacity of vegetarians and omnivores. Methylation capacity reflects chemical reactions that occur in the body and can be determined using a blood sample.

DESCRIPTION OF RESEARCH STUDY
You have indicated to us that you are 18-65 years of age and healthy. Those of you who follow a vegetarian diet have done so for at least 6 months. If female, you are not pregnant, or recently pregnant, or lactating. This study will involve the completion of a brief health history questionnaire to demonstrate the absence of other conditions that may contraindicate participation.

This research entails that you visit our test facilities on one or two occasions on ASU's Downtown Phoenix campus. At your **first visit** (lasting ~60 minutes) you will complete a health history questionnaire and diet questionnaire. Your height, weight, and waist circumference will be measured. If you are fasting, a blood sample (2 tablespoons) will be drawn. A fast is defined as no food or drink with the exception of water for 12 hours. If you are not fasting at the first visit, a second visit will be scheduled within a few days and a fasting blood sample will be draw then.

RISKS
Mild discomfort due to the venous blood draw may occur. Blood sampling may be associated with nausea, dizziness, faintness, and bruising at the site of needle insertion. A trained phlebotomist will collect the blood and manage participant reaction as appropriate.

BENEFITS
You may not benefit from this study, but once the study is complete you will be provided with your test results if desired. You will need to sign a release form to receive the test results.

NEW INFORMATION
If the researchers find new information during the study that would reasonably change your decision about participating, then they will provide this information to you.

CONFIDENTIALITY
All information obtained in this study is strictly confidential unless disclosure is required by law. The results of this research study may be used in reports, presentations, and publications, but your name or identity will not be revealed. In order to maintain confidentiality of your records, Dr. Johnston will use subject codes on all data collected, maintain a master list separate and

secure from all data collected, and limit access to all confidential information to the study investigators.

WITHDRAWAL PRIVILEGE
You may withdraw from the study at any time for any reason without penalty or prejudice toward you. Your decision will not affect you any manner.

COSTS
There are no costs associated with participation in this study.

COMPENSATION FOR ILLNESS AND INJURY
If you agree to participate in the study, then your consent does not waive any of your legal rights. However, in the event of harm, injury, or illness arising from this study, neither Arizona State University nor the researchers are able to give you any money, insurance coverage, free medical care, or any compensation for such injury. Major injury is not likely but if necessary, a call to 911 will be placed.

VOLUNTARY CONSENT
Any questions you have concerning the research study or your participation in the study, before or after your consent, will be answered by Dr. Carol Johnston, 500 N. 3rd St., Phoenix, AZ 85004. [602-827-2265]

If you have questions about your rights as a subject/participant in this research, or if you feel you have been placed at risk, you can contact the Chair of the Human Subjects Institutional Review Board, through the ASU Research Compliance Office, at 480-965 6788.

This form explains the nature, demands, benefits and any risk of the project. By signing this form you agree knowingly to assume any risks involved. Remember, your participation is voluntary. You may choose not to participate or to withdraw your consent and discontinue participation at any time without penalty or loss of benefit. In signing this consent form, you are not waiving any legal claims, rights, or remedies. A copy of this consent form will be given to you.

Your signature below indicates that you consent to participate in the above study.

_____	_____	_____
Subject's Signature	Printed Name	Date

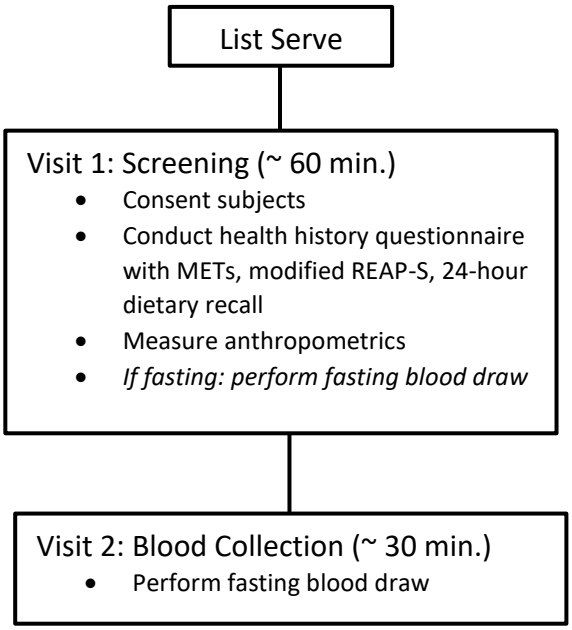
_____	_____
Contact phone number	Email

INVESTIGATOR'S STATEMENT
"I certify that I have explained to the above individual the nature and purpose, the potential benefits, and possible risks associated with participation in this research study, have answered any questions that have been raised, and have witnessed the above signature. These elements of Informed Consent conform to the Assurance given by Arizona State University to the Office for Human Research Protections to protect the rights of human subjects. I have provided the subject/participant a copy of this signed consent document."

Signature of Investigator_____ Date_____

APPENDIX C

STUDY DESIGN


List Serve
Visit 1: Screening (~ 60 min.)
Consent subjects
Conduct health history questionnaire with METs, modified REAP-S, 24-hour dietary recall
Measure anthropometrics
If fasting: perform fasting blood draw
Visit 2: Blood Collection (~ 30 min.)
Perform fasting blood draw

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APPENDIX D

HEALTH HISTORY QUESTIONNAIRE WITH METS EVALUATION

HEALTH HISTORY QUESTIONNAIRE ID#_____

1. Gender: M F

2. Age: _____

3. Have you lost or gained more than 10 lbs in the last 6 months? Yes No
   If yes, how much lost or gained? _____ How long ago? _____

4. Ethnicity: (please circle one) Native American African-American Caucasian Hispanic Asian Other

5. Education (please circle) High school diploma AA/vocational degree College degree MS degree PhD degree

6. Do you smoke? No, never _____
   Yes _____ # Cigarettes per day = _____
   I used to, but I quit _____ months/years (circle) ago

7. If female, have you ever been pregnant? _____
   If yes, date of last pregnancy? _____

8. If female, what were the dates of your last menstrual cycle? _____

9. Do you take any medications regularly? Yes No *If yes, list type and frequency:*

Medication	Dosage	Frequency

10. Do you currently take supplements (vitamins, minerals, herbs, etc.) ? Yes No
If yes, list type and frequency:

Supplement	Dosage	Frequency

11. Have been hospitalized in the past 6 months? _____ If yes, for what?_____
12. How much alcohol do you drink? (average #drinks per week) _____

13. Do you have any food allergies? Yes No If yes, explain: _____

14. Please ANSWER (YES/NO) if **<u>you</u> have <u>ever</u>** been clinically diagnosed with any of the following diseases or symptoms:

	YES	NO		YES	NO
Coronary Heart Disease			Chest Pain		
High Blood Pressure			Shortness of Breath		
Heart Murmur			Heart Palpitations		
Rheumatic Fever			Any Heart Problems		
Irregular Heart Beat			Coughing of Blood		
Varicose Veins			Feeling Faint or Dizzy		
Stroke			Lung Disease		
Diabetes			Liver Disease		
Low Blood Sugar			Kidney Disease		
Bronchial Asthma			Thyroid Disease		
Hay Fever			Anemia		
Leg or Ankle Swelling			Hormone Imbalances		
Eating Disorder			Depression		

Please elaborate on any condition listed previously.

_____

_____

15. How would you rate your lifestyle?

Not active _____ Active _____

Somewhat active _____ Very Active _____

16. Please circle the total time you spend <u>in each category</u> for an average **week.**

Light activities such as:

Slow walking, golf, slow cycling, doubles tennis, easy swimming, gardening

Hours per week: 0 1 2 3 4 5 6 7 8 9 10+

Moderate activities such as:

Mod. Moderate walking, cycling, singles tennis, moderate swimming, weight lifting

Hours per week: 0 1 2 3 4 5 6 7 8 9 10+

Vigorous activities such as:
Fast walking/jogging, fast cycling, court sports, fast swimming, heavy weight lifting
Hours per week: 0 1 2 3 4 5 6 7 8 9 10+

17. How long have you been following your current diet? _____

18. How many times per week do you consume meat, fish, or poultry? _____

19. How many times per week do you consume dairy products (milk, cheese, butter, yogurt)? _____

20. How many eggs do you consume per week? _____

21. Are you fasting today? (e.g., you have not eaten any food or beverage other than water for 12
hours) Yes No

APPENDIX E

MODIFIED REAP-S QUESTIONNAIRE

Diet Questionnaire ID#

In an <u>average week</u>, how often do you:	Usually/ Often	Sometimes	Rarely/ Never	
1. Skip breakfast?	O	O	O	
2. Eat <u>4 or more</u> meals from sit-down or takeout restaurants?	O	O	O	
3. Eat <u>2 or more servings</u> of whole grain products or high fiber starches a day? **Serving** = 1 slice of 100% whole grain bread; 1 cup whole grain cereal like Shredded Wheat; 3-4 whole grain crackers; ½ cup brown rice or whole wheat pasta; boiled or baked potatoes, yuca, yams or plantain.	O	O	O	*
4. Eat <u>2 or more servings</u> of fruit a day? **Serving** = ½ cup or 1 med. Fruit or ¾ cup 100% fruit juice.	O	O	O	*
5. Eat <u>2 or more servings</u> of vegetables a day? **Serving** = ½ cup vegetables, or 1 cup leafy raw vegetables.	O	O	O	*
6. Eat or drink <u>2 or more servings</u> of milk, yogurt, or cheese a day? **Serving** = 1 cup milk or yogurt; 1 ½-2 ounces cheese.	O	O	O	I follow a vegan diet O *
7. Eat <u>more than 2 servings</u> (see size below) of meat, chicken, turkey, or fish per day? **Serving** = 3 ounces of meat or chicken (size of a deck of cards) (e.g., 1 regular hamburger, 1 chicken breast or leg [thigh and drumstick], or 1 pork chop)	O	O	O	I follow a vegetarian/vegan diet O
8. Use <u>processed meats</u> (bologna, salami, corned beef, hotdogs, sausage or bacon) instead of low fat processed meals (roast beef, turkey, lean ham, low-fat cold cuts/hotdogs)?	O	O	O	I follow a vegetarian/vegan diet O
9. Eat <u>fried foods</u> (French fries, fried plantains, tostones, fried yuca, fried chicken, or fried fish)?	O	O	O	
10. Eat <u>regular potato chips, nacho chips, corn chips, crackers, regular popcorn?</u>	O	O	O	
11. <u>Add butter or margarine</u> to bread, potatoes, rice or vegetables at the table?	O	O	O	
12. Eat <u>sweets</u> like cake, cookies, pastries, donuts, muffins, chocolate and candies more than once per day.	O	O	O	
13. <u>Drink 16 ounces or more</u> of non-diet soda, fruit drink/punch or Kool-Aid a day?	O	O	O	

Note: *1 can of soda = 12 ounces*				
14. Prepare your meals from scratch as opposed to eating take-out, prepared, or convenience meals?	O	O	O	*
15. Eat processed foods such as frozen pizza and microwavable dinners?	O	O	O	
16. Eat or drink 2 or more servings of dairy-free milk, yogurt, or cheese a day?	O	O	O	
17. Eat 2 or more servings of meat-alternatives such as vegetable burgers, tofu, seitan, or tempeh a day?	O	O	O	

APPENDIX F

24-HOUR DIETARY RECALL GUIDE

24 Hour Dietary Recall Guide

Subject # _____ Date _____

Did you consume any supplements yesterday?

Supplement	Quantity	Time of Day

Upon waking, what food and beverages did you consume?

Food/Beverage	Quantity	Portion Size

What was the next thing you ate or drank?

Food/Beverage	Quantity	Portion Size

What did you have to eat and drink for lunch?

Food/Beverage	Quantity	Portion Size

Please turn over →

Did you have any snacks or beverages next?

Food/Beverage	Quantity	Portion Size

What did you have to eat and drink for dinner?

Food/Beverage	Quantity	Portion Size

Did you eat or drink anything else throughout the day or night?

Food/Beverage	Quantity	Portion Size

Is there any condiment, topping, seasoning or food you may have missed, such as: sugar, butter, ketchup, salt, cream cheese, etc.?

Think for a minute. Was there any food, beverage or anything else you may have missed that you consumed yesterday?

Was this a typical day in terms of dietary choices and eating patterns? What differs?