Comparing Glutathione in the Plasma of Vegetarian and Omnivore Populations

by

Rachel Christine Manley

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Graduate Supervisory Committee

Carol Johnston, Chair
Simin Levinson
Christina Barth

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ABSTRACT

Background: The Academy of Nutrition and Dietetics states that it is possible for a vegetarian to obtain the recommended amount of nutrients with a properly planned diet but nutrient deficiencies, such as vitamin B12 deficiency, may occur if diet planning is not optimal. An early indicator of B12 deficiency is raised homocysteine concentrations in blood which can cause health issues.

Objective: The amino acid methionine is consumed via dietary protein. Methionine is used in the biosynthesis of other proteins. After a removal of a methyl group, it makes homocysteine. Slightly raised homocysteine may promote greater synthesis of glutathione, an important endogenous antioxidant protectant. It can then be recycled back into methionine or converted into cysteine with the addition of various B-vitamins such as vitamin B12, folic acid, and vitamin B6. Cysteine then uses outside sources of glutamate and glycine to create glutathione (GSH). With the catalyst glutathione peroxidase it donates an electron and becomes the oxidized form, glutathione disulfide (GSSG). It can then convert back to GSH with the aid of glutathione reductase by using the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor. This study will examine glutathione levels in omnivores and vegetarians and see if it is related to vitamin B12 and homocysteine levels.

Design: This cross-sectional study encompassed 16 omnivores and 17 vegetarians from Phoenix, Arizona. A vegetarian diet was defined as one that excludes red meat, poultry, pork and seafood but allows dairy products and/or eggs; the diet had to be followed for at least one year. An omnivore diet is defined as eats meat daily. Participants completed a

diet questionnaire and a vitamin B12, B6 and folate food questionnaire and provided a fasting blood sample.

Results: The mean plasma B12 and homocysteine did not differ between diet groups. Glutathione was significantly lower among vegetarians in comparison to omnivores, 1.9±0.5 and 2.3±0.7 mmol respectively ($p=0.046$).

Conclusions: The hypothesis was shown to be incorrect that vegetarians would have a higher glutathione level than omnivores as a result of their modest consumption of vitamin B12. The implications of a reduced glutathione status are discussed.

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CHAPTER 1

INTRODUCTION

Over half a million National Institutes of Health-AARP participants aged 50-71 years completed food frequency questionnaires in a prospective cohort study. The study authors found that the data revealed that men and women who consumed large quantities of red (68.1 g/1000 kcal) and processed meat (19.4 g/1000 kcal) in comparison to people who consumed small amounts of red (9.3 g/1000 kcal) and processed meat (5.1 g/1000 kcal) had a significantly elevated risk for overall mortality. For men who were in the highest quartile and consumed large quantities of red meat, they had over double the number of deaths as men in the lowest quartile.[1] In a meta-analysis of observational studies, the findings revealed a 29% increased mortality rate for people in the highest category of total red meat consumption in comparison to the lowest category.[2] Many other studies have also confirmed these findings.[3,4] Hence, the current focus on plant-based diets by major U.S. health agencies is warranted.[5–7]

Vegetarianism is growing in popularity in the U.S. and worldwide.[8] According to a 2016 National Poll conducted by Harris Poll, there are approximately 4.3 million vegetarian adults in the United States, approximately 3.4% of the population. Although much research has demonstrated benefits associated with plant-based diets, vegetarian diets that completely restrict animal flesh can lead to nutrient inadequacies.[9] The Academy of Nutrition and Dietetics states that it is possible for a vegetarian to obtain the recommended amount of nutrients with a properly planned diet but nutrient deficiencies may occur if diet planning is not optimal.[10] One of the nutrients that vegetarians are typically deficient in is vitamin B12, or cobalamin. B12 deficiency can impact the

neurological system and is ultimately fatal if not corrected. An early indicator of B12 deficiency is raised homocysteine concentrations in blood.[11] Generally, high homocysteine in blood is considered an irritant creating oxidative stress and damaging blood vessels. It has been linked to risk for cardiovascular disease, stroke, and blood clots.[12–14] However, homocysteine is a metabolite in cellular reactions that produce glutathione which protects cells against oxidative stress.[15] Recent research has even linked this pathway with longevity in mice.[16] Possibly, the lower B12 status of vegetarians may increase homocysteine concentrations and promote a greater degree of glutathione production. Clearly, a B12 deficiency should be avoided at all costs; however, slightly raised homocysteine may promote greater synthesis of glutathione.[16] This study will examine glutathione levels in omnivores and vegetarians and to determine if glutathione concentration is related to vitamin B12 and homocysteine levels.

PURPOSE OF STUDY

This cross-sectional study compares the vitamin B12, homocysteine and glutathione serum levels in age and gender matched omnivores and vegetarians in Phoenix, Arizona.

REASEARCH AIM AND HYPOTHESIS

We hypothesized that there will be an inverse correlation between blood vitamin B12 and glutathione concentrations. We also hypothesized that since vegetarians have a lower vitamin B12 intake, they will have higher homocysteine and glutathione concentrations.

DEFINITION OF TERMS

- Vegetarian: An individual whose diet does not include meat, poultry, fish, and other seafood but does include some animal products including eggs, milk, and other dairy products.[17]
- Omnivores: Individuals who consume animal products daily.

DELIMITATIONS AND LIMITATIONS

- Limitations of this study include possible small sample size and possible misinformation given. Participants may not have adhered to a vegetarian or omnivore diet for more than 1 year.
- Delimitations include apparently healthy, between the ages of 18 and 65 years of age, non-smokers, not taking any medications that affect the methylation processes, adherence to a vegetarian or omnivore diet for more than 1 year and on stable medication for three or more months.

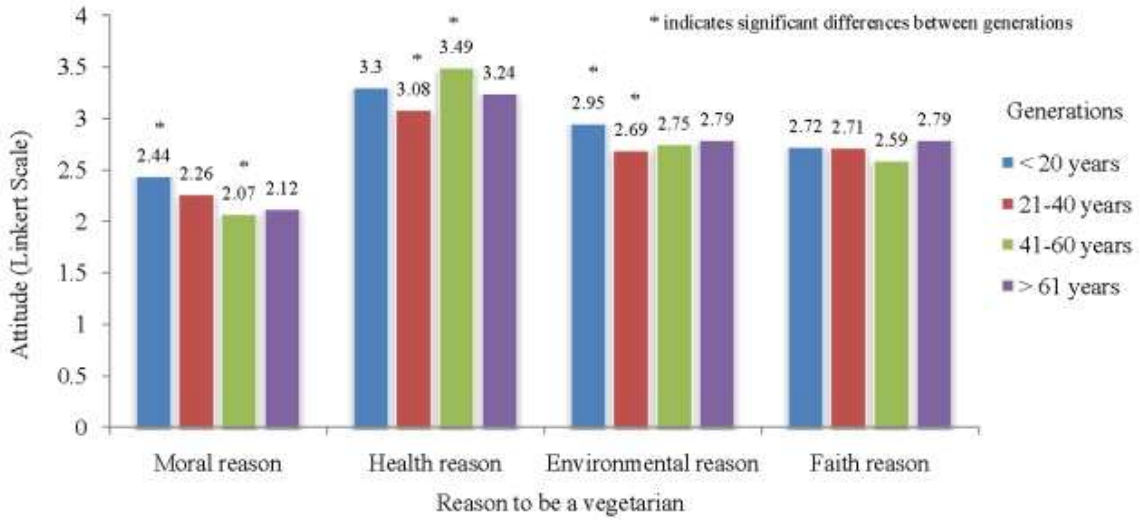
CHAPTER 2

REVIEW OF LITERATURE

Background on Vegetarian Diets

A vegetarian diet is defined as a person who does not eat meat, poultry or seafood or products containing those items. There are variations of vegetarian diets as well. Lacto-ovo vegetarians avoid all meat, fish and poultry but will eat dairy products and eggs. The lacto-vegetarian does not consume meat, fish or poultry but consumes dairy products but avoids eggs. The vegan does not eat any products of animal origin which includes eggs, dairy products, honey, meat, fish, poultry.[10,18] Pesco-vegetarians avoid meat and poultry, but do eat fish. Flexitarians is a combination of the words "flexible" and "vegetarian." It refers to the individual who mainly consumes a vegetarian diet but does not strictly follow it. This means that they occasionally eat meat, poultry or fish.[19] The reasons they do this is due to health issues associated with high meat consumption and animal welfare.[20] There is a wide range of reasons as to why people are adopting this vegetarian diet aside from health reasons.[20] One study took a Food Frequency Questionnaire and found that out of 609 participants, 4% were vegans, 25% were lacto-ovo vegetarians, 4% pesco-vegetarians, and 67% non-vegetarians (omnivores). The results are demonstrated in the following Figure 1:

Figure 1: Reasons to Be a Vegetarian[18]

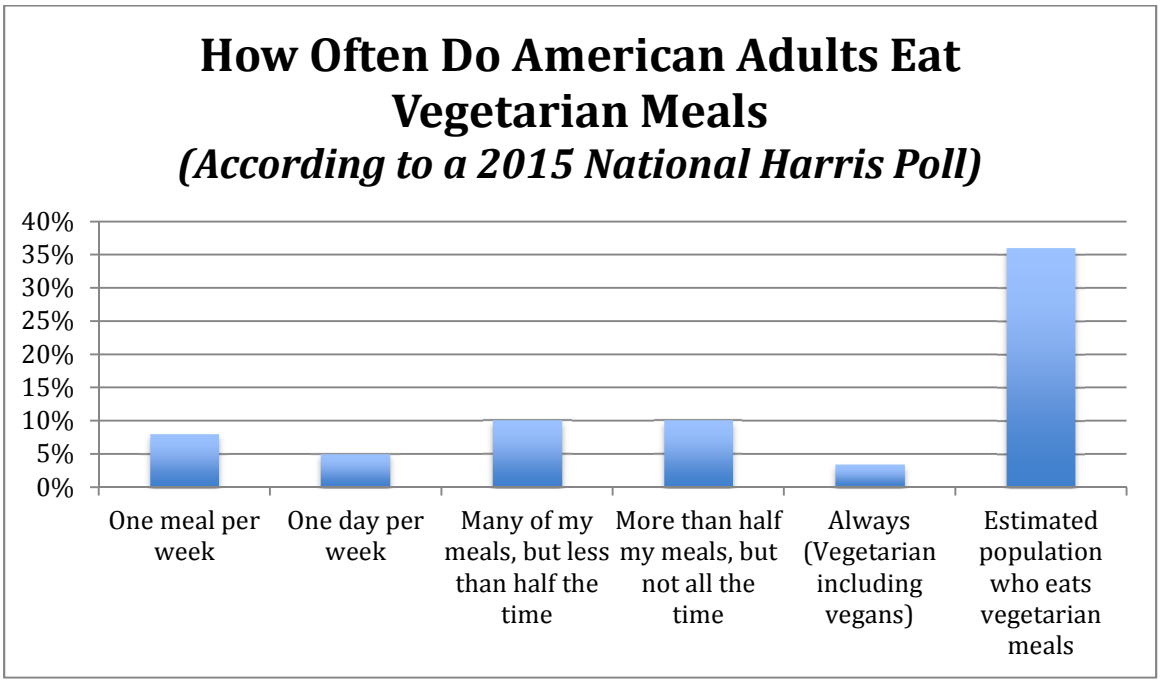


Researchers found that younger people adopt a vegetarian lifestyle for moral and environmental reasons but middle-aged people adopt it for health reasons. The reason for this is because younger people do not see their health as a priority concern. Although, as they age and they approach middle-age, their health becomes a priority. This leads them to focus on following specific diets that aid in their health.[18] Other research from NatCen's British Social Attitudes survey revealed how more people in the 65 to 79 years olds (39%) reduced their meat intake when compared to 18-24 year olds (19%). Over half of these people reduced their meat intake because of health reasons.[21]

The Vegetarian Resource Group commissioned Harris Interactive to conduct a nationally representative online 2015 National Harris Poll to determine out of 2,017 adults aged 18 and over, how many were vegetarian and vegan. They defined vegetarian as people that never eat meat, fish, seafood, or poultry. Vegans were defined as never eating meat, fish, seafood, poultry, dairy or eggs. The survey was conducted online on March 16th through 18th in 2016. Only people aged 18 and older were able to take the survey. 2,015 adults

submitted their responses, which were analyzed. The Figure 2 titled How Often Do American Adults Eat Vegetarian Meals displays how out of the population who eats vegetarian meals, a small percentage of that always eats vegetarian meals, vegans included. A larger percentage of the population (20%) who eats vegetarian meals responded with many of their meals are vegetarian, either less than half or more than half but not all the time. 8% of the population who eats vegetarian meals, eats only one meal per week and 5% of the population who eats vegetarian meals eats vegetarian one entire day per week.[9] These data are confirmed when looking at the increasing number of flexitarians who are attempting to reduce the amount of meat consumed.[19]

Figure 2: How Often Do American Adults Eat Vegetarian Meals?



The following table displays the percentage of the specific populations who never eat meat, fish or poultry and follow a vegetarian diet. This includes vegans as well. Male and females present similar numbers of vegetarians with females being 0.7% higher.[9]

NatCen's British Social Attitudes survey revealed that in Britain, 29% of the population has reduced their meat intake of all meats excluding fish in the past 12 months. The survey also showed that females were more likely to reduce their meat intake than males.[21] The age group that has the largest number of vegetarians is 18-34 years old. 35-44 years old and 45-54 years old have the same percentage of vegetarians, which is 3%. In addition 2% of 55-64 years old and 65+ years old are vegetarians. The largest numbers of vegetarians reside in the northeastern United States. After analyzing all these data, one can see that the number of strict vegetarians is not as high as people who occasionally follow the vegetarian diet.[9]

Table 1: People Who Never Eat Meat, Fish, or Poultry

People Who Never Eat Meat, Fish or Poultry (Total Number of Vegetarians, including Vegans)	
Category	**Number (%)**
Total	3.4%
Male	3%
Female	3.7%
18-34 years old	6%
35-44 years old	3%
45-54 years old	3%
55-64 years old	2%
65+ years old	2%
Reside in Northeastern	5%

United States	
Reside in Midwestern United States	3%
Reside in Southern United States	3%
Reside in Western United States	3%
Hispanic	3%
Black	8%
Below $50,000 household income	7%
$50,000-75,000 family income	2%
$75,000-100,000 family income	1%
Over $100,000 family income	2%[9]

Pros of Vegetarian Diets

Emerging data alludes to the fact that there are health risks associated with red meat, and an increasing number of people have started to reconsider consuming large amounts of meat. Substantial evidence has come out that shows that consumption of

meat, particularly red and processed meat, is associated with increased risks of diabetes, cardiovascular disease (CVD), and various types of common cancers such as breast, colorectal, and prostate.[22–24] The benefits of following a vegetarian diet when compared to a conventional diet can assist people with diabetes in many ways but not limited to (1) helping them control their weight, (2) aiding in glycemic control, (3) helping to control blood lipids, (4) increasing insulin sensitivity, and (5) decreasing oxidative stress markers.[25–28] Another study's results indicated how a vegetarian diet led to a greater improvement in mood and quality of life because the patients felt less constrained than the patients who were consuming a conventional diet. The vegetarian diet was also shown to decrease disinhibition and hungry feelings in the patients.[26] In a pilot study design, the researchers found a 28% significant mean reduction in fasting serum glucose in the group that consumed a low-fat vegan diet in comparison with the group that consumed the low-fat conventional diet (12% decrease).[28] Another study compared a calorie-restricted vegetarian and conventional diabetic diet in combination with exercise. They looked at how both affected insulin resistance, visceral fat, and oxidative stress markers in people with Type 2 diabetes. They found that the insulin sensitivity significantly increased in the calorie-restricted vegetarian diet when compared to the conventional diabetic diet. They also found a greater reduction in oxidative stress markers, visceral fat and subcutaneous fat in the calorie-restricted vegetarian diet.[25] The results from another study indicated that a low-fat vegan diet had greater improvement of $HbA_{1C,}$ reduction of diabetic medications, decrease in body weight and aided in better lipid control when compared with the American Diabetes Association diet.[27] The vegetarian diet is high in fruits and

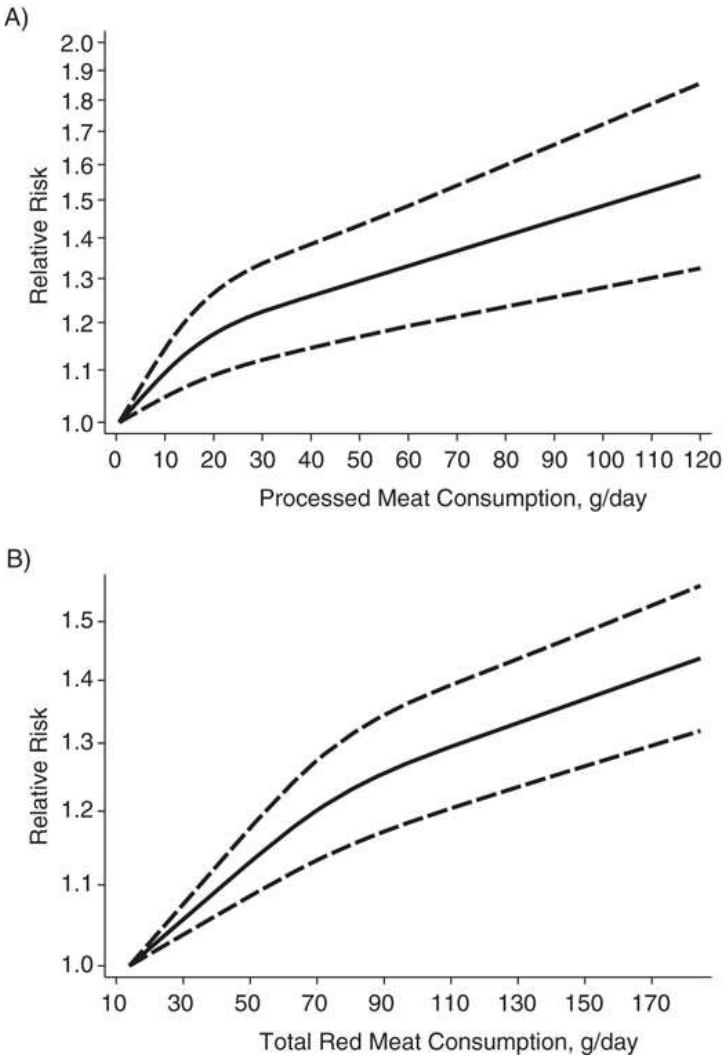
vegetables that contain plant-based antioxidant compounds, which likely partially explain why vegetarian diets help lower disease risk.

Researchers found that when comparing vegetarians with a group of age- and sex-matched omnivores, the vegetarians had higher fasting plasma ascorbic acid concentrations and lower concentrations of triglyceride, uric acid, and high-sensitivity C-reactive protein. These are all plasma biomarkers of antioxidant stress, oxidative stress, inflammation and risk for coronary heart disease. This lead researchers to the assumption that a long-term vegetarian diet will improve antioxidant status and lower risk of coronary heart disease better than a person who follows an omnivore diet.[29] In a 2012 meta-analysis that reviewed seven studies and a total of 124,706 subjects, the vegetarian group had a significantly lower mortality rate from coronary disease (29%) than the group that was non-vegetarian.[30] When researchers look at populations in rural Africa and Asia who follow a traditional plant-based diet, they see a low occurrence of coronary heart disease.[31] Not only does the vegetarian diet decrease risk of cardiovascular disease, but it also has the ability to reverse coronary heart disease. In a randomized clinical trial, patients with angiographically documented coronary heart disease were divided into two groups: a control group which received standard care from their doctors including dietary advice and a group that followed a low-fat vegan diet, exercised, managed their stress, and did not smoke. In the intervention group, 82% of the patients displayed measureable reversal of their coronary artery blockages and improvement in stenosis flow reserve. In the control group, they experienced further progression of their coronary heart disease.[32]

The dietary fat in meat, particularly the saturated fat, has been thought to be the reason for the meat and cancer connection. One study's results indicated that exposure to

Heterocyclic amines (HCAs), which are a group of mutagenic compounds, found in well-done meat may actually be the reason for increasing the risk of certain cancer in humans. Researchers looked at 2 prospective cohort studies (the Health Professionals Follow-up study and the Nurses' Health Study), which took data from 37,698 mean and 83,644 women. By using time-dependent Cox proportional hazards regression models, they assessed the association of red meat consumption with cause-specific and total mortality risks during follow-up. The researchers found that a higher consumption of unprocessed and processed red meat is associated with a higher total, cardiovascular disease, and cancer mortality. Processed red meat posed a greater risk of mortality. The researchers defined unprocessed red meat as beef, pork, or lamb as main dish or hamburger, beef, pork or lam as a sandwich or mixed dish. Processed meat was defined as bacon, hot dogs, sausage, salami, bologna, and other processed meats.[3] The World Cancer Research Fund recommended to the public that in order to reduce overall cancer risk, they should eat 500 g or less of cooked weight of red meats per week which included beef, pork and lamb. In regards to processed meat, they recommended avoiding these processed meats which they defined as ham, bacon, salami, hot dogs, and sausages.[33] A meta-analysis also confirmed these results that all-cause mortality risk is increased by consumption of red meat. The all-cause mortality was increased by 23% in the highest category of processed meat when compared with those in the lowest category. In addition, those in the highest category for total red meat consumption had an increased risk of all-cause mortality than those in the lowest category. This is revealed in the following Figure 3 from a study by Susanna Larsson and Nicola Orsini:

Figure 3: Relative Mortality Risk for Processed Meat and Total Red Meat Consumption[2]



EPIC is a large prospective cohort study which looked at 448,568 men and women from 10 European countries including France, Italy, Spain, The Netherlands, United Kingdom, Greece, Germany, Sweden, Norway and Denmark. The analysis of the results revealed a positive association between processed meat consumption and mortality. It was thought to be due to cardiovascular diseases and cancer.[4] In comparison with red meat, other sources of protein such as fish, poultry, nuts, legumes, low-fat dairy products, and whole grains, were associated with lower risk of mortality.[3] One study took

74 patients with Type 2 diabetes mellitus and randomly assigned them to either an experimental group or a control group. The experimental group received a vegetarian, isocaloric, calorie restricted (~500 kcal/day) diet and the control group received a conventional diabetic, isocaloric, calorie restricted (~500 kcal/day) diet. Out of the 24 weeks total of the experiment, the last 12 weeks were combined with aerobic exercise. The researchers found that a calorie-restricted vegetarian diet improved insulin sensitivity more than when compared with a conventional diabetic diet over 24 weeks. Researchers concluded that the greater loss of visceral fat and improvements in oxidative stress markets and plasma concentrations of adipokines may be the reason for the improvement of insulin resistance. When exercise training was added in the last 12 weeks, it further improved the outcomes of the vegetarian diet.[25]

Overall, vegetarians are noted as having a lower body mass index. Vegetarian diets are rich in dietary fiber, magnesium, potassium, vitamin C, vitamin E, folate, carotenoids, flavonoids, and other phytochemicals.[10] With all these benefits being documented in large numbers of published research, rising health care costs, and the increasing prevalence of obesity that leads to an increased risk of diabetes, hypertension, and cardiovascular disease, physicians and reputable health organizations such as the American Cancer Society, American Diabetes Association and Academy of Nutrition and Dietetics are recommending plant based diets.[5–7,10] For example, diabetes affects more than 11% of US adults aged 20 years and older and majority of that population suffer from type 2 diabetes.[23] A plant-based diet is a cost-effective intervention to improve health outcomes. A healthy, plant-based diet focuses on increasing consumption of nutrient-dense plant foods, minimizing processed foods, oils and animal foods (including

dairy products and eggs), and keeping intake of fat low. The main focus is to increase vegetable, fruit, beans, peas, lentils, soybeans, seeds, and nuts (in moderation). Plant-based is used synonymously with vegetarian or vegan. In one case study, which took a 63-year old man with a history of hypertension who complained to his physician of fatigue, nausea, and muscle cramps. His labs revealed a total cholesterol of 283 mg/dL, blood pressure of 132/66 mmHg, and body mass index (BMI) of 25 kg/m^2. He was taking a variety of pharmaceutical medications including lisinopril, hydrochlorothiazide, amlodipine, atorvastatin, metformin, glipizide, and neutral protamine Hagedom insulin. His physician prescribed a low-sodium, plant-based diet. The plant-based diet excluded all animal products and refined sugars, limited bread products, rice, potatoes and tortillas, and he was able to consume nuts and unlimited nonstarchy vegetables, legumes and beans. His regimen also included exercising 15 minutes twice a day. His results revealed the health benefits of a plant-based diet including improvements on biometric outcomes such as blood pressure, diabetes, and lipid profile. His $HbaA_{1C}$ went from 11.1% to 6.3% in 3 months.[7]

Current research even shows that for athletes, a well-planned and appropriately supplemented vegetarian diet can effectively support athletic performance. A variety of plant based proteins mixed with animal based protein such as dairy products and eggs needs to be consumed to provide adequate nutritional needs for total nitrogen and essential amino acids and if this is done, a vegetarian diet is reported to provide adequate protein for an athlete.[17] The healthy macronutrient ranges for vegetarian and non-vegetarian athletes is 45-65% for carbohydrate, 20-35% for fat and 10-35% for protein.[23] The United States market for process foods is making it much simpler to replace meat or

other animal products with vegetarian and vegan friendly products. There is an exponentially growing number of new products hitting the market currently that include fortified foods, dietary supplements and convenience foods. With all these new products on the market to be selected from, it makes it drastically easier for vegetarians to meet their needs for key nutrients such as calcium, iron, zinc, vitamin B12, vitamin D, riboflavin and long-chain n-3 fatty acids. Because of this, the Academy of Nutrition and Dietetics reveals how the nutritional status of a current day vegetarian is much better due to these new fortified foods than that of a vegetarian 1-2 decades ago.[10]

Cons of a Vegetarian Diet

With all the current interest and benefits of a vegetarian diet, one might wonder why every person not following it? The reason for this is due to the fact that there are some negatives to a vegetarian diet. It is vital to ensure that a vegetarian is getting adequate nutrition and some might say it takes additional work to ensure this.

Protein

Because most plant sources of protein do not contain all the essential amino acids, a variety of plant foods that contain protein need to be eaten to ensure that all essential amino acids and adequate nitrogen retention are provided.[34] The FAO/WHO created the protein digestibility-corrected amino acid score (PDCAAS). This scoring method calculates the protein quality by accounting for protein digestibility and indispensable amino acid pattern.[35] Also, protein needs for a vegetarian who is consuming limited protein sources such as cereals and legumes that are less well digested may need to be

higher than the Recommended Dietary Allowance of protein.[10] *The Dietitians Guide* looked at protein intakes of lacto-ovo-vegetarians and vegan females. Although some of the vegan females had protein intake that was borderline, the intakes of lacto-ovo-vegetarians and vegans appeared to meet and actually exceed the protein recommendations.[34] Due to the fact that non-animal protein has a decreased bioavailability, if a vegetarian is not consuming the correct amounts of animal protein, which is 45-50% of total protein, they may need to increase their protein intake. Researchers propose that they should add an addition 12-15 grams of protein daily to make up for the decreased bioavailability of non-animal protein.[36] In a prospective cohort study that took peri- and postmenopausal women who were Seventh Day Adventist. The women who never consumed meat had the highest risk of wrist fracture.[37] The risk for the fractures decreased when the vegetarian female increased animal and/or plant-based proteins. Other studies have also shown a link between protein intake and bone mineral density in vegetarian women.[38,39]

n-3 Fatty Acids

Vegetarian diets tend to be rich in n-6 fatty acids and they also tend to be low in n-3 fatty acids. Both n-3 fatty acid intakes and blood concentrations of n-3 fatty acids were lower in vegetarians when compared with omnivores.[40,41] One study looked at 12 Dutch vegans and compared the data with 15 age-and sex-matched Dutch omnivores. The researchers found that the omega-3 index in vegans was half the amounts detected in the omnivores as evidenced in Table 2.[42]

Table 2: Omega-3 Index in Omnivores vs. Vegans

	Omega-3 Index
	% of total fatty acids
Omnivores (n=15)	**4.45%**
Vegans (n=12)	**2.26%**[42]

Although the status of n-3 fatty acids in vegans and vegetarians is less than omnivores, vegetarians and vegans both respond to supplemental eicosapentaenoic acid and docosahexaenoic acid. Omnivores do as well.[40] Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are long-chain n-3 fatty acids, are low in diets that do not include eggs, fish, and/or algae. There are products on the market now such as soymilk and breakfast bars that are fortified with DHA making it easier to ensure adequacy. Because the plant based n-3 fatty acid, alpha-linoleic acid (ALA), has a conversion rate to EPA of about 10% in humans, vegetarians must make sure that they consume sources of ALA in foods such as flaxseed, walnuts, canola oil, and soy.[10]

Iron

Iron is another nutrient of concern form vegetarians because the iron found in plant foods is nonheme iron, which is easily affected by inhibitors and enhancers of iron absorption. That means that when nonheme iron is consumed with inhibitors such as phytates, calcium and the polyphenolics in tea, coffee, herb teas, and cocoa, it decreases iron absorption. Some enhancers include vitamin C and other fruit and vegetable based organic acids can increase absorption.[10] Heme iron is mainly found in animal products

such as poultry, meat, and fish.[43] A study looking at 35 vegetarians and 32 nonvegetarians in Taiwan found that even when vegetarian subjects had sufficient iron intake , they had difficulty maintaining iron balance due to the poor bioavailability of plant-based iron sources.[44] Another study compared Australian male vegetarians with Australian male omnivores aged between 20 and 50 years old. The researchers found that even though the vegetarians had a higher intake of iron, their iron status was actually significantly lower compared to that of the omnivores.[45] Although vegetarian adults have lower iron intakes compared to nonvegetarians, serum ferritin levels are within the normal range.[10] It is recommended that to lower the risk of iron deficiency amongst the vegetarian population, dietitians and health professionals should recommend that vegetarians consume iron-fortified food with enhancers, such as vitamin C or animal tissues, and to decrease the consumption of foods that inhibit iron absorption, such as calcium phosphate, phytates, bran or polyphenols that are in tea and coffee. Since a vegetarian diet does not include the consumption of animal tissues which contain readily available heme iron and increase non-heme iron absorption, that is why they are a population at risk for iron deficiency.[43] Importantly, the recommended Dietary Allowance of iron for vegetarians is higher than an omnivore because the bioavailability of iron from vegetarian sources is lower than from omnivore sources (33 versus 18 mg for adult women; 14 versus 8 for adult men).

Zinc

Dietary zinc needs are also higher in vegetarians as compared to omnivores. This is due to the fact that vegetarian sources of zinc contain phytic acid, which inhibits zinc.[10]

Other nondietary factors that increase the risk of zinc deficiency include inflammation/infection, bacterial overgrowth and parasitic infections, overweight or obesity, *Helicobacter pylori*-induced hypochlorhydria, chronic diarrhea, environmental enteropathy, Hookworm, *Trichuris trichiura*, and *H. pylori* infections, and genetic hemoglobin disorders, all because they have a negative impact upon zinc absorption.[46] Research on zinc intakes of vegetarians is inconclusive because some reveal that their intake is adequate while others reveal that it is inadequate. That is why vegetarians need to makes sure they consume adequate sources of zinc such as soy, legumes, grains, cheese, and nuts.[10] Many of the vegetarian foods have a high content of inhibitors of zinc absorption.[46] In the pediatric population, if a zinc deficiency is present in early childhood, it is associated with anorexia, poor linear growth, and impaired immune competence. All of these issues increase the risk of childhood illnesses and overall mortality.[47] Based upon fasting blood samples or serum zinc concentrations, lower serum zinc concentrations were reported among the young women who were vegetarians when compared with omnivores.[48]

Iodine

Plant-based diets are typically low in iodine, which is why vegetarians and vegans need to make sure they are consuming adequate iodine.[10] Vegetarian diets, especially vegan diets, have a low amount of iron because animal products such as eggs, cheese, milk, meat, fish, and other poultry are high in iron. Also, depending upon the iodine content of the soil that the produce is grown in, the plant foods will have a lower iodine content as well. Research shows that vegans who do not consume iodine supplements,

seaweed and/or iodine-rich products have inadequate intakes of dietary iodine. In a group of North American vegetarians who only used uniodized sea salt, they had significantly lower serum iodine concentrations. Researchers have found that iodine content is higher in non-vegetarian diets than in vegetarian diets.[49] One study looked at the iodine intake and iodine deficiency among thirty vegans. The researchers found that vegans fall into the 'at risk' for iodine deficiency category.[50] Other research also confirms that due to the low iodine content of vegetables and fruits, vegetarians and others following restrictive diets are at a higher risk of iodine deficiency. Vegetarians and vegans that are pregnant or plan to become pregnant, should consume iodine-containing prenatal vitamins to decrease their risk of iodine deficiency.[51] In addition, iodine has a positive correlation with the antioxidant glutathione (GSH). This was demonstrated in a study involving rats. The researchers concluded that acute iodine ingestion will lead to an increase in intrathyroidal glutathione.[52] Another study demonstrated how when dietary iodine was added in the thyroid, glutathione perosidase (GSH-Px) secretion was increased. This led to a further effect on thyroid hormone synthesis.[53]

Calcium

Lacto-ovo-vegetarians are noted as having calcium intakes similar to or even higher than nonvegetarians. Vegans, since they do not eat any products derived from animals, tend to fall below the recommended intakes. Various plants such as low-oxalate greens (e.g., bok Choy, broccoli, Chinese cabbage, collards, and kale) can be good sources of calcium that is highly bioavailable.[10] The bioavailability of calcium from plant foods can be affected by their content of inhibitors of calcium absorption content. Such

inhibitors include oxalate and phytate.[54] In a cross-sectional survey, calcium status was analyzed in 236 children from India aged 7-19 years old. The researchers found that calcium intake was lower in vegetarians. The study also looked at how if non-dairy-based, calcium-rich products were consumed, it helped to reduce calcium deficiency in Indian adolescents.[55] Inadequate calcium intake is a risk factor for bone loss. Due to the fact that sodium and calcium both utilize the same transport systems in the proximal tubule, when sodium is excreted from the kidney, it pulls calcium out with it.[54] Researchers from a study looking at Irish individuals found that for each 1-gram of sodium excreted, 26.3 milligrams of calcium per day was lost in the urine. Therefore, 1 gram extra of sodium consumed in the diet will lead to an additional 1% per year of bone loss.[56] In addition, dietary protein plays a role on calcium balance. When a person increases the amount of protein consumed, an increased amount of calcium is lost in the urine due to decreased fractional tubular reabsorption.[57] If a well-balanced diet with adequate dietary calcium is consumed, a vegetarian can obtain adequate calcium levels. Obtaining adequate dietary calcium intake can easily be done by consuming dairy products, calcium-fortified products, or supplements. For the vegetarians that get most of their calcium from plant sources or vegans, they need to be aware of the various factors such as protein and salt intake, that affect calcium levels.[54]

Vitamin D

Low serum 25-hydroxyvitamin D, low vitamin D intakes, and reduced bone mass has been reported in some vegan and macrobiotic groups who did not supplement with vitamin D supplements or fortified foods.[10] Macrobiotic diet is a diet consisting mainly of

whole grains and beans but also may include fish.[51] This can play a role in bone healthy which is why both vegans and vegetarians need to ensure they are meeting their vitamin D needs.[10] One study looked 1388 meat eaters, 210 fish eaters, 420 vegetarians, and 89 vegans from the European Prospective Investigation into Cancer and Nutrition-Oxford cohort and they measured their plasma 25-hydroxyvitamin D concentrations. The goal was to compare the differences in vitamin D intake and plasma concentrations among the four groups. The researchers found that plasma 25-hydroxyvitamin D concentrations were lower in vegetarians and vegans than in meat and fish eaters. This led the researchers to conclude that diet is an important factor to determine the plasma levels of 25-hydroxyvitamin D in this specific British population.[58] Another study took 23 vegetarian and 16 non-vegetarian Asians and compared their plasma 25-hydroxyvitamin D concentrations. The researchers found that the vegetarian Asians had lower levels than the non-vegetarian Asians.[59] Another study found that out of 297 adult Asians and 68 white subjects, the subjects following a vegetarian diet had an impaired seasonal peak during the summer season in 25-hydroxyvitamin D status which put them at risk for metabolic bone disease.[60]

Creatinine

Creatinine is used for energy metabolism in the body. Creatinine is a product of when creatine phosphate in muscle breaks down. Serum creatinine is measured by commercially available assays and can be used as an indirect marker of renal function.[61] When comparing vegetarians with non-vegetarians, muscle creatinine stores are lower in vegetarians. This might be explained by most sources of creatine are found in meat, fish

and poultry.[62] Many people including sports enthusiasts supplement creatinine claiming that it helps maintain energy phosphates during vigorous exercise and that it increase muscle mass during training.[63] Almost two thirds of the total creatine in the muscles serves as an energy source during exercise in the storage form of creatine. Creatine supplementation may be required because it has been shown to increase muscle creatine concentration by >30%. It has been hypothesized that vegetarians are more responsive to supplements that aid in sport performance because they have lower muscle creatine concentration.[62] In another study, when subjects were put on a vegetarian diet, there was no abnormal increase in creatinine and serum creatinine was within normal range.[61]

Vitamin B12

Vegetarians tend to have an inadequate level of vitamin B12 due to low dietary sources of vitamin B12.[10] Vitamin B12 occurs naturally in foods of animal origin such as meat and fish.[64] Vitamin B12 is found in dairy sources or fortified foods in a vegetarian diet but must be consumed regularly. The risk with inadequate vitamin B12 intake is serious which is why vegetarians and vegans need to ensure they consume enough vitamin B12.[10] If a person experiences a vitamin B12 deficiency, the side effects are rather severe. It can manifest as gastrointestinal, hematological, and/or neuropsychiatric signs. Some common signs and symptoms of a vitamin B12 deficiency are depression, dementia, catatonia, and delirium.[65] In a cross-sectional analysis of 226 omnivores, 231 vegetarians, and 232 vegans from the European Prospective Investigation into Cancer and Nutrition Oxford cohort, omnivores had the highest mean serum vitamin B12 status. Vegans had the lowest mean serum vitamin B12 levels. 52% of vegans and 7% of

vegetarians were classified as being vitamin B12 deficient as defined by having a serum vitamin B12 level of less than 118 pmol/L.[64] In a reported clinical case, a female vegetarian patient was experiencing delirium due to a vitamin B12 deficiency. The patient was given weekly injections of vitamin B12 (cyanocobalamin). Her vitamin B12 level and mental status normalized rapidly after the vitamin B12 supplementation.[65]

Due to these possible nutrient deficiencies, it is recommended in various studies that meeting with a health professional is recommended to avoid or minimize risks. This is demonstrated by studies that show how vegetarians who plan well-balanced meals and are informed have less nutritional deficiencies than those who have poor meal planning.[66] Vegetarian dietary needs change throughout the lifecycle but a well-planned vegetarian or vegan diet can meet a person's needs at any stage throughout their life including infancy, childhood, pregnancy, and lactation periods.[10] With all these nutrients of concern for vegetarians noted above, there is real concern regarding unhealthful outcomes for individuals following vegetarian diets. One study conducted in the Sahel region of Chad states that due to the low intake of protein and sulfur amino acids, vegetarians are at risk for subclinical protein malnutrition. Because of the subclinical protein malnutrition, this population also had hyperhomocysteinemia and atherogenesis. The vegetarian population deficient in sulfur amino acids had normal B vitamin status, normal serum lipids, and decreased lean body mass. The researchers stated that this all led to the down regulation of the trans-sulfuration pathway, hyperhomocysteinemia, and oxidative stress. The study stated how plant-based regimens do not fulfill the nutritional requirements of populations living in developing countries as evidenced by their hyperhomocysteinemia. This study

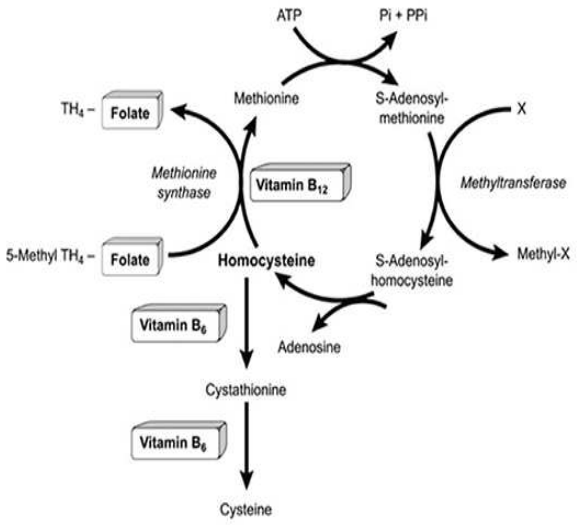
was limited due to their small number of participants of 24 males in the experiment group and 15 in the control group but reveals some interesting counter ideas.[67] Another study was conducted at Deenanath Mangeshkar Hospital and Research Centre on 119 young, healthy graduate Indian vegetarians. Half were deficient in B12 but had no clinical signs of vitamin B12 deficiency. 92% of males and half of females had hyperhomocysteinemia. One of the limitations of this study is that plasma methylmalonic acid (MMA) had not been measured which can reveal vitamin B12 deficiency.[68]

Information about Vitamin B12

Vitamin B12 is one of the eight water-soluble vitamins, which also include thiamin (vitamin B1), riboflavin (vitamin B2), niacin (vitamin B3), vitamin B6 (pyridoxine), folate (folic acid), biotin, and pantothenic acid. It is found naturally in meat and animal products and is available as dietary supplement and prescription medication. The metabolism of vitamin B12 is shown in Figure 4.

Figure 4: Vitamin B12 and Homocysteine Metabolism[69]

Figure 1. Vitamin B_{12} and Homocysteine Metabolism



Methionine synthase is a vitamin B_{12}-dependent enzyme that catalyzes the formation of methionine from homocysteine using 5-methyltetrahydrofolate (5-methyl TH_4), a folate derivative, as a methyl donor. Another pathway catalyzed by betaine homocysteine methyltransferase also remethylates homocysteine to methionine using betaine as a methyl donor (not shown here). Methionine, in the form of S-adenosylmethionine, is required for most biological methylation reactions, including DNA methylation.

Dietary vitamin B12 is bound to protein in the food and in the active absorption mechanism, it is released by hydrochloric acid and gastric protease in the stomach and bound to a salivary R-binder. It is released into the upper small intestine and attached to the intrinsic factor (IF). The vitamin B12-IF complex travels to the lower end of the small intestine and is absorbed by certain ileal receptors.[70] Cubilin, a peripheral membrane protein, is the receptor present in the epithelium of the intestine that facilitates the uptake of vitamin B12-IF. Cubilin is dependent upon the membrane protein amnionless (AMN) in order to efficiently absorb the vitamin B12-IF into the intestine. Issues or a deficiency of cubilin can lead to various health issues and diseases.[71,72] Vitamin B12 is transported by the transport proteins haptocorrin and transcobalamin in human plasma.[70] Transcobalamin II is essential for the transport of plasma cobalamin into all cells. It is a nonglycoprotein secretory protein.[73] For the passive mechanism of vitamin B12

absorption, it is absorbed throughout the absorptive surface of the gastrointestinal tract but only about a limited 1-2% of an oral dose can be absorbed.[70(p12)] Synthetic vitamin B12 found in fortified foods and dietary supplements is in free form and does not need to be separated like the vitamin B12 found in food sources. Vitamin B12 contains the mineral cobalt, which is why it is also called cobalamin with other compounds using vitamin B12 activity. Vitamin B12 is needed for erythropoiesis, neurological function, DNA synthesis, methionine synthase and L-methylmalonyl-CoA mutase. Vitamin B12 aids in the conversion of homocysteine to methionine, which is a required to form the universal methyl donor S-adenosylmethionine. S-adenosylmethionine is a donor for about 100 substrates including but not limited to DNA, RNA, hormones, proteins, and lipids. The recommended intake for vitamin B12 is shown in Table 3.

Table 3: Recommended Dietary Allowance (RDAs) for Vitamin B12

Recommended Dietary Allowances (RDAs) for Vitamin B12				
Age	**Male**	**Female**	**Pregnancy**	**Lactation**
0-6 months*	0.4 mcg	0.4 mcg		
7-12 months*	0.5 mcg	0.5 mcg		
1-3 years	0.9 mcg	0.9 mcg		
4-8 years	1.2 mcg	1.2 mcg		
9-13 years	1.8 mcg	1.8 mcg		
14+ years	2.4 mcg	2.4 mcg	2.6 mcg	2.8 mcg

** Adequate intake is provided by the Food and Nutrition Board (FNB) and is equivalent to the mean intake of vitamin B12 in healthy, breastfed infants.*

Groups that are at risk for vitamin B12 deficiency are older adults, individuals with pernicious anemia, individuals with gastrointestinal disorders, individuals who have had gastrointestinal surgery, vegetarians, pregnant and lactating women who follow strict vegetarian diets and their infants.[74] The reason for this is either due to inadequate dietary intake or malabsorption such as malabsorption from food, pernicious anemia, postsurgical malabsorption.[70,74,75]

Signs of a vitamin B12 deficiency are unusual fatigue, digestion problems, and frequent upper respiratory infections. Signs of cobalamin malabsorption are hematologic (pernicious anemia) and neurologic symptoms.[76(p12)] The most frequent genetic disorder of vitamin B12 is cobalamin deficiency type C with methylmalonic aciduria and homocysteinuria. The Cbl-C defect impairs the conversion of dietary cobalamin into the two metabolically active forms called methycobalamin and adenosylcobalamin. This then will lead to an increase in homocysteine and methylmalonic acid. There will also be a reduction in the synthesis of methionine. Due to the difficulty in diagnosing Cbl-C defect, the defect can present itself anywhere between the neonatal period to childhood or later in life.[77,78]

Table 4: The clinical and neuroradiological signs in Cbl-C defect according to the age of disease onset[77]

	Early Onset	Late Onset
General Signs		
Blood cytopenia	+	+/-

Acidosis	+	-
Feeding difficulties	++	-
Failure to thrive	++	-
Hemolytic uremic syndrome	+	+/-
Renal failure	+/-	+/-
Dysmorphic features	+	-
Cardiopulmonary signs	+	-
Thromboembolic events	-	+/-
Atrophic gastritis	+/-	-
Neurological signs		
Hypotonia	++	-
Developmental delay/mental retardation	++	+/-
Seizures	+	-
Psychiatric signs	-	++
Microcephaly	+	-
Spasticity	-	+
Myelopathy	-	++

Gait abnormalities	-	++
Brain MRI		
Hydrocephalus	+	-
White matter alterations	+	+
Brain atrophy	++	+
Basal ganglia lesions	+	-
Ocular signs		
Nystagmus	++	-
Visual impairment	++	+/-
Optic atrophy	+	-
Pigmentary retinopathy	+	+/-

Researchers believe that the Cbl-C patients can affect such a variety of systems in the body could be due to methylmalonate toxicity, homocysteine toxicity, and/or methionine deficiency. These particular patients are treated with hydroxocobalamin and folic acid. The hydroxocobalamin (OHCbl) increases intracellular cobalamin and maximized deficient enzyme activity. Folic acid enhances the remethylation pathway.[77,78]

Homocysteine

Studies have revealed that vegetarians, especially vegans who consume no animal products, have a lower serum vitamin B12 and higher homocysteine concentrations than omnivores.[11,76,79–86,87(p),88–94] Elevated plasma homocysteine can be one of the detectors of vitamin B12 deficiency but not the only one because vitamin B6 and folate deficiencies also elevate plasma homocysteine concentrations. Homocysteine is an amino acid. It mainly comes from dietary sources of methionine, an essential amino acid. If folate, vitamin B12, and vitamin B2 are available, homocysteine can be re-methylated to methionine. If vitamin B6 is available, it can also be converted to cysteine in the transsulfuration pathway. Other factors can also cause a change in homocysteine metabolism such as several SNPs that affect folate metabolism, genetic defects, renal failure, or deficiencies of folate, vitamin B12, vitamin B6, and vitamin B2.[70]

Increasing evidence has revealed that low serum cobalamin status results in elevated serum homocysteine in all ages.[95–99] Studies show lower vitamin B12 and higher homocysteine concentrations in vegetarians when compared to omnivores.[70] Studies from India revealed that there was vitamin B12 deficiency and elevated homocysteine levels in both vegetarians and omnivores. The researchers studied 441 middle-aged vegetarian and nonvegetarian men from rural areas, slum dwellers, and urban areas. The results showed that 67% of all the men recruited had low vitamin B12 concentration and elevated homocysteine levels. Vegetarians had 4.4 times higher risk of vitamin B12 deficiency and 3.0 times greater risk of hyperhomocysteinemia when compared to those who frequently ate non-vegetarian foods. They also found that low vitamin B12 concentration and hyperhomocysteinemia was more common in urban middle class residents. The study limitation was that they were solely looking at men from India but the results solidify

how vitamin B12 and homocysteine are inversely related. This means that the lower the vitamin B12 status, the higher the level of homocysteine.[76] Since the vegetarian diet is consumed in all geographic areas, another study looked at b-vitamin status and concentrations of homocysteine in Austrians. 4% (320,000 people) of Austrian adults stated that they are vegetarian according to the European Vegetarian Union. 118 participants ranging in ages from 19 to 65 years old were studied. The researchers defined vegetarian as a person who does not eat meat, fish or poultry. A vegan was described as someone who avoids all products of animal origin. The researchers found that vegans had a significantly higher mean intake of folate when compared to vegetarians and omnivores. Vegans had a mean intake of vitamin B12 that was significantly lower than vegetarians and omnivores. For vegetarians, they found that the mean plasma vitamin B12 concentrations were in the normal range, which was also seen in the omnivore population. The researchers predicted that it was due to the consumption of dairy products and eggs in the vegetarian diet. Vitamin B12 deficiency was only seen in 2.4% of the vegans. Moderate hyperhomocysteinemia was seen in over 65% of the vegans, 52% of the vegetarians and 45% of the omnivores. Significant inverse correlations between homocysteine and vitamin B12 were demonstrated by the authors of this study within the vegetarian and omnivore groups.[11] Many other studies reveal these findings that vegans have a lower serum concentration of vitamin B12 than omnivores and vegetarians. The EPIC study consisted of 689 men-226 omnivores, 231 vegetarians, and 232 vegans. It found that the mean serum vitamin B12 concentration was 33% lower in vegans than in vegetarians. Vegans were 57% lower than omnivores. Vegetarians were 35% lower than omnivores when looking at mean serum vitamin B12 concentration. In

regards to deficiency of <118 pmol/l, 52% of vegans, 7% of vegetarians and one omnivore fell into that category.[64]

Pathogenesis of Hyperhomocysteinemia

Homocysteine, a sulfur-containing, non-protein amino acid, is at the intersection of remethylation to methionine requiring folate and vitamin B12 and transsulfuration to cystathionine requiring pyridoxal-5'-phosphate. In the metabolic pathway of remethylation, homocysteine accepts a methyl group from N-5-methyltetrahydrofolate or from betaine, an enzyme, to produce methionine. Both are dependent upon vitamin B12 in order to have a methyl group to transfer to from homocysteine to methionine. With the aid of ATP, methionine becomes activated to form S-adenosylmethionine (SAM) which is a universal methyl donor. The by-product of the methylation reactions is S-adenosylhomocysteine (SAH). SAH is then hydrolyzed and recreates homocysteine, which will again begin a new methyl-group transfer cycle.[100] The normal values for homocysteine are 12.4 [11.8-13.0] μmol/L.[101] The issues associated with elevated plasma homocysteine levels include an increased risk of neural tube defects.[102,103] Another study analyzing a chicken embryo model revealed that elevated homocysteine levels causes dysmorphogenesis of the heart, neural tube, and ventral wall.[104] A variety of other studies reveal an association with hyperhomocysteinemia and Alzheimer's disease, dementia, and loss of cognitive function.[105–108] The following problems can cause an increase in homocysteine levels.

Lack of N-5-Methyltetrahydrofolate Synthesis

A folate deficiency or a defect in the enzyme MTHFR can cause a decrease in the synthesis of methionine. This is because N-5-methyltetrahydrofolate must be available in order to create methionine. If this occurs, then homocysteine does not go to the remethylation pathway and will head to the transsulfuration pathway.[100,109] Folate deficiencies have been noted in alcoholics and folate deficiencies encourage hyperhomocysteinemia which can lead to various health issues noted above[100,110,111]. This additional homocysteine causes issues because there is an decreased SAM concentration within the cell and the fact that there is not enough N-5-methyltetrahydrofolate it will cause the glycine N-methyltransferase (GNMT) to be active. This will further decrease SAM concentration and increase homocysteine as a by-product of the methylation of glycine. This will create an increased homocysteine and decreased SAM concentration. The decreased SAM concentration prohibits the initial reaction of transulfuration, which is the activation of cystathionine synthesis, to occur. Homocysteine will then increase within the cell and spill out into the blood causing hyperhomocysteinemia.[100]

Issues with Homocysteine Transsulfuration

When the Transsulfuration pathway is severely impaired, it affects the remethylation pathway because there is a redirection of homocysteine toward the remethylation pathway. When this occurs, greater amounts of methionine are produced and SAM concentration increases. The increase in SAM will eventually

inhibit MTHFR and the entire remethylation system is stopped. Both pathways to break down homocysteine are affected and hyperhomocysteinemia occurs.[100]

Issues associated with Hyperhomocysteinemia

Hyperhomocysteinemia creates oxidative stress in the body due to an increase in the production of reactive oxygen species and can lead to serious health issues. When there is an increase in oxidative stress, such as with elevated homocysteine concentrations, it will lead to increased activation of macrophages. Macrophages play a roll in inducing atherosclerotic plaque. Hyperhomocysteinemia leads to endothelial dysfunction, which promotes the progression of atherosclerosis. Hyperhomocysteinemia also creates vascular changes.[15] The reason for concern when looking at elevated homocysteine concentrations is because it has been positively associated with the risk of ischemic heart disease, deep vein thrombosis, pulmonary embolism, and stroke.[12,112,113] All of these are linked to cardiovascular disease as revealed in current research. The researchers found that by reducing homocysteine concentrations with increasing folic acid intake by 3 umol/L, it reduces the risk of disease (see Table 5)

Table 5: Health Issue and Reduction in Risk with folate supplementation

Health Issue	**Reduction in Risk**
Ischemic heart disease	16%
Deep vein thrombosis with or without pulmonary embolism	25%
Stroke	24%[12]

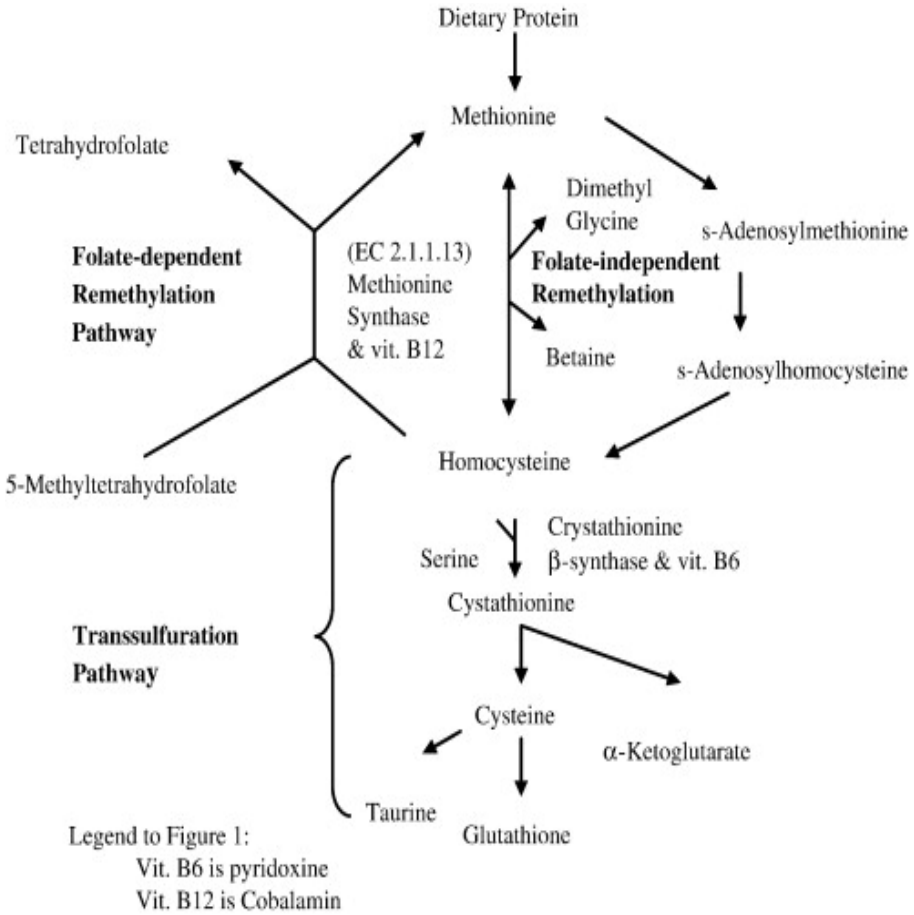
This is an easy and painless way to prevent hyperhomocysteinemia. The results from another study, which looked at 133 asymptomatic patients, indicate that participants with an elevated homocysteine concentration of ≥12 umol/L presented with a mean increase of coronary calcium by 35% per year. Patients with a homocysteine concentration of <12 umol/L only had a 17% progression of coronary calcium per year. That led the researchers to determine that when homocysteine concentrations are >12 umol/L, it can strongly predict the progression of atherosclerotic plaque burden which is a precursor in predicting future cardiovascular events.[14] Homocysteine has also been a risk factor for physical impairment, neurogenetive disease, and alcoholic liver disease. Alcohol related hyperhomocysteinemia has been seen in human and rodents as they develop alcoholic steatohepatitis. On the other hand, homocysteine and glutathione has been demonstrated to play crucial reduction-oxidation mediators for each other. Homocysteine provides oxidative stress upon the body and glutathione reduces that oxidative stress.[114(p2)]

Glutathione

Glutathione is a thiol-containing tripeptide and antioxidant. It plays a vital role in intracellular defense. The reference range for healthy adults ages 18-73 years were 3.39 +/- 1.04 mumol/L for plasma GSH.[115] Decreased levels of glutathione are correlated with increased occurrence of chemically reactive chemical species containing oxygen mediated mitochondrial damage.[116–119] Glutathione is made in the transsulfuration pathway and is present in all mammalian tissues with the highest concentrations in the

liver.[120,121] When there is oxidative stress, cystathionine-beta-synthase is activated via glucocorticoids and signals the need for the conversion of methionine to cysteine to glutathione. Insulin inhibits the transcription of methionine to glutathione. Homocysteine provides a sulfur group from the methionine cycle and creates cysteine. Cysteine then turns into glutathione.[120] Within the liver cells, two types of intracellular cysteine aids in making glutathione. The first one is from bringing in the amino acid that is outside the cell. The second one is via the homocysteine-dependent transsulfuration pathway.[122]

Figure 5: Homocysteine removal via the remethylation and transsulfuration pathways[109]



Oxidative stress in macrophages will lead to a depletion and compensatory increase in the creation of antioxidants, such as glutathione. For example, when homocysteine concentrations increase, GSH concentrations will go up within the cell. This reveals how homocysteine may actually play a beneficial role in antioxidant reactions due to the fact that homocysteine is needed to make glutathione. Researchers think this is either due to transsulfuration pathway that creates the precursor for GSH, cysteine. Another explanation is that it can be caused by an increase in de novo synthesis.[120] Another study's results indicated that homocysteine causes oxidative stress because it induces glutamate-cysteine ligase (Gcl) within mouse macrophages and it occurs as a result of an antioxidant response within the catalytic Gclc promoter triggered by the antioxidant response elements (AREs). The increase in Gclc expression when homocysteine is present is due to the increased de novo gene expression. This was revealed by the increase in luciferase activity from Gclc promoter/luciferase reporter construct.[15] One study's results indicate that increased homocysteine concentrations were associated with higher systemic blood pressures but it also showed that when glutathione concentrations were high, it led to lower blood pressures. This might be a demonstration of the protective balance that elevated glutathione has upon elevated homocysteine levels.[123] Researchers saw that when oxidative stress oxidative metabolites such as homocysteine and oxLDL increase in the body, the levels of glutathione (GSH) become depleted. When a significant amount of GSH has been depleted, it will trigger a compensatory effect which causes an increase in GSH levels.[15] Methionine and other metabolites of methionine have been shown to play a role in antioxidative processes. A group of researchers studied Ames dwarf mice. The Ames dwarf mice live 49-64%

longer than their normal counterparts and do not have growth hormone, prolactin, and thyroid-stimulating hormone. The study's goal was to determine if methionine metabolism was altered in Ames dwarf mice and if that alteration explained their extended lifespan when compared to wild type mice. Current research has shown how reduced growth hormone (GH) signaling is linked to lower oxidative stress and therefore longer lifespan in dwarf mice. [16]

CHAPTER 3

METHODS

Healthy (disease-free by self-report), non-smoking men and women between the ages of 18 and 65 years of age were recruited from a campus population in Phoenix Arizona in the spring of 2018. 16 omnivores and 17 vegetarians were recruited for this study. Medication use was permitted if use was consistent for >3 months; however, adults taking medications that altered the methylation process were excluded from the study. Both vegetarians and omnivores were recruited. A vegetarian diet was defined as one that excludes red meat, poultry, pork and seafood but allows dairy products and/or eggs; the diet had to be followed for at least one year. An omnivore diet includes all animal products. Exclusion criteria included those unable to consent, unwilling to come to the lab for testing and/or unwilling to follow the study protocol, and women who were pregnant, pregnant within the past 3 months, or lactating. Recruitment began after IRB approval, and all participants provided written consent.

Trial was conducted between January 2018 and April 2018. Methods of recruitment were list serves and fliers posted at farmers markets and Seventh Day Adventists churches. List serves at Arizona State University and businesses and organizations in the Phoenix, Arizona area were utilized to send ads. Interested parties completed an online survey to determine if they met the inclusion criteria. People who qualified were contacted by investigators and invited to 1-2 visits at the Arizona Biomedical Collaborative building on the downtown ASU campus. The first visit was the screening, which took up to 30 minutes to ensure eligibility was met. If the participant was fasting testing, blood samples were drawn. If the participant was not fasting, they

returned for a second visit to obtain a fasting blood sample. During the visit, the participant provided written consent and completed a diet questionnaire and a vitamin B12, B6 and folate food questionnaire. Height, weight, and waist circumference were taken using a stadiometer (SECA 213 Portable Stadiometer, SECA North America, Chino, California), electronic weighing scale (Tanita TBF 300A, Tanita, Arlington Heights, Illinois), Gulick tape measure (Gulick Tape Measure). For the participants that verified they were fasting through verbal consent (i.e., no food or drink, except for water for 10 hours), about 2 tablespoons of the participant's blood was collected. Blood samples were labeled with the subject numbers and dates and stored at -80° until analyses.

Fasting blood samples were analyzed for blood B12, homocysteine, and glutathione. Plasma vitamin B12 and homocysteine concentrations were obtained from the parent study titled 'Assessing the Relationship Between Cobalamin Deficiency and Methylation Capacity in a Vegetarian Population'. Total glutathione (GSSG/GSH) was found from the participant blood samples using the OxiSelect Total Glutathione (GSSG/GSH) Assay Kit. The glutathione was analyzed using the Gen5 2.0 All-in-One Microplate Reader Software by BioTek. The plate reader was a Synergy #1 hybrid reader by BioTek. BioTek is located in Winooski, Vermont. Risks included mild discomfort, possible nausea, dizziness, fainting and bruising at the site of needle infection due to the venous blood draw which is why a trained phlebotomist collected blood samples and monitored participants. For statistical analysis, results were expressed as means ± SD. After checking for normality, t-tests were used to compare vegetarian and omnivore groups (SPSS Version 24, SPSS Inc., Chicago, Illinois). Data was considered significant

at $p \leq 0.05$. Deidentified data was used, entered into excel/SPSS files and stored on password-protected computers.

CHAPTER 4

RESULTS

A total of 16 omnivores and 17 vegetarians completed the trial. The mean age of omnivores was 29.4 ± 9.1 years old in comparison to the mean age of vegetarians, which was younger at 27.7±9.0 years old. The BMI of omnivores was 23.2±2.8 kg/m^2 in comparison with vegetarian mean BMI being 21.9±2.5 kg/m^2.

Table 6: Demographics Chart

	Units	**Omnivores: Mean**	**Vegetarians: Mean**	**p-Value**
Age	Years	29.4±9.1	27.7±8.9	0.464
Weight	Kilograms	64.8±9.4	62.0±8.2	0.369
Height	Centimeters	166.9±6.4	168.2±7.6	0.616
BMI	Kilogram/meters^2	23.2±2.8	21.9±2.5	0.171
Waist Circumference	Centimeters	77.6±7.7	76.4±15.1	0.776
Physical Activity	METS	50.5±30.7	53.9±24.6	0.729
Diet Quality	Score	37.7±3.1	37.8±2.8	0.895
Glutathione	mmol	2.3±0.7	1.9±0.5	0.046

Mean plasma B12 and homocysteine concentrations did not differ by diet group. The mean glutathione was higher in omnivores in comparison with vegetarians (2.3±0.7

and 1.9±0.5 mmol respectively). Using a non-parametric Mann-Whitney U Test due to small sample size, glutathione was significantly lower among vegetarians as compared to omnivores with a p-value of 0.046. The reference range for GSH in plasma can be seen in Table 7.[115]

Table 7: Literature values of plasma GSH concentrations according to sex and/or age[115]

Reference Number	Sex	Age (Years)	GSH (umol)
24	M; F	10-17	3.57±0.74
124	M; F	18-73	3.39±1.04
5	M	33.0±6	3.90±1.07
5	F	29.6±8	2.79±0.96
9	M	40.2±11.5	2.22±0.49
5	M	27-35	11.36±0.72
12	M	36-57	7.60±0.25

CHAPTER 5

DISCUSSION

The present study examined the difference in glutathione concentration in omnivores compared with vegetarians. The vitamin B12 and homocysteine levels were normal in the vegetarians and omnivores and did not differ between groups. Research currently reveals a strong, protective, antioxidant effect associated with elevated glutathione concentrations. It was hypothesized that vegetarians would have lower serum vitamin B12, a higher homocysteine concentration, and hence, an elevated glutathione level. This hypothesis was rejected because the omnivores displayed significantly elevated glutathione concentration compared to vegetarians. These data suggest that omnivores may have a greater antioxidant protection than vegetarians. However, current literature shows higher total antioxidant activity in vegetarians as compared to omnivores. Researchers looked at the common antioxidant vitamins including vitamin C, vitamin E, beta-carotene in vegetarians compared with omnivores. Researchers found that vegetarians have significantly higher levels of the antioxidant vitamins in the plasma or serum than omnivores. The researchers attribute this to the high amount of fruits, vegetables, and nuts that vegetarians consume. All of which are high in antioxidants.[124] Another study that looked at the vegetarians in a sample size of 35,372 women in the UK found that vitamin and mineral intakes were high in the vegetarian group according to the food frequency questionnaires.[125] When plasma carotenoid levels were looked at in 31 vegetarians and 58 omnivores in northern Ireland, vegetarians had 15% higher levels. Carotenoids have protective effects such as anti-cacinogenic and anti-atherogenic.

However, in a separate study the overall antioxidant status was shown to be similar between vegetarians and omnivores.[126]

There is currently some research on the level of glutathione in vegetarians compared with omnivores. Most of the research looked at rural or undernourished populations. In a study looking at rural vegetarians in comparison with urban living vegetarians in Chad, the data revealed that rural living participants had a significantly lower glutathione level in blood than urban living vegetarians. It is likely that the vegetarians from the rural areas were undernourished as revealed by their body mass index, weight, and other dietary markers. Due to the undernourished body with assumedly elevated oxidative stress, their antioxidant stores were more depleted than the nourished population living in the urban areas.[67] This is why it is vital to look at where the studies were conducted. Most of the results show that omnivores and vegetarians do not have significantly different levels of glutathione. One study looked at 31 vegetarians and 58 omnivores in Northern Ireland. The researchers found that reduced glutathione concentrations were similar in vegetarians and omnviores.[126] Researchers in India analyzed 23 vegetarians and 22 fish eaters. Fish eaters were defined as subjects who consumed 4-6 fish dishes per week along with vegetables and occasionally other meats. The glutathione levels were similar in vegetarians when compared to the fish eaters.[127] When looking at antioxidant status, many researchers choose to look at glutathione peroxidase because it is a representation of selenium status, which is another antioxidant. A study from Korea took 45 vegetarians who had maintained a vegetarian diet for a minimum of 15 years and compared them to 30 omnivores. The researchers did not analyze glutathione levels but they did look at their diacron reactive oxygen metabolite

(d-ROM) which reflects oxidative stress. The researchers found that d-ROM concentrations was significantly lower in vegetarians than that in omnivores. This represents how vegetarians in this study were shown to have lower oxidative stress levels.[128] In addition to looking at oxidative stress, more research needs to be done into what exactly affects glutathione levels. Among Seventh Day Adventists from Georgia, USA, men were shown to have significantly higher glutathione than women. Their results displayed significantly higher plasma glutathione levels than the nonvegetarians. In addition, among the white participants, plasma glutathione concentrations increased with age in men but decreased with age in women. Oral contraceptives was somewhat associated with a decreased plasma glutathione concentration. The researchers of this study revealed how the total plasma glutathione concentrations in humans can be affected by demographic and health-related factors. All of these factors need to be taken into account when further research is conducted.[129] A similar study has been done but used participants from the Slovak Republic in comparison to the college students used from Phoenix, Arizona in this study. The researchers analyzed serum glutathione concentrations in 103 vegetarians and 123 non-vegetarians. Researchers found that the glutathione in blood and glutathione peroxidase activity in plasma and erythrocytes were both significantly lower than in non-vegetarians. The researchers were shocked as a result of the high level of antioxidants in a typical vegetarian diet. The researchers concluded that the vegetarians in the Slovak Republic consume a nutritionally imbalanced diet which includes minimal antioxidants such as selenium or high oxidative stress from the high environmental pollution levels in Slovakia.[130] It is interesting to see that even with

two very different populations; the glutathione status in vegetarians was lower than the non-vegetarians or omnivores.

One limitation that can be noted in this study is the small sample size of 34 participants. In addition, it was a cross-sectional study which means the data can only suggest a relationship. It would be beneficial to conduct an intervention to prove the connection. One example of a possible intervention would be to put omnivores on a plant-based diet, control their antioxidants, and then take a look at their antioxidant levels, including glutathione, to compare with the initial antioxidant levels. Another limitation is the fact that only one antioxidant was looked at. If other antioxidant concentrations, such as vitamin C or selenium, were low then the glutathione would need to be used to combat oxidation and protect the body. The assay or assay reader had some issues which means there could have been instrumentation issues and it could have affected the results. Most of the participants were females and oral contraceptive use was not checked. This is important because as the research shows, oral contraceptive use affects glutathione levels.[129] When looking at antioxidants, it is important to check the level of oxidative stress that the participant is undergoing because this will affect the antioxidant levels. This presents another limitation because oxidative stress levels were not checked.

The delimitations allow the results to be generalized to a healthy, campus population since most of the participants were college students in their 20's. In addition it was mostly women which can limit the results to women. It would have been beneficial to have a more varied sample pool to draw from in order to relate the results to a wider population of people.

CHAPTER 6

CONCLUSION

After the result of this study, it can be concluded that an omnivore diet is more likely to have more glutathione in their diet when compared to vegetarians. The reason for this is unknown and more research needs to be done to reveal those facts. It is important for the public to understand how to get an adequate amount of vitamin B12 in their diet so their body can be able to make enough glutathione via the transsulfuration pathway.

The hypothesis was shown to be incorrect that vegetarians would have a higher glutathione level than omnivores as a result of their minimal consumption of vitamin B12. This was shown to be incorrect due to the significantly larger concentrations of glutathione in the omnivores used in this study. At this time, a vegetarian diet cannot be recommended for increasing glutathione levels in the body based upon this research. As stated in the introduction, there are plenty of health benefits for eating a vegetarian diet but increased glutathione concentrations is not one of them. Continuation of this research may eventually reveal why this is the case.

More research needs to be done looking at a well-nourished population and check more of their antioxidant levels including glutathione. In addition it would be beneficial to check oral contracptive use, oxidative stress levels, and a greater number of participants. This would make these results more applicable to the general population. In conducting more research on this topic, the general public could get more insights into how to maintain elevated antioxidant levels and the numerous benefits.

REFERENCES

1. Sinha R, Cross AJ, Graubard BI, Leitzmann MF, Schatzkin A. Meat Intake and Mortality: A Prospective Study of Over Half a Million People. *Archives of Internal Medicine*. 2009;169(6):562. doi:10.1001/archinternmed.2009.6

2. Larsson SC, Orsini N. Red Meat and Processed Meat Consumption and All-Cause Mortality: A Meta-Analysis. *American Journal of Epidemiology*. 2014;179(3):282-289. doi:10.1093/aje/kwt261

3. Sun Q. Red Meat Consumption and Mortality: Results From 2 Prospective Cohort Studies. *Archives of Internal Medicine*. 2012;172(7):555. doi:10.1001/archinternmed.2011.2287

4. Rohrmann S, Overvad K, Bueno-de-Mesquita HB, et al. Meat consumption and mortality - results from the European Prospective Investigation into Cancer and Nutrition. *BMC Medicine*. 2013;11(1). doi:10.1186/1741-7015-11-63

5. American Diabetes Association. Standards of Medical Care in Diabetes-2017. *The Journal of Clinical and Applied Research and Education*. 2017;40(1):S34-41.

6. American Cancer Society. ACS Guidelines for Nutrition and Physical Activity. https://www.cancer.org/healthy/eat-healthy-get-active/acs-guidelines-nutrition-physical-activity-cancer-prevention/guidelines.html. Accessed August 8, 2018.

7. Tuso P. Nutritional Update for Physicians: Plant-Based Diets. *The Permanente Journal*. 2013;17(2):61-66. doi:10.7812/TPP/12-085

8. ReportBuyer. Top Trends in Prepared Foods 2017: Exploring trends in meat, fish and seafood; pasta, noodles and rice; prepared meals; savory deli food; soup; and meat substitutes. https://www.reportbuyer.com/product/4959853/top-trends-in-prepared-foods-2017-exploring-trends-in-meat-fish-and-seafood-pasta-noodles-and-rice-prepared-meals-savory-deli-food-soup-and-meat-substitutes.html. Published June 2017. Accessed August 8, 2018.

9. The Vegetarian Resource Group (VRG). HOW MANY ADULT VEGETARIANS IN THE U.S.? https://www.vrg.org/press/201511press.htm. Accessed August 8, 2018.

10. Position of the American Dietetic Association: Vegetarian Diets. *Journal of the American Dietetic Association*. 2009;109(7):1266-1282. doi:10.1016/j.jada.2009.05.027

11. Majchrzak D, Singer I, Männer M, et al. B-Vitamin Status and Concentrations of Homocysteine in Austrian Omnivores, Vegetarians and Vegans. *ANM*. 2006;50(6):485-491. doi:10.1159/000095828

12. Wald DS. Homocysteine and cardiovascular disease: evidence on causality from a meta-analysis. *BMJ*. 2002;325(7374):1202-1206. doi:10.1136/bmj.325.7374.1202

13. Lowering Homocysteine in Patients With Ischemic Stroke to Prevent Recurrent Stroke, Myocardial Infarction, and Death: The Vitamin Intervention for Stroke Prevention (VISP) Randomized Controlled Trial | Cerebrovascular Disease | JAMA | JAMA Network. https://jamanetwork-com.ezproxy1.lib.asu.edu/journals/jama/fullarticle/198139. Accessed August 8, 2018.

14. Rasouli ML, Nasir K, Blumenthal RS, Park R, Aziz DC, Budoff MJ. Plasma homocysteine predicts progression of atherosclerosis. *Atherosclerosis*. 2005;181(1):159-165. doi:10.1016/j.atherosclerosis.2005.01.001

15. Bea F, Hudson FN, Neff-LaFord H, et al. Homocysteine stimulates antioxidant response element-mediated expression of glutamate-cysteine ligase in mouse macrophages. *Atherosclerosis*. 2009;203(1):105-111. doi:10.1016/j.atherosclerosis.2008.06.024

16. Uthus E. Altered methionine metabolism in long living Ames dwarf mice. *Experimental Gerontology*. 2003;38(5):491-498. doi:10.1016/S0531-5565(03)00008-1

17. Barr SI, Rideout CA. Nutritional considerations for vegetarian athletes. *Nutrition*. 2004;20(7-8):696-703. doi:10.1016/j.nut.2004.04.015

18. Pribis P, Pencak RC, Grajales T, Pribis P, Pencak RC, Grajales T. Beliefs and Attitudes toward Vegetarian Lifestyle across Generations. *Nutrients*. 2010;2(5):523-531. doi:10.3390/nu2050523

19. Derbyshire EJ. Flexitarian Diets and Health: A Review of the Evidence-Based Literature. *Front Nutr*. 2017;3. doi:10.3389/fnut.2016.00055

20. Forestell CA. Flexitarian Diet and Weight Control: Healthy or Risky Eating Behavior? *Front Nutr*. 2018;5. doi:10.3389/fnut.2018.00059

21. Are we eating less meat a british social attitudes report. The British Library. https://www.bl.uk/collection-items/are-we-eating-less-meat-a-british-social-attitudes-report. Accessed February 21, 2019.

22. Micha R, Wallace SK, Mozaffarian D. Red and Processed Meat Consumption and Risk of Incident Coronary Heart Disease, Stroke, and Diabetes Mellitus: A Systematic Review and Meta-Analysis. *Circulation*. 2010;121(21):2271-2283. doi:10.1161/CIRCULATIONAHA.109.924977

23. Pan A, Sun Q, Bernstein AM, et al. Red meat consumption and risk of type 2 diabetes: 3 cohorts of US adults and an updated meta-analysis. *The American Journal of Clinical Nutrition*. 2011;94(4):1088-1096. doi:10.3945/ajcn.111.018978

24. Zheng W, Lee S. Well-done meat intake, heterocyclic amine exposure, and cancer risk. *Nutrition & Cancer*. 2009;61(4):437-446. doi:10.1080/01635580802710741

25. Kahleova H, Matoulek M, Malinska H, et al. Vegetarian diet improves insulin resistance and oxidative stress markers more than conventional diet in subjects with Type 2 diabetes: Vegetarian diet in Type 2 diabetes, a randomized study. *Diabetic Medicine*. 2011;28(5):549-559. doi:10.1111/j.1464-5491.2010.03209.x

26. Kahleova H, Hrachovinova T, Hill M, Pelikanova T. Vegetarian diet in type 2 diabetes – improvement in quality of life, mood and eating behaviour. *Diabetic Medicine*. 2013;30(1):127-129. doi:10.1111/dme.12032

27. Barnard ND, Cohen J, Jenkins DJA, et al. A Low-Fat Vegan Diet Improves Glycemic Control and Cardiovascular Risk Factors in a Randomized Clinical Trial in Individuals With Type 2 Diabetes. *Diabetes Care*. 2006;29(8):1777-1783. doi:10.2337/dc06-0606

28. Nicholson AS, Sklar M, Barnard ND, Gore S, Sullivan R, Browning S. Toward Improved Management of NIDDM: A Randomized, Controlled, Pilot Intervention Using a Lowfat, Vegetarian Diet. *Preventive Medicine*. 1999;29(2):87-91. doi:10.1006/pmed.1999.0529

29. Szeto YT, Kwok TC, Benzie IF. Effects of a long-term vegetarian diet on biomarkers of antioxidant status and cardiovascular disease risk. *Nutrition; Kidlington*. 2004;20(10):863-866. doi:http://dx.doi.org.ezproxy1.lib.asu.edu/10.1016/j.nut.2004.06.006

30. Huang T, Yang B, Zheng J, Li G, Wahlqvist ML, Li D. Cardiovascular Disease Mortality and Cancer Incidence in Vegetarians: A Meta-Analysis and Systematic Review. *ANM*. 2012;60(4):233-240. doi:10.1159/000337301

31. Mendis S. Global progress in prevention of cardiovascular disease. *Cardiovasc Diagn Ther*. 2017;7(Suppl 1):S32-S38. doi:10.21037/cdt.2017.03.06

32. Ornish D, Brown SE, Scherwitz LW, et al. Can lifestyle changes reverse coronary heart disease? The Lifestyle Heart Trial. *Lancet*. 1990;336(8708):129-133.

33. Food, nutrition, physical activity, and the prevention of cancer: a global perspective. *Choice Reviews Online*. 2008;45(09):45-5024-45-5024. doi:10.5860/CHOICE.45-5024

34. V M, R M, M M. *The Dietitian's Guide to Vegetarian Diets: Issues and Applications*. 2nd ed. Sudbury, MA: Jones and Bartlett Publishers; 2004.

35. Schaafsma G. The protein digestibility-corrected amino acid score. *J Nutr*. 2000;130(7):1865S-7S. doi:10.1093/jn/130.7.1865S

36. Kniskern MA, Johnston CS. Protein dietary reference intakes may be inadequate for vegetarians if low amounts of animal protein are consumed. *Nutrition; Kidlington*. 2011;27(6):727-730. doi:http://dx.doi.org.ezproxy1.lib.asu.edu/10.1016/j.nut.2010.08.024

37. Thorpe DL, Knutsen SF, Beeson WL, Rajaram S, Fraser GE. Effects of meat consumption and vegetarian diet on risk of wrist fracture over 25 years in a cohort of peri- and postmenopausal women. *Public Health Nutr*. 2008;11(6):564-572. doi:10.1017/S1368980007000808

38. Ho SC, Woo J, Lam S, Chen Y, Sham A, Lau J. Soy protein consumption and bone mass in early postmenopausal Chinese women. *Osteoporos Int*. 2003;14(10):835-842. doi:10.1007/s00198-003-1453-9

39. Lau EM, Kwok T, Woo J, Ho SC. Bone mineral density in Chinese elderly female vegetarians, vegans, lacto-vegetarians and omnivores. *Eur J Clin Nutr*. 1998;52(1):60-64.

40. Harris WS. Achieving optimal n–3 fatty acid status: the vegetarian's challenge... or not. *Am J Clin Nutr*. 2014;100(suppl_1):449S-452S. doi:10.3945/ajcn.113.071324

41. Sanders TAB. DHA status of vegetarians. *Prostaglandins, Leukotrienes and Essential Fatty Acids*. 2009;81(2):137-141. doi:10.1016/j.plefa.2009.05.013

42. Fokkema MR, Brouwer DAJ, Hasperhoven MB, Hettema Y, Bemelmans WJE, Muskiet FAJ. Polyunsaturated fatty acid status of Dutch vegans and omnivores. *Prostaglandins, Leukotrienes and Essential Fatty Acids (PLEFA)*. 2000;63(5):279-285. doi:10.1054/plef.2000.0215

43. Huang Y-C. Nutrient intakes and iron status of vegetarians. *Nutrition*. 2000;16(2):147-148. doi:10.1016/S0899-9007(99)00253-1

44. Huang Y-C, Lin W-J, Cheng C-H, Su K-H. Nutrient intakes and iron status of healthy young vegetarians and nonvegetarians. *Nutrition Research*. 1999;19(5):663-674. doi:10.1016/S0271-5317(99)00031-7

45. Wilson A, Ball M. Nutrient intake and iron status of Australian male vegetarians. *European Journal of Clinical Nutrition*. 1999;53(3):189-194. doi:10.1038/sj.ejcn.1600696

46. Gibson RS, Heath A-LM, Szymlek-Gay EA. Is iron and zinc nutrition a concern for vegetarian infants and young children in industrialized countries? *Am J Clin Nutr*. 2014;100(suppl_1):459S-468S. doi:10.3945/ajcn.113.071241

47. International Zinc Nutrition Consultative Group (IZiNCG), Brown KH, Rivera JA, et al. International Zinc Nutrition Consultative Group (IZiNCG) technical document #1. Assessment of the risk of zinc deficiency in populations and options for its control. *Food Nutr Bull*. 2004;25(1 Suppl 2):S99-203.

48. Iron and zinc status of young women aged 14 to 19 years consuming vegetarian and omnivorous diets.: Journal of the American College of Nutrition: Vol 14, No 5. https://www-tandfonline-com.ezproxy1.lib.asu.edu/doi/abs/10.1080/07315724.1995.10718537. Accessed February 25, 2019.

49. Remer T, Neubert A, Manz F. Increased risk of iodine deficiency with vegetarian nutrition. *British Journal of Nutrition*. 1999;81(1):45-49. doi:10.1017/S0007114599000136

50. Lightowler HJ, Davies GJ. Iodine intake and iodine deficiency in vegans as assessed by the duplicate-portion technique and urinary iodine excretion. *British Journal of Nutrition*. 1998;80(06):529-535. doi:10.1017/S0007114598001627

51. Fields C, Borak J. Chapter 54 - Iodine Deficiency in Vegetarian and Vegan Diets: Evidence-Based Review of the World's Literature on Iodine Content in Vegetarian Diets. In: Preedy VR, Burrow GN, Watson R, eds. *Comprehensive Handbook of Iodine*. San Diego: Academic Press; 2009:521-531. doi:10.1016/B978-0-12-374135-6.00054-6

52. Allen EM. Acute iodine ingestion increases intrathyroidal glutathione. *J Endocrinol Invest*. 1993;16(4):265-270. doi:10.1007/BF03348827

53. Qin F, Pan X, Yang J, et al. Dietary Iodine Affected the GSH-Px to Regulate the Thyroid Hormones in Thyroid Gland of Rex Rabbits. *Biol Trace Elem Res*. 2018;181(2):251-257. doi:10.1007/s12011-017-1064-5

54. Weaver CM, Proulx WR, Heaney R. Choices for achieving adequate dietary calcium with a vegetarian diet. *Am J Clin Nutr*. 1999;70(3):543s-548s. doi:10.1093/ajcn/70.3.543s

55. Sanwalka NJ, Khadilkar AV, Chiplonkar SA. Development of non-dairy, calciumrich vegetarian food products to improve calcium intake in vegetarian youth. *Current Science (00113891)*. 2011;101(5):657-663.

56. Shortt C, Madden A, Flynn A, Morrissey PA. Influence of dietary sodium intake on urinary calcium excretion in selected Irish individuals. *Eur J Clin Nutr*. 1988;42(7):595-603.

57. Heaney RP. Protein intake and the calcium economy. *J Am Diet Assoc*. 1993;93(11):1259-1260.

58. Crowe FL, Steur M, Allen NE, Appleby PN, Travis RC, Key TJ. Plasma concentrations of 25-hydroxyvitamin D in meat eaters, fish eaters, vegetarians and vegans: results from the EPIC–Oxford study. *Public Health Nutrition*. 2011;14(2):340-346. doi:10.1017/S1368980010002454

59. Dent CE, Gupta MM. PLASMA 25-HYDROXYVITAMIN-D LEVELS DURING PREGNANCY IN CAUCASIANS AND IN VEGETARIAN AND NON-VEGETARIAN ASIANS. *The Lancet*. 1975;306(7944):1057-1060. doi:10.1016/S0140-6736(75)90430-4

60. Finch PJ, Ang L, Colston KW, Nisbet J, Maxwell JD. Blunted seasonal variation in serum 25-hydroxy vitamin D and increased risk of osteomalacia in vegetarian London Asians. *Eur J Clin Nutr*. 1992;46(7):509-515.

61. Pimenta E, Jensen M, Jung D, Schaumann F, Boxnick S, Truebel H. Effect of Diet on Serum Creatinine in Healthy Subjects During a Phase I Study. *J Clin Med Res*. 2016;8(11):836-839. doi:10.14740/jocmr2738w

62. Venderley AM, Campbell WW. Vegetarian Diets: Nutritional Considerations for Athletes. *Sports Medicine*. 2006;36(4):293-305.

63. Holland RD, Gehring T, Taylor J, Lake BG, Gooderham NJ, Turesky RJ. Formation of a Mutagenic Heterocyclic Aromatic Amine from Creatinine in Urine of Meat Eaters and Vegetarians. *Chemical Research in Toxicology*. 2005;18(3):579-590. doi:10.1021/tx049675w

64. Gilsing AMJ, Crowe FL, Lloyd-Wright Z, et al. Serum concentrations of vitamin B12 and folate in British male omnivores, vegetarians and vegans: results from a cross-sectional analysis of the EPIC-Oxford cohort study. *European Journal of Clinical Nutrition*. 2010;64(9):933-939. doi:10.1038/ejcn.2010.142

65. Mavrommati K, Sentissi O. Delirium as a result of vitamin B12 deficiency in a vegetarian female patient. *European Journal of Clinical Nutrition*. 2013;67(9):996-997. doi:10.1038/ejcn.2013.128

66. Leitzmann C. Vegetarian diets: what are the advantages? *Forum Nutr*. 2005;(57):147-156.

67. Ingenbleek Y, McCully KS. Vegetarianism produces subclinical malnutrition, hyperhomocysteinemia and atherogenesis. *Nutrition*. 2012;28(2):148-153. doi:10.1016/j.nut.2011.04.009

68. Naik S, Mahalle N, Bhide V. Identification of vitamin B_{12} deficiency in vegetarian Indians. *British Journal of Nutrition*. 2018;119(6):629-635. doi:10.1017/S0007114518000090

69. Vitamin B12. Linus Pauling Institute. https://lpi.oregonstate.edu/mic/vitamins/vitamin-B12. Published April 22, 2014. Accessed March 29, 2019.

70. Elmadfa I, Singer I. Vitamin B-12 and homocysteine status among vegetarians: a global perspective. *The American Journal of Clinical Nutrition*. 2009;89(5):1693S-1698S. doi:10.3945/ajcn.2009.26736Y

71. Kozyraki R, Kristiansen M, Silahtaroglu A, et al. The human intrinsic factor-vitamin B12 receptor, cubilin: molecular characterization and chromosomal mapping of the gene to 10p within the autosomal recessive megaloblastic anemia (MGA1) region. *Blood*. 1998;91(10):3593-3600.

72. Christensen EI, Nielsen R, Birn H. From bowel to kidneys: the role of cubilin in physiology and disease. *Nephrol Dial Transplant*. 2013;28(2):274-281. doi:10.1093/ndt/gfs565

73. Seetharam B, Li N. Transcobalamin II and its cell surface receptor. *Vitam Horm*. 2000;59:337-366.

74. Office of Dietary Supplements - Vitamin B12. https://ods.od.nih.gov/factsheets/VitaminB12-HealthProfessional/. Accessed October 18, 2018.

75. Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes and its Panel on Folate, Other B Vitamins, and Choline. *Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline*. Washington (DC): National Academies Press (US); 1998. http://www.ncbi.nlm.nih.gov/books/NBK114310/. Accessed October 18, 2018.

76. Yajnik CS, Deshpande SS, Lubree HG, et al. Vitamin B12 deficiency and hyperhomocysteinemia in rural and urban Indians. *J Assoc Physicians India*. 2006;54:775-782.

77. Martinelli D, Deodato F, Dionisi□Vici C. Cobalamin C defect: natural history, pathophysiology, and treatment. *Journal of Inherited Metabolic Disease*. 2011;34(1):127-135. doi:10.1007/s10545-010-9161-z

78. Pastore A, Martinelli D, Piemonte F, et al. Glutathione metabolism in cobalamin deficiency type C (cblC). *Journal of Inherited Metabolic Disease*. 2014;37(1):125-129. doi:10.1007/s10545-013-9605-3

79. Koebnick C, Garcia AL, Dagnelie PC, et al. Long-term consumption of a raw food diet is associated with favorable serum LDL cholesterol and triglycerides but also with elevated plasma homocysteine and low serum HDL cholesterol in humans. *J Nutr*. 2005;135(10):2372-2378. doi:10.1093/jn/135.10.2372

80. Waldmann A, Koschizke JW, Leitzmann C, Hahn A. Homocysteine and cobalamin status in German vegans. *Public Health Nutr*. 2004;7(3):467-472. doi:10.1079/PHN2003540

81. Herrmann W, Schorr H, Obeid R, Geisel J. Vitamin B-12 status, particularly holotranscobalamin II and methylmalonic acid concentrations, and hyperhomocysteinemia in vegetarians. *Am J Clin Nutr*. 2003;78(1):131-136. doi:10.1093/ajcn/78.1.131

82. Huang Y-C, Chang S-J, Chiu Y-T, Chang H-H, Cheng C-H. The status of plasma homocysteine and related B-vitamins in healthy young vegetarians and nonvegetarians. *European Journal of Nutrition*. 2003;42(2):84-90. doi:10.1007/s00394-003-0387-5

83. Su T-C, Jeng J-S, Wang J-D, et al. Homocysteine, circulating vascular cell adhesion molecule and carotid atherosclerosis in postmenopausal vegetarian women and omnivores. *Atherosclerosis*. 2006;184(2):356-362. doi:10.1016/j.atherosclerosis.2005.04.022

84. Hung C-J, Huang P-C, Lu S-C, et al. Plasma homocysteine levels in Taiwanese vegetarians are higher than those of omnivores. *J Nutr*. 2002;132(2):152-158. doi:10.1093/jn/132.2.152

85. Bissoli L, Di Francesco V, Ballarin A, et al. Effect of vegetarian diet on homocysteine levels. *Ann Nutr Metab*. 2002;46(2):73-79. doi:10.1159/000057644

86. Kazimírová A, Barancoková M, Krajcovicová-Kudlácková M, et al. The relationship between micronuclei in human lymphocytes and selected micronutrients in vegetarians and non-vegetarians. *Mutat Res*. 2006;611(1-2):64-70. doi:10.1016/j.mrgentox.2006.08.001

87. Koebnick C, Hoffmann I, Dagnelie PC, et al. Long-term ovo-lacto vegetarian diet impairs vitamin B-12 status in pregnant women. *J Nutr*. 2004;134(12):3319-3326. doi:10.1093/jn/134.12.3319

88. Ambroszkiewicz J, Klemarczyk W, Chełchowska M, Gajewska J, Laskowska-Klita T. Serum homocysteine, folate, vitamin B12 and total antioxidant status in vegetarian children. *Adv Med Sci*. 2006;51:265-268.

89. Karabudak E, Kiziltan G, Cigerim N. A comparison of some of the cardiovascular risk factors in vegetarian and omnivorous Turkish females. *J Hum Nutr Diet*. 2008;21(1):13-22. doi:10.1111/j.1365-277X.2007.00831.x

90. Haddad EH, Berk LS, Kettering JD, Hubbard RW, Peters WR. Dietary intake and biochemical, hematologic, and immune status of vegans compared with nonvegetarians. *Am J Clin Nutr*. 1999;70(3 Suppl):586S-593S. doi:10.1093/ajcn/70.3.586s

91. Hokin BD, Butler T. Cyanocobalamin (vitamin B-12) status in Seventh-day Adventist ministers in Australia. *Am J Clin Nutr*. 1999;70(3 Suppl):576S-578S. doi:10.1093/ajcn/70.3.576s

92. Leung SS, Lee RH, Sung RY, et al. Growth and nutrition of Chinese vegetarian children in Hong Kong. *J Paediatr Child Health*. 2001;37(3):247-253.

93. Refsum H, Yajnik CS, Gadkari M, et al. Hyperhomocysteinemia and elevated methylmalonic acid indicate a high prevalence of cobalamin deficiency in Asian Indians. *Am J Clin Nutr*. 2001;74(2):233-241. doi:10.1093/ajcn/74.2.233

94. Dhonukshe-Rutten RAM, van Dusseldorp M, Schneede J, de Groot LCPGM, van Staveren WA. Low bone mineral density and bonemineral content are associated withlow cobalamin status in adolescents. *Eur J Nutr*. 2005;44(6):341-347. doi:10.1007/s00394-004-0531-x

95. Rogers LM, Boy E, Miller JW, Green R, Sabel JC, Allen LH. High prevalence of cobalamin deficiency in Guatemalan schoolchildren: associations with low plasma holotranscobalamin II and elevated serum methylmalonic acid and plasma homocysteine concentrations. *Am J Clin Nutr*. 2003;77(2):433-440. doi:10.1093/ajcn/77.2.433

96. Kim MK, Ordovas JM, Selhub J, Campos H. B vitamins and plasma homocysteine concentrations in an urban and rural area of Costa Rica. *J Am Coll Nutr*. 2003;22(3):224-231.

97. Gonzalez-Gross M, Sola R, Albers U, et al. B-vitamins and homocysteine in Spanish institutionalized elderly. *Int J Vitam Nutr Res*. 2007;77(1):22-33. doi:10.1024/0300-9831.77.1.22

98. Taneja S, Bhandari N, Strand TA, et al. Cobalamin and folate status in infants and young children in a low-to-middle income community in India. *Am J Clin Nutr*. 2007;86(5):1302-1309. doi:10.1093/ajcn/86.5.1302

99. Ueland PM, Monsen ALB. Hyperhomocysteinemia and B-vitamin deficiencies in infants and children. *Clin Chem Lab Med*. 2003;41(11):1418-1426. doi:10.1515/CCLM.2003.218

100. Selhub J. Homocysteine Metabolism. *Annual Review of Nutrition*. 1999;19(1):217.

101. Vayne C, Deroche L, Drillaud N, et al. An UPLC-MSMS method to measure plasma homocysteine concentration. *Ann Biol Clin (Paris)*. 2017;75(3):334-338. doi:10.1684/abc.2017.1248

102. Kirke PN, Molloy AM, Daly LE, Burke H, Weir DG, Scott JM. Maternal plasma folate and vitamin B12 are independent risk factors for neural tube defects. *Q J Med*. 1993;86(11):703-708.

103. Steegers-Theunissen RP, Boers GH, Blom HJ, et al. Neural tube defects and elevated homocysteine levels in amniotic fluid. *Am J Obstet Gynecol*. 1995;172(5):1436-1441.

104. Rosenquist TH, Ratashak SA, Selhub J. Homocysteine induces congenital defects of the heart and neural tube: effect of folic acid. *Proc Natl Acad Sci USA*. 1996;93(26):15227-15232.

105. Riggs KM, Spiro A, Tucker K, Rush D. Relations of vitamin B-12, vitamin B-6, folate, and homocysteine to cognitive performance in the Normative Aging Study. *Am J Clin Nutr*. 1996;63(3):306-314. doi:10.1093/ajcn/63.3.306

106. McCaddon A, Davies G, Hudson P, Tandy S, Cattell H. Total serum homocysteine in senile dementia of Alzheimer type. *Int J Geriatr Psychiatry*. 1998;13(4):235-239.

107. Joosten E, Lesaffre E, Riezler R, et al. Is metabolic evidence for vitamin B-12 and folate deficiency more frequent in elderly patients with Alzheimer's disease? *J Gerontol A Biol Sci Med Sci*. 1997;52(2):M76-79.

108. Bell IR, Edman JS, Selhub J, et al. Plasma homocysteine in vascular disease and in nonvascular dementia of depressed elderly people. *Acta Psychiatr Scand*. 1992;86(5):386-390.

109. Berlin KN, Cameron LM, Gatt M, Miller RR. Reduced de novo synthesis of 5-methyltetrahydrofolate and reduced taurine levels in ethanol-treated chick brains. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*. 2010;152(3):353-359. doi:10.1016/j.cbpc.2010.06.002

110. Cravo ML, Glória LM, Selhub J, et al. Hyperhomocysteinemia in chronic alcoholism: correlation with folate, vitamin B-12, and vitamin B-6 status. *Am J Clin Nutr*. 1996;63(2):220-224. doi:10.1093/ajcn/63.2.220

111. Geer BW, Miller RR, Heinstra PWH. Genetic and Dietary Control of Alcohol Degradation in Drosophila. In: Watson RR, ed. *Liver Pathology and Alcohol*. Drug and Alcohol Abuse Reviews. Totowa, NJ: Humana Press; 1991:325-373. doi:10.1007/978-1-4612-0421-3_13

112. Boushey CJ, Beresford SAA, Omenn GS, Motulsky AG. A Quantitative Assessment of Plasma Homocysteine as a Risk Factor for Vascular Disease: Probable Benefits of Increasing Folic Acid Intakes. *JAMA*. 1995;274(13):1049-1057. doi:10.1001/jama.1995.03530130055028

113. Danesh J, Lewington S. Plasma homocysteine and coronary heart disease: systematic review of published epidemiological studies. *J Cardiovasc Risk*. 1998;5(4):229-232.

114. Zhang B, Dong J-L, Chen Y-L, et al. Nrf2 mediates the protective effects of homocysteine by increasing the levels of GSH content in HepG2 cells. *Molecular Medicine Reports*. 2017;16(1):597-602. doi:10.3892/mmr.2017.6633

115. Michelet F, Gueguen R, Leroy P, Wellman M, Nicolas A, Siest G. Blood and plasma glutathione measured in healthy subjects by HPLC: relation to sex, aging, biological variables, and life habits. *Clinical Chemistry*. 1995;41(10):1509-1517.

116. Merad-Boudia M, Nicole A, Santiard-Baron D, Saillé C, Ceballos-Picot I. Mitochondrial impairment as an early event in the process of apoptosis induced by

glutathione depletion in neuronal cells: relevance to Parkinson's disease. *Biochemical Pharmacology*. 1998;56(5):645-655. doi:10.1016/S0006-2952(97)00647-3

117. Mårtensson J, Meister A. Mitochondrial damage in muscle occurs after marked depletion of glutathione and is prevented by giving glutathione monoester. *Proc Natl Acad Sci USA*. 1989;86(2):471-475.

118. Jha N, Jurma O, Lalli G, et al. Glutathione Depletion in PC12 Results in Selective Inhibition of Mitochondrial Complex I Activity IMPLICATIONS FOR PARKINSON′S DISEASE. *J Biol Chem*. 2000;275(34):26096-26101. doi:10.1074/jbc.M000120200

119. Vitvitsky V, Mosharov E, Tritt M, Ataullakhanov F, Banerjee R. Redox regulation of homocysteine-dependent glutathione synthesis. *Redox Report*. 2003;8(1):57-63. doi:10.1179/135100003125001260

120. McBean GJ. The transsulfuration pathway: a source of cysteine for glutathione in astrocytes. *Amino Acids*. 2012;42(1):199-205. doi:10.1007/s00726-011-0864-8

121. Lu SC. GLUTATHIONE SYNTHESIS. *Biochim Biophys Acta*. 2013;1830(5):3143-3153. doi:10.1016/j.bbagen.2012.09.008

122. Mosharov E, Cranford MR, Banerjee R. The Quantitatively Important Relationship between Homocysteine Metabolism and Glutathione Synthesis by the Transsulfuration Pathway and Its Regulation by Redox Changes. *Biochemistry*. 2000;39(42):13005-13011. doi:10.1021/bi001088w

123. Sergueeva AI, Miasnikova GY, Okhotin DJ, et al. Elevated homocysteine, glutathione and cysteinylglycine concentrations in patients homozygous for the Chuvash polycythemia VHL mutation. *Haematologica*. 2008;93(2):279-282. doi:10.3324/haematol.11851

124. Rauma A-L, Mykkänen H. Antioxidant status in vegetarians versus omnivores. *Nutrition*. 2000;16(2):111-119. doi:10.1016/S0899-9007(99)00267-1

125. Cade J, Burley V, Greenwood D, UK Women's Cohort Study Steering Group. The UK Women's Cohort Study: comparison of vegetarians, fish-eaters and meat-eaters. *Public Health Nutrition*. 2004;7(07). doi:10.1079/PHN2004620

126. Haldar S, Rowland IR, Barnett YA, et al. Influence of habitual diet on antioxidant status: a study in a population of vegetarians and omnivores. *Eur J Clin Nutr*. 2007;61(8):1011-1022. doi:10.1038/sj.ejcn.1602615

127. Poornima K, Cariappa M, Asha K, Kedilaya HP, Nandini M. Oxidant and antioxidant status in vegetarians and fish eaters. *Indian J Clin Biochem*. 2003;18(2):197-205. doi:10.1007/BF02867387

128. Kim MK, Cho SW, Park YK. Long-term vegetarians have low oxidative stress, body fat, and cholesterol levels. *Nutr Res Pract*. 2012;6(2):155-161. doi:10.4162/nrp.2012.6.2.155

129. Flagg EW, Coates RJ, Jones DP, et al. Plasma total glutathione in humans and its association with demographic and health-related factors. *Br J Nutr*. 1993;70(3):797-808.

130. Nagyova A, Ginter E, Kovacikova Z. Low glutathione levels and decreased glutathione peroxidase activity in the blood of vegetarians. *Int J Vitam Nutr Res*. 1995;65(3):221.

APPENDIX A

IRB APPROVAL

Instructions and Notes:

- Depending on the nature of what you are doing, some sections may not be applicable to your research. If so mark as "NA".
- When you write a protocol, keep an electronic copy. You will need to modify this copy when making changes.

1 Protocol Title

Include the full protocol title: Assessing the Relationship Between Cobalamin Deficiency and Methylation Capacity in a Vegetarian Population

2 IRB Review History

If you have submitted this protocol for review by an external IRB, provide the previous study identification number and provide details of the review including the IRB name, date of review, and IRB contact information.

NA

3 Background and Objectives

Provide the scientific or scholarly background for, rationale for, and significance of the research based on the existing literature and how will it add to existing knowledge.

- Describe the purpose, specific aims, or objectives.
- State the hypotheses to be tested.
- Describe the relevant prior experience and gaps in current knowledge.
- Describe any relevant preliminary data.

Background

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.[1] Nutrient deficiencies can prove detrimental because they directly inhibit certain metabolic pathways that are essential for life. Perhaps the most critical nutrient that is commonly lacking in vegetarian diets is cobalamin. Cobalamin acts as a methylation cofactor in various biological reactions.[2-4] This cofactor role impacts many aspects of normal physiology, including food metabolism, neurological functions, and oxygen perfusion via the circulatory system.[2,5,6] To date, the correlation between cobalamin status 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 S-adenosylmethionine, have been neglected in the literature. It is possible that the widespread cobalamin deficiency experienced within the vegetarian population can lead to an overall decreased methylation capacity, which could have important physiological consequences.

Purpose of Study

This cross-sectional study will examine the relationship between vitamin B12 status and methylation capacity in vegetarians and omnivores in Phoenix, Arizona. Serum homocysteine, methylmalonic acid, and S-adenosylmethionine will be used to assess total methylation capacity in both diet groups.

Research Aim and Hypothesis

H1: There is a direct correlation between low serum cobalamin levels and low methylation capacity.
H2: Vegetarians will have significantly lower serum cobalamin levels and lower methylation capacity compared to omnivores.

References:

1. Majchrzak D, Singer I, Manner M, et al. B-vitamin status and concentrations of homocysteine in Austrian omnivores, vegetarians and vegans. Ann Nutr Metab. 2006;509(6):485-491.
2. Moll R, Davis B. Iron, vitamin B12 and folate. Medicine. 2017;45(4):198-203.
3. Stabler SP, John WE, Macdonald IA, Zeisel SH. Vitamin B12 in Present Knowledge in Nutrition, Tenth Edition. Oxford, UK. Wiley-Blackwell; 2012.
4. Rizzo G, Lagana AS, Rapisarda AMC, et al. Vitamin B12 among vegetarians: status, assessment and supplementation. Nutrients. 2016;8(12):767.
5. Kumar N. Neurologic aspects of cobalamin (B12) deficiency. Handb Clin Neurol. 2014;120:915-926.
6. Bizzaro N, Antico A. Diagnosis and classification of pernicious anemia. Autoimmun Rev. 2014;13(4-5):565-568.

This will be a secondary data analysis titled Comparing Cobalamin and Glutathione in Vegetarian and Omnivore Populations

4 Inclusion and Exclusion Criteria

Describe the inclusion and the exclusion criteria for the study.

Describe how individuals will be screened for eligibility.

How many participants do plan to enroll into the study?

- Provide a rationale for the proposed enrollment number
- What percentage of screened individuals will likely qualify for the study?

Indicate if you are specifically recruiting individuals of the following populations as participants. If this is the case, these populations must be indicated in your inclusion criteria.

- Adults unable to consent
- Pregnant Women
- Individuals who are not yet adults (individuals who are under age 18)
- Native Americans

Inclusion criteria:

Participants were accepted into the study if they are:

- apparently healthy
- adhered to a vegetarian or omnivore diet for more than 1 year
- between the ages of 18 and 65 years of age
- non-smokers
- not taking medications that alter the methylation processes
- on stable medication [if taking medication, use will be stable for three or more months]

Exclusion criteria:

- those unable to consent
- unwilling to the come to the lab for testing or follow the study protocol
- women who are pregnant, recently pregnant (past 3 months), or lactating

Enrollment:

- Seventy participants between the ages of 18 and 65 years of age will be enrolled in the study (35 vegetarians and 35 omnivores). Preliminary studies examining homocysteine concentrations in vegetarians and omnivores provided data for sample size calculations.

5 Recruitment Methods

- Describe when, where, and how potential participants will be identified and recruited.
- Describe materials that will be used to recruit participants. (Attach copies of these documents with the application.)

Participants will be recruited by list serves and fliers. List serves are obtained from Arizona State University and surrounding businesses and organizations. Recruitment fliers will be placed at farmers markets and at Seventh Day Adventists churches. Individuals who are interested in participating in the study are directed to an online survey to assess general health and adherence to the inclusion criteria. Individuals who meet the inclusion criteria will be invited to ASU to participate in the study.

6 Study Timelines

Describe:

- The duration of an individual participant's participation in the study.
- The duration anticipated to enroll all study participants.
- The estimated date for the investigators to complete this study (up to and including primary analyses).

The study entails 1-2 visits: (1) a screening visit (≤30 minutes) to finalize eligibility and conduct testing and possibly an additional visit (2) to obtain a fasting blood sample. If the individual is fasting at the first visit, all testing can be completed in one visit. Visits will take place at the Arizona Biomedical Collaborative building on the downtown ASU campus. We will start recruiting once IRB approval is finalized; we anticipate that the trial will begin in January 2018 and extend through March or April 2018. The timeline for study completion (the primary analyses through manuscript acceptance) may extend 1 year after subject recruitment is complete.

7 Procedures Involved

Describe and explain the study design. Provide a description of all research procedures being performed and when they are performed. Describe procedures including:

- The documents/ measures / devices/ records /sampling that will be used to collect data about participants. (Attach all surveys, scripts, and data collection forms.)
- What data will be collected including long-term follow-up.
- All drugs and medical devices used in the research and the purpose of their use, and their regulatory approval status.

Study design

The study is a cross-sectional, differential research design that spans 1-2 visits within a week. After recruitment, eligible participants are invited to Visit 1. This visit is up to 45 minutes. During this visit, the participant will provide written consent and complete a diet questionnaire and Vitamin B12, B6 and Folate food frequency questionnaire. Height, weight, and waist circumference are measured using a stadiometer, electronic weighing scale, Gulick tape measure, respectively. If the participant is fasted, a blood sample will be collected (2 tablespoons). If necessary, the participant will return in the next day or so to provide a fasting blood sample. Participants will provide verbal confirmation that they are fasted (no food or drink with the exception of water).

Fasting blood samples will be tested for blood B12 and folate levels and for methylation capacity.

8 Withdrawal of Participants

Describe anticipated circumstances under which participants will be withdrawn from the research without their consent.

Describe procedures that will be followed when participants withdraw from the research, including partial withdrawal from procedures with continued data collection.

Participants may withdraw from the trial at any time for any reason. We will ask participants to tell us why they are withdrawing so this information can be recorded and reported. A participant will be withdrawn from the study if it becomes clear to the investigators that the participant is not complying with the research protocol.

9 Risks to Participants

List the reasonably foreseeable risks, discomforts, hazards, or inconveniences to the participants related the participants' participation in the research. Include as may be useful for the IRB's consideration, the probability, magnitude, duration, and reversibility of the risks. Consider physical, psychological, social, legal, and economic risks. Reference this information when appropriate.

- If applicable, indicate which procedures may have risks to an embryo or fetus should the participant be or become pregnant.
- If applicable, describe risks to others who are not subjects.

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.

10 Potential Benefits to Participants

Realistically describe the potential benefits that individual subjects may experience from taking part in the research. Include the probability, magnitude, and duration of the potential benefits.

Indicate if there is no direct benefit. Do not include benefits to society or others.

We anticipate no direct benefit to the participants

11 Vulnerable Populations

If the research involves individuals who are vulnerable to coercion or undue influence, describe additional safeguards included to protect their rights and welfare.

- If the research involves pregnant women, review "CHECKLIST: Pregnant Women (HRP-412)" to ensure that you have provided sufficient information.
- If the research involves persons who have not attained the legal age for consent to treatments or procedures involved in the research ("children"), review the "CHECKLIST: Children (HRP-416)" to ensure that you have provided sufficient information.
- If the research involves cognitively impaired adults, review "CHECKLIST: Cognitively Impaired Adults (HRP-417)" to ensure that you have provided sufficient information.

NA

12 Setting

Describe the sites or locations where your research team will conduct the research.

- Identify where research procedures will be performed.
- For research conducted outside of the ASU describe:
  - Site-specific regulations or customs affecting the research.
  - Local scientific and ethical review structures in place.

The ABC1 building on the ASU downtown campus, the home of the ASU nutrition laboratories. If we can get approval from several Seventh Day Adventists churches, we will collect blood at these sites to eliminate the need for participants to travel to the ABC site in Phoenix. Phlebotomists from our labs will carry all blood collection supplies, biohazard waste containers, and sanitation items to the church and blood will be collected in a private room. Blood tubes will be placed on ice in ice chests for transport back to ASU.

13 Multi-Site Research

If this is a multi-site study where you are the lead investigator, describe the processes you will use to ensure communication among sites, such as:

- Each site has the most current version of the protocol, consent document, and HIPAA authorization.
- Required approvals have been obtained at each site (including approval by the site's IRB of record).
- Describe processes you will use to communicate with participating sites.
- Participating sites will safeguard data as required by local information security policies.
- Local site investigators conduct the study appropriately.

NA

14 Sharing of Results with Participants

Describe whether results (study results or individual participant results, such as results of genetic tests or research findings) will be shared with participants or others (e.g., the participant's primary care physicians) and if so, describe how it will be shared.

Personal blood work and assessment results will be available to participants on request but will require that they sign a release form. The aggregate results of the trial will be reported to participants if they are interested.

15 Resources Available

Describe the qualifications (e.g., training, experience, oversight) of you and your staff as required to perform your roles. When applicable describe knowledge of the local study sites, culture, and society. Provide enough information to convince the IRB that you have qualified staff for the proposed research.

Describe other resources available to conduct the research: For example, as appropriate:

- Describe your facilities.
- Describe the availability of medical or psychological resources that participants might need as a result of any anticipated consequences of the human research.
- Describe your process to ensure that all persons assisting with the research are adequately informed about the protocol, the research procedures, and their duties and functions.

The nutrition laboratories are housed in the School of Nutrition and Health Promotion's Central Research Facility in the ABC Building on the ASU Downtown Phoenix Campus. All blood analyses will be performed in these labs. Three full time staff (research nurse, sonographer, and radiology technician) oversee the labs and assist with phlebotomy, blood processing, and chemical analyses. All staff and investigators have completed CITI training. If we can get approval from several Seventh Day Adventists churches, we will collect blood at these sites to eliminate the need for participants to travel to the ABC site in Phoenix. Phlebotomists from our labs will carry all blood collection supplies, biohazard waste containers, and sanitation items to the church and blood will be collected in a private room. Blood tubes will be placed on ice in ice chests for transport back to ASU. This protocol is followed for other community trials conducted by SNHP investigators and is not uncommon.

16 Prior Approvals

Describe any approvals that will be obtained prior to commencing the research. (E.g., school, external site, funding agency, laboratory, radiation safety, or biosafety approval.)

Biosafety disclosure: Johnston: 12-421

17 Privacy

Describe the steps that will be taken to protect subjects' privacy interests. "Privacy interest" refers to a person's ability to place limits on who they interact or who can have access to personal information they provide.

Describe what steps you will take to make the subjects feel at ease with the research situation in terms of the nature of questions being asked and the procedures being performed. "At ease" does not refer to physical discomfort, but the sense of intrusiveness a subject might experience in response to questions, examinations, and procedures.

Indicate how the research team is authorized to access any sources of information about the participants.

All participants will be given a study ID number once the consent forms are signed, and the participant will assume that number as a means of identification during trial and for all data collected. Consent forms will be stored separate from data sheets (in Johnston's office, ABC 139); the master list will be kept separate from the data sheets and this list will be destroyed at the completion of the study. Only the investigators will have access to identifying information during the study and after the study is over.

18 Data Management and Confidentiality

Describe the data analysis plan, including procedures for statistical analysis.

Describe the steps that will be taken to secure the data during storage, use, and transmission.

- Training, authorization of access, password protection, encryption, physical controls, certificates of confidentiality, and separation of identifiers and data

Describe how data and any specimens will be handled:

- What personal identifiers will be included in that data or associated with the specimens?
- Where and how data or specimens will be stored?
- How long the data or specimens will be stored?
- Who will have access to the data or specimens?
- Who is responsible for receipt or transmission of the data or specimens?
- How will data and specimens be transported?
- If data or specimens will be banked for future use, describe where the specimens will be stored, how long they will be stored, how the specimens will be accessed, and who will have access to the specimens.
- Describe the procedures to release data or specimens, including: the process to request a release, approvals required for release, who can obtain data or specimens, and the data to be provided with specimens.

Data will be reported as mean ± SE. Data will be examined for normality and log transformed if necessary. Correlation analyses will be performed to assess the strength of the relationship between variables. T-tests will be utilized to examine differences between diet groups. Significance will be set at $P \leq 0.05$ and the Statistical Package for Social Sciences (SPSS 21 for Windows, 2010, Chicago IL) will be used for analysis. Deidentified data will be entered into excel and SPSS files and stored on password protected computers. Hard copies of the deidentified data will be stored for up to 10 years in the locked office of Carol Johnston. Documents with Identifying information (consents and master list) will not be kept on computers; consent forms will be filed separate from the data sheets in the locked office of Carol Johnston. The master list will be shredded when the study is complete. Blood samples will be identified only by the subject number and date, and blood samples are stored in freezers in the Nutrition labs at the ABC building for up to 5 years. Only the study investigators and the laboratory staff will access these samples.

19 Safety Monitoring

This is required when research involves more than Minimal Risk to participants. The plan might include establishing a data monitoring committee and a plan for reporting data monitoring committee findings to the IRB and the sponsor. Describe:

- The plan to periodically evaluate the data collected regarding both harms and benefits to determine whether participants remain safe.
- What data are reviewed, including safety data, untoward events, and efficacy data?
- How the safety information will be collected (e.g., with case report forms, at study visits, by telephone calls with participants). Who will review the data?

NA

20 Compensation and Costs

Describe the available compensation (monetary or credit that will be provided to research participants).

Describe any costs that participants may be responsible for because of participation in the research.

There are minimal costs to participation in this trial. Participants may need to drive to the ASU downtown campus which will be associated with travel and parking costs. We will provide blood tests including the common biomarkers glucose and cholesterol at no charge.

21 Consent Process

Indicate how you will be obtaining consent. Describe:

- Where will the consent process will take place
- Any waiting period available between informing the prospective participant and obtaining the consent.
- Any process to ensure ongoing consent.
- Whether you will be following "SOP: Informed Consent Process for Research (HRP-090)." If not, describe:
  - The role of the individuals listed in the application as being involved in the consent process.
  - The time that will be devoted to the consent discussion.
  - Steps that will be taken to minimize the possibility of coercion or undue influence.
  - Steps that will be taken to ensure the participants' understanding.

Non-English Speaking Participants

- Indicate what language(s) other than English are understood by prospective participants or representatives.
- If participants who do not speak English will be enrolled, describe the process to ensure that the oral and written information provided to those participants will be in their language. Indicate the language that will be used by those obtaining consent.

Waiver or Alteration of Consent Process (consent will not be obtained, required information will not be disclosed, or the research involves deception)

- Review the "CHECKLIST: Waiver or Alteration of Consent Process (HRP-410)" to ensure you have provided sufficient information for the IRB to make these determinations.

Participants who are not yet adults (infants, children, teenagers)

- Describe the criteria that will be used to determine whether a prospective participant has not attained the legal age for consent to treatments or procedures involved in the research under the applicable law of the jurisdiction in which the research will be conducted. (E.g., individuals under the age of 18 years.)
- Describe whether parental permission will be obtained from:
  - Both parents unless one parent is deceased, unknown, incompetent, or not reasonably available, or when only one parent has legal responsibility for the care and custody of the child.
  - One parent even if the other parent is alive, known, competent, reasonably available, and shares legal responsibility for the care and custody of the child.
- Describe whether permission will be obtained from individuals other than parents, and if so, who will be allowed to provide permission. Describe the process used to determine these individuals' authority to consent to each child's general medical care.
- Indicate whether assent will be obtained from all, some, or none of the children. If assent will be obtained from some children, indicate which children will be required to assent.
- When assent of children is obtained describe whether and how it will be documented.

Cognitively Impaired Adults

- Describe the process to determine whether an individual is capable of consent. The IRB allows the person obtaining assent to document assent
- on the consent document and does not routinely require assent documents and does not routinely require children to sign assent documents.

Adults Unable to Consent

- List the individuals from whom permission will be obtained in order of priority. (E.g., durable power of attorney for health care, court appointed guardian for health care decisions, spouse, and adult child.)
  - For research conducted in the state, review "SOP: Legally Authorized Representatives, Children, and Guardians (HRP-013)" to be aware of which individuals in the state meet the definition of "legally authorized representative."
  - For research conducted outside of the state, provide information that describes which individuals are authorized under applicable law to consent on behalf of a prospective participant to their participation in the procedure(s) involved in this research. One method of obtaining this information is to have a legal counsel or authority review your protocol along the definition of "legally authorized representative" in "SOP: Legally Authorized Representatives, Children, and Guardians (HRP-013)."
- Describe the process for assent of the participants. Indicate whether:
  - Assent will be required of all, some, or none of the participants. If some, indicated, which participants will be required to assent and which will not.
  - If assent will not be obtained from some or all participants, an explanation of why not.
  - Describe whether assent of the participants will be documented and the process to document assent. The IRB allows the person obtaining assent to document assent on the consent document and does not routinely require assent documents and does not routinely require participants to sign assent documents.

The study protocol, design, risks and benefits will be discussed with volunteers; those wishing to participate will read and sign the consent form - the investigators will be present to answer any questions. Volunteers completing the screening survey and appear to be eligible for the study will be invited to study visit #1. Participants will be told they may withdraw from the study at any time; any concerns/questions will be addressed at the visits, and participants will have the investigators' contact information if questions arise.

21 Process to Document Consent in Writing

Describe whether you will be following "SOP: Written Documentation of Consent (HRP-091)." If not, describe whether and how consent of the participant will be documented.

If your research presents no more than minimal risk of harm to participants and involves no procedures for which written documentation of consent is normally required outside of the research context, the IRB will generally waive the requirement to obtain written documentation of consent.

(If you will document consent in writing, attach a consent document. If you will obtain consent, but not document consent in writing, attach a consent script. Review "CHECKLIST: Waiver of Written Documentation of Consent (HRP-411)" to ensure that you have provided sufficient information. You may use "TEMPLATE CONSENT DOCUMENT (HRP-502b)"to create the consent document or script.)

A consent form (IRB approved) will be read and signed by all participants, and participants will receive a copy of the consent form to take with them.

22 Sharing of Results with Participants

If the drug is investigational (has an IND) or the device has an IDE or a claim of abbreviated IDE (non-significant risk device), include the following information:

- Identify the hold of the IND/IDE/Abbreviated IDE.
- Explain procedures followed to comply with FDA sponsor requirements for the following:

	Applicable to:		
FDA Regulation	**IND Studies**	**IDE studies**	**Abbreviated IDE studies**
21 CFR 11	X	X	
21 CFR 54	X	X	
21 CFR 210	X		
21 CFR 211	X		
21 CFR 312	X		
21 CFR 812		X	X
21 CFR 820		X	

NA

23 CITI

Provide the date that the members of the research team have taken the CITI training for human participants. This training must be taken within the last 3 years. Additional information can be found at: http://researchintegrity.asu.edu/training/humans

Carol Johnston March 2016
Noel Ugarte August 2014