Vitamin C is Not Related to Resting Fat Oxidation in Healthy, Non-Obese Adults

by

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ABSTRACT

Vitamin C plays an important role in fatty acid metabolism because it is required for carnitine synthesis. Vitamin C has been shown to have an inverse relationship with weight and body fat percent in a number of studies. However, there has been limited research exploring the relationship between vitamin C status and fat oxidation. This cross-sectional study investigates the relationship between plasma vitamin C and fat oxidation in 69 participants and between plasma vitamin C and body fatness in 82 participants. Participants were measured for substrate utilization via indirect calorimetry while at rest and measured for body fatness via DEXA scan. Participants provided a single fasting blood draw for analysis of plasma vitamin C. Results did not show a significant association between vitamin C and fat oxidation while at rest, therefore the data do not support the hypothesis that vitamin C status affects fat oxidation in a resting state. However, a significant inverse association was found between vitamin C and both total body fat percent and visceral fat.

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TABLE OF CONTENTS

Page

LIST OF TABLES v

LIST OF FIGURES vi

CHAPTER

1 INTRODUCTION 1

Study Purpose 2

Definitions 2

Delimitations 3

Limitations 3

2 REVIEW OF LITERATURE 4

Overview 4

Vitamin C Overview 4

Physiological Roles 4

Absorption and Transport 5

Sources 6

Dietary Recommendations 7

Deficiency 9

Fat Oxidation Overview 9

Role in metabolism 9

Measurement 11

Association with weight and body composition 12

Defining Obesity 13

Fat types and distribution 13

Assessment 14

CHAPTER Page

Vitamin C and Weight....15

Research overview....15

Supplementation and weight loss....19

Vitamin C and Fat Oxidation....21

The role of carnitine....21

Research Overview....22

3 METHODS....25

Participants....25

Study Design....25

Study Protocol....26

Measurements....27

Laboratory Analysis....27

4 STATISTICAL ANALYSIS....27

5 RESULTS....28

6 DISCUSSION....34

REFERENCES....39

APPENDIX

A INSTITUTIONAL REVIEW BOARD APPROVAL....44

B DETERMINING SUBSTRATE UTILIZATION USING INDIRECT CALORIMETRY DATA....46

C VITAMIN C ASSAY....49

LIST OF TABLES

Table Page

1. Dietary Recommendations for Vitamin C.....8

2. Descriptive Measures Grouped by Diet Type and Gender.....29

3. Correlations Between Plasma Vitamin C and Measures of Substrate Utilization.....30

4. Correlations Between Plasma Vitamin C and Body Composition.....31

LIST OF FIGURES

Figure Page

1. Reduction of Dehydroascorbate to Ascorbate.....6

2. Process of Fatty Acid Oxidation.....10

3. Simple Scatter Plot Depicting the Trend Between Body Fat Percent and Plasma Vitamin C.....32

4. Simple Scatter Plot Depicting the Trend Between Visceral Fat and Plasma Vitamin C.....33

5. Bar Graph Depicting the Trend Between Visceral Fat and Plasma Vitamin C.....34

Introduction

The prevalence of overweight and obesity has risen at an alarming rate over the past couple of decades in the United States (1). Consequently, the incidence of weight-related health complications such as heart disease, diabetes, and cancer has risen as well (1,2), leading to a dramatic increase in healthcare costs. In fact, the average annual healthcare cost of an obese individual in the United States is over $1000 higher than that of a normal weight individual (1). Considering the urgency of this problem, it is clear that further research is essential in order to better understand the physiological mechanisms responsible for weight loss and healthy weight maintenance.

Research has repeatedly shown an inverse relationship between vitamin C and both weight and body fatness (2-4). In fact, there are multiple hypotheses attempting to explain this relationship. One hypothesis argues that levels of vitamin C are decreased in response to the state of oxidative stress that is caused by excess weight (2). Another theory maintains that low levels of vitamin C cause a decrease in carnitine production, thereby slowing the rate of β-oxidation, otherwise known as fat oxidation (4). Vitamin C serves as a cofactor that maintains the necessary enzyme activity for carnitine synthesis. Carnitine is essential to the process of fat β-oxidation, acting as a shuttle that moves long-chain fatty acids from the cell cytosol into the mitochondria to be metabolized (5). Several studies have shown that in a state of vitamin C depletion, there is a decrease in carnitine production (6,7).

Very few studies have been conducted exploring the relationship between vitamin C and fat oxidation, and each of those studies experienced limitations. A randomized controlled trial by Johnston and colleagues showed a decreased rate of fat oxidation in control participants concomitant with low concentrations of plasma vitamin C, yet sample sizes were low (n=3 and 5 for the control and vitamin C groups, respectively) (8).

Another study by Ji and colleagues found a similar correlation between vitamin C and fat oxidation results. However, the study was conducted among black sea bream rather than in humans, therefore the results are not generalizable to humans (9).

There is a need for research that is aimed at further exploring the relationship between vitamin C, fat oxidation and body fatness in humans utilizing a large sample size. Efficient fat oxidation is necessary for weight loss and the maintenance of a healthy weight and body composition (10,11). Increased dietary intake or supplementation of vitamin C could prove to be a safe, easy and affordable way to help maintain an efficient rate of fat oxidation, potentially improving the success of weight loss attempts.

Study Purpose

The objective of this study was to examine the relationships between plasma vitamin C concentration, fat oxidation and body fatness in healthy adults. The study utilized a convenience sample of omnivorous, vegetarian and vegan adults aged 18-50 years who were participating in an investigation examining substrate utilization in adults adhering to meatless diets. The differences between diet groups will not be taken into account for the purposes of this study. The first hypothesis was that there would be a significant relationship between vitamin C and fat oxidation. The second hypothesis was that there would be a significant inverse relationship between vitamin C and body fatness. These hypotheses were made based on the results found in prior studies (2-4,8,9).

Definitions

There are two terms that may require further definition. A cofactor is a substance that aids in biochemical transformations by participating in enzymatic reactions. Fat

oxidation is the metabolism of fat in the cell mitochondria for the production of adenosine triphosphate (ATP), which is a molecule used for energy.

Delimitations

Participants were healthy men and premenopausal women aged 18-50 years. Qualifying participants had a BMI <30, no history of chronic disease, were free from prescription medications with the exception of oral contraception, were not endurance-trained athletes and women were not pregnant.

Limitations

The study had several major limitations. Muscle carnitine was not measured, therefore a correlation could not be made between vitamin C levels and carnitine production. Participants were recruited from a small campus community, as well as vegan and vegetarian social networking websites. Therefore, the study results can only be generalized to these specific populations and not to the general public. Obese individuals were excluded from the trial; hence, the relationship between vitamin C status and body fatness were examined only in normal weight and overweight individuals. Lastly, the study design was cross-sectional, therefore the results cannot prove causation. A randomized controlled trial should be conducted in the future to improve internal validity and to more clearly illustrate a causal relationship.

Review of Literature

Overview

Overweight and obesity have become a major topic of concern in the biomedical and healthcare fields. Adding to this concern is the inability of scientists and medical professionals to come to a consensus as to what is the safest and most effective way to lose weight and keep it off. Further complicating the obesity problem is the fact that there is a genetic component that causes increased susceptibility to weight gain and retention (12,13). Thus, further research is essential in order to understand the major components that cause weight gain and to determine the major causes of the struggle of losing weight once it has been gained. The physiological roles of vitamin C, the physiological role of fat oxidation, the assessment of fat distribution, the relationship between vitamin C and weight, and potential benefits of vitamin C supplementation in weight loss will be discussed in this paper. However, the relationships between vitamin C and fat oxidation and vitamin C and body fatness will be the focal points.

Vitamin C Overview

Physiological roles. Vitamin C, also known as ascorbic acid or ascorbate, is a water-soluble vitamin and an important cofactor in a number of physiological processes. It is required for the synthesis of collagen, carnitine, tyrosine, and the neurotransmitters norepinephrine and serotonin. It is necessary for a number of immune reactions and also functions as an important antioxidant in the blood and cells (5). For the purposes of this study, the role of vitamin C as a cofactor in carnitine biosynthesis as it relates to the role of carnitine in fat oxidation will be discussed further.

Absorption and transport. Vitamin C in the ascorbate form does not require digestion and is readily absorbed across the brush border in each section of the small intestine. Absorption is facilitated by SVCT1 and SVCT2, which are both sodium-dependent transporters. These transporters are also present in most organs and tissues, sometimes separately, and are therefore thought to be a major mode of absorption for vitamin C throughout the body. In addition to active transport, simple diffusion also occurs in the stomach and small intestine when a large amount of vitamin C is ingested. Ascorbate in its oxidized form is called dehydroascorbate, which is absorbed into the intestinal cells by the glucose transporters GLUT1 and GLUT2 (14). Once absorbed, dehydroascorbate is immediately reduced back to the ascorbate form by the enzyme dehydroascorbate reductase. Reduced glutathione (GSH) is required to reduce the dehydroascorbate, and in the process the glutathione is converted to its oxidized state (GSSG). The semidehydroascorbic acid radical is a free radical that is formed during the oxidation of ascorbate. This free radical has a short half-life and does not react well with oxygen, therefore it does not typically generate damaging reactive oxygen species. Typically, the semidehydroascorbic acid radical is either oxidized to dehydroascorbate, or forms ascorbate upon reacting with another ascorbate free radical (15).

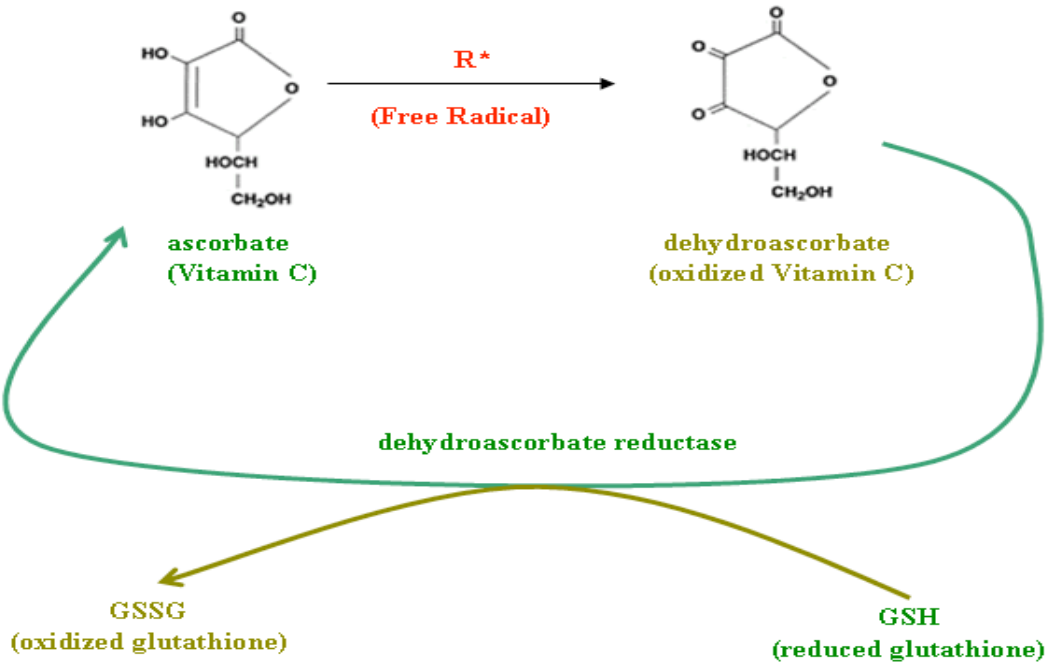


Figure 1. Reduction of Dehydroascorbate to Ascorbate (16)

Sources. The most common dietary sources of vitamin C include fruits and vegetables such as cantaloupe, bell peppers, oranges, lemons, kale, strawberries, broccoli, and citrus juices (5). It is important to note that a number of factors can cause a decrease in the vitamin C content of foods, including an increased shelf life, increased transport time, storing fruits and vegetables after cutting or juicing, and boiling fruits or vegetables (17,18). Because of this, it is recommended that fruits and vegetables and their juices are consumed fresh and with minimal cooking in order to avoid vitamin C losses (19).

In addition to dietary sources, vitamin supplements are considered a safe and effective source of vitamin C, especially if dietary intake is inadequate. However, when consuming vitamin C from supplements, it is important to consider that the bioavailability of vitamin C decreases as the dose increases. Therefore, a high dose of

vitamin C will not be as readily absorbed as it would from a lower dose supplement. In fact, the bioavailability of vitamin C from a supplement is 80% for a 100 mg dose, 72% for 200 mg, 63% for 500 mg, and as low as 16% for a 12 g dose (20).

Dietary Recommendations. The vast majority of plants and animals are able to synthesize vitamin C. However, a few mammals are unable to synthesize this vitamin, including humans, apes, guinea pigs, and fruit bats. This is due to a genetic mutation in the coding of the enzyme gulonolactone oxidase that is required to catalyze the final reaction in the process of vitamin C synthesis (21). Thus, vitamin C is an essential nutrient for these mammals and must be consumed regularly through diet or supplementation.

The current Recommended Dietary Allowances for vitamin C are based on a number of factors, including dietary availability, bioavailability, urinary excretion, beneficial effects, and prevention of deficiency (22). It is important to note that pregnant and lactating women have an increased need for vitamin C in order to provide adequate nutrition to both mother and child (23). Additionally, smokers have an increased need for vitamin C; it is recommended that smokers consume an additional 35 mg/d of vitamin C above the RDA for nonsmoking adults in order to decrease the oxidative stress caused by cigarette smoke (24). In fact, a recent study by Dey and colleagues found that vitamin C prevented the proliferation of lung cells that had been exposed to cigarette smoke, while the smoke-exposed cells that were not treated with vitamin C continued to proliferate. These findings support the importance of vitamin C supplementation for smokers (25).

Even for non-smokers, vitamin C is depleted relatively quickly. In fact, it would only take one month to develop scurvy if vitamin C intake was discontinued completely (19). Table 1 shows the RDA for vitamin C across various groups and age ranges.

Table 1. 2010 Dietary Recommendations for Vitamin C (23)

Age/Group	RDA (mg/d)
Birth - 6 months	40
7 - 12 months	50
1-3 years	15
4-8 years	25
9-13 years	45
14-18 years (male)	75
14-18 years (female)	65
Adults 19+ (male)	90
Adults 19+ (female)	75
Pregnant teens (14-18 y)	80
Pregnant women (19+)	85
Lactating teens (14-18 y)	115
Lactating women (19+)	120
Smoking males	125
Smoking females	110

The Tolerable Upper Intake Level (UL) has been set at 2000 mg/day for adults due to the possibility of diarrhea and abdominal discomfort when vitamin C is consumed at higher levels. There are no known serious or life-threatening side effects associated with consuming more than 2 g of vitamin C per day (23).

Deficiency. A dietary deficiency of vitamin C (plasma concentration < 11 μmol/L) can result in scurvy, which is a disease that causes fatigue, pain in the joints, irritability, large bruise-colored spots on the limbs, and swollen and bleeding gums. If left untreated, scurvy can result in tooth loss, joint and bone pain leading to decreased mobility, and even death (26,27). Vitamin C deficiency is still quite prevalent in the United States. In the 2003-2004 National Health and Nutrition Examination Survey (NHANES), data was collected from 7277 Americans. It was found that about 13% of Americans in the study were vitamin C deficient, with a higher prevalence among low-income individuals and young adults (28). While a marginal vitamin C status (plasma concentration 11-28 μmol/L) will not typically induce the severe physical symptoms of scurvy, it is associated with impaired immune function and fatigue, and could potentially cause a decrease in carnitine and collagen synthesis (29).

Fat Oxidation Overview

Role in metabolism. The process of fatty acid oxidation occurs in the cell mitochondria of most body tissues. While short- and medium-chain fatty acids are able to diffuse directly through the mitochondrial membrane, long-chain fatty acids must be transported through the inner mitochondrial membrane via the carnitine shuttle. Outside the mitochondria, the activated fatty acid, also known as fatty acyl CoA, is covalently joined to carnitine by the enzyme carnitine acyltransferase I (CAT I). The

shuttle passes the inner mitochondrial membrane and the fatty acid is released into the mitochondrial matrix by the enzyme carnitine acyltransferase II (ACAT II) (30).

Once the fatty acid is in the matrix, beta oxidation can occur. Initially, a double bond is formed between the α- and β-carbons by the enzyme acyl CoA dehydrogenase. A molecule of water is added by acyl CoA and the β-hydroxy group is oxidized, converting the fatty acyl CoA into β-ketoacyl CoA. The β-ketoacyl CoA is then cleaved at the β-carbon, which results in a saturated fatty acyl CoA that is two carbons shorter than the original fatty acid. Each of these reactions is then repeated, resulting in two carbons being cleaved from the chain each time. One acetyl CoA is produced each time the sequence is repeated, therefore an 18-carbon fatty acid would produce 9 acetyl CoA. Each acetyl CoA then goes on to produce 12 ATP in the TCA cycle. Additionally, each cleavage of a carbon-carbon bond that occurs produces 5 ATPs by the oxidation of FADH2 and NADH in the electron transport chain (30).

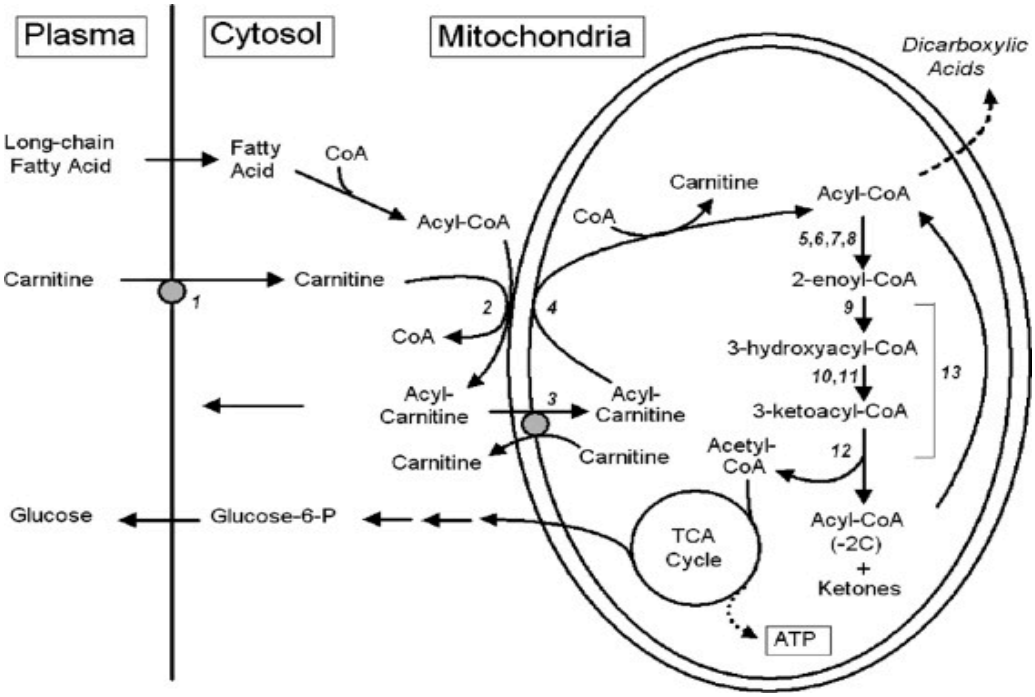


Figure 2. Process of Fatty Acid Oxidation (31)

Measurement. Fat oxidation is required to metabolize long-chain fatty acids for use as energy. The amount or proportion of fat that is oxidized can be determined by measuring an individual's respiratory exchange values. This method can also be used to determine the extent of utilization of carbohydrate and protein as fuel. In research settings, this is typically done via indirect calorimetry using a metabolic cart. In order to determine respiratory quotient (RQ), the metabolic cart measures the consumption of oxygen (O_2) and the elimination of carbon dioxide (CO_2). The RQ is determined by dividing the amount of CO_2 eliminated by the amount of O_2 consumed. It is a measure of the types of substrates that are being metabolized for energy production. An RQ of 1.0 indicates carbohydrate metabolism, 0.82 indicates protein metabolism, and 0.71 indicates fat metabolism (32).

Metabolic carts have been repeatedly validated as an accurate means of the measurement of substrate utilization. In a study by Phang and colleagues of two popular metabolic cart models, it was found that there was ≤2.4% error in CO_2 measurements, and ≤3.2% error in O_2 measurements (33). Additionally, a study by Blond and colleagues showed that two different popular metabolic cart models produced highly reliable measurements, with coefficients of variation for repeatability at 1.2% for both machines (34).

Collecting a participant's Urinary Urea Nitrogen (UUN) and calculating the nonprotein RQ provides more accurate data regarding the types and amounts of substrates that are being oxidized. This value can be calculated by using an individual's UUN output to calculate the amounts of CO_2 produced and O_2 consumed by the metabolism of protein. This is because 1 gram of nitrogen equates to approximately 4.8 liters of CO_2 expired and 6.0 liters of oxygen consumed. Subtracting the calculated protein values from the CO_2/O_2 ratio will produce the nonprotein RQ. The amount of

oxidized fat and carbohydrate in grams per liter and by percentage can then be determined using the nonprotein RQ table (35).

In order to obtain data utilizing a metabolic cart, the participant is fitted with a mask connected to two tubes. Both tubes are connected to the metabolic cart; one tube is for inspired O2 and the other is to measure the CO2 that is expired by the participant. Before beginning data collection, the participant is allowed to rest in a darkened room, allowing them to achieve a resting metabolic state. After a period of rest, data collection begins; O2 consumption and CO2 elimination are measured. The RQ is then calculated from those values as previously discussed (35).

Association with weight and body composition. Fat oxidation has been shown in human and animal models to have an inverse relationship with weight and body fatness (10,11). In fact, in a study of Pima Indians by Zurlo et al, lower fat oxidation was significantly correlated with weight gain (11). This finding suggests that when fat is oxidized efficiently, it can lead to the maintenance of a healthier body weight (10). Conversely, when fat oxidation is inefficient, the body will begin using protein as an energy source, which contributes to muscle wasting and a higher percent body fat (36).

An illustration of the effect of impaired fat oxidation can be observed in individuals with an inborn error of metabolism called primary carnitine deficiency. Individuals with this condition have a mutation in the SLC22A5 gene, causing a defect in the OCTN2 carnitine transporter. Thus, depending on the particular mutation, individuals with primary carnitine deficiency either have a decreased ability or an inability to shuttle long-chain fatty acids into the cell mitochondria for fat oxidation. Individuals with this condition often present with symptoms such as cardiac and skeletal myopathy and hepatic steatosis due to an accumulation of long-chain fatty acids in these tissues,

hypoketotic hypoglycemia, and a general feeling of fatigue due to the inability to properly metabolize long-chain fatty acids (36,37).

Defining Obesity

Fat types and distribution. There are two distinct types of fat in the human body, known as brown adipose tissue and white adipose tissue. Brown adipose tissue is unique to mammals. Its function is to metabolize the macronutrients from food into energy for the production of heat. Additionally, children have a significantly higher proportion of brown adipose tissue than adults, which lends itself to the increased metabolic activity of childhood. Due to its role in energy expenditure, the presence of brown adipose tissue is protective against obesity and does not increase the risk for chronic disease (38). The composition of brown adipocytes is also quite different than that of white adipocytes; brown adipocytes are smaller, contain less fat and more water, and are rich in mitochondria and vasculature. On the contrary, the sole purpose of white adipose tissue is fat storage. White adipose tissue becomes more prevalent with age and is not metabolically active. Therefore, the accumulation of excess white adipose tissue is associated with an increased risk for chronic disease (39).

The risk associated with body fat is not only based on the amount present, but on the location of the accumulated fat. A number of research studies have shown a significant correlation between abdominal fat and chronic diseases such as heart disease and type II diabetes (40,41). In a study by Canoy and colleagues, the data from 24,508 men and women in the European Prospective Investigation Into Cancer and Nutrition in Norfolk Cohort was examined. A significant correlation was found between waist-to-hip ratio and subsequent cardiovascular events that occurred throughout the cohort study (40).

In recent years, it has been found that visceral fat is a stronger predictor of chronic disease risk than abdominal fat alone. Visceral fat accumulates in the intra-abdominal cavity surrounding the internal organs. It is thought that the accumulation of visceral fat occurs due to a dysfunction in adipose tissue storage. Further, some researchers hypothesize that the adipocyte dysfunction that is present in visceral adiposity is involved in the pathogenesis of type 2 diabetes (42). In a prospective cohort study of 520 Japanese Americans by Bokyo and colleagues, it was found that visceral fat was a significant predictor of the development of type 2 diabetes. This effect was independent of other risk factors such as glycemia, insulin secretion, fasting insulin, total and regional adiposity, and family history (43).

On the contrary, overall body fat is not typically considered a serious risk factor for chronic disease without the presence of significant abdominal adiposity (44). However, in a study by Meisinger and colleagues examining data from the Monitoring Trends and Determinants on Cardiovascular Diseases (MONICA) Augsburg survey, both abdominal and overall body fat were found to be significantly related to subsequent development of type II diabetes (41).

Assessment. Body fat percent is often measured for the purposes of determining risk for chronic disease. The American Council on Exercise classifies males as obese when they have ≥25% body fat, and females as obese when they have ≥32% body fat. Although BMI is commonly used to determine health and disease risk, research has shown that body fat percent is a better indicator for disease risk than BMI (45,46). In fact, in a recent study by Goonsageran and colleagues, it was found that BMI falsely classified a significant number of both male and female participants as overweight or obese although they had healthy levels of body fat (45).

Body fat percent can be measured using a number of techniques, with varying levels of accuracy. The gold standard method of body fat measurement is computed tomography (CT). However, CT is quite expensive, and is therefore impractical for most research studies (46). The most common methods used for research purposes are dual-energy X-ray absorptiometry (DEXA), bioelectrical impedance, and anthropometric skinfold measurements. All of these are valid and reliable methods for measuring total body fat percent (46,47).

Waist circumference measurement is another commonly used method of defining overweight and assessing risk for chronic disease. Specifically, waist circumference measurement is used to determine abdominal obesity. In fact, in a study by Abbasi and colleagues, greater than normal waist circumferences were positively correlated with markers for chronic disease risk such as blood pressure, blood glucose, and triglycerides (48). The waist circumference cut points for increased relative risk are >88 cm (>35 in.) in women, and >102 cm (>40 in.) in men (49).

Currently, the most valid and reliable methods of measuring visceral fat are DEXA and CT (46,47). Because visceral fat is located in the intra-abdominal cavity, it cannot be accurately assessed superficially. Therefore, it must be measured using imaging technologies. The DEXA scan is the most recent method of assessing body fat, as it was initially developed exclusively for the measurement of bone density. The DEXA scan has repeatedly been shown to be a precise and reliable method of measuring body composition (50).

Vitamin C and Weight

Research overview. Vitamin C has been shown repeatedly to have an inverse relationship with both body weight and body fatness. In the European Prospective

Investigation into Cancer and Nutrition Norfolk (EPIC-Norfolk) cohort study, a very large-scale study of 19,068 adult participants, a significant inverse relationship between vitamin C and abdominal obesity was observed. After controlling for a number of factors, such as BMI, age, cigarette smoking, and supplement use, the data remained significant (40). Similarly, in a study by Singh et. al., a significant inverse relationship was observed between body fat percent and plasma vitamin C concentration in a cross-sectional study of Indian men (51). Because these are not experimental studies, they do not show causation for the observed effects. However, the significant relationships found between vitamin C status and body fat in these large-scale studies indicate that vitamin C may play a role in weight and the accumulation of body fat.

There is not enough evidence at this point to determine whether vitamin C deficiency could be an underlying cause of overweight and obesity, or a consequence of these conditions. However, many researchers maintain that the energy-dense and often nutrient-poor "Westernized" diet is causing widespread micronutrient deficiencies in our society (52). The micronutrient deficiencies resulting from the consumption of this type of diet could cause a number of metabolic changes in the body, possibly resulting in an increase in weight gain and retention. In fact, recent evidence has emerged that indicates that a reduction in energy intake alone is not as effective for weight loss when micronutrient intake is not sufficient (2,3). This data suggests that micronutrients such as vitamin C could be key factors in achieving and maintaining a healthy weight. There are a number of hypotheses attempting to explain the relationship between vitamin C deficiency and overweight and obesity. In fact, it is likely that there are a number of physiological factors involved. The associations between vitamin C and various physiological mechanisms involved in weight gain and the accumulation of body fat will be discussed briefly.

One theory attempting to explain the inverse association between vitamin C and weight maintains that inadequate vitamin C could cause changes in leptin levels, causing weight gain and increased inflammation. Leptin is a hormone that regulates energy intake by acting as an appetite suppressant. Adequate plasma vitamin C has been associated with increased leptin sensitivity (2,3). If sufficient plasma vitamin C is able to counteract the effect of leptin resistance, a deficient level of vitamin C could promote a state of leptin resistance, which would increase adiposity and the risk of overweight and obesity. When an individual is sensitive to leptin, they are able to receive normal cues telling them that they are full, and plasma leptin levels remain low. Overweight and obese individuals tend to have impaired leptin sensitivity, requiring much higher plasma concentrations of leptin in order to receive cues of satiety. This causes them to eat more, resulting in increased weight gain (53). In a cross-sectional study by Garcia and colleagues, the researchers hypothesized that there would be a link between plasma leptin concentrations, plasma vitamin C, and weight. They made this hypothesis based on the fact that there is a very high prevalence of obesity among Mexican women and also a high rate of micronutrient deficiencies, with vitamin C being the highest of these at 50%. Nearly 600 Mexican women were tested for plasma levels of glucose, leptin, zinc, and antioxidant vitamins A, C, and E. Additionally, anthropometric measures were taken to determine BMI and waist circumference. The researchers found that vitamin C had a significant inverse relationship with BMI, waist circumference, and plasma leptin concentrations among obese Mexican women (3). The relationships found in this study support the hypothesis that the plasma vitamin C could play a role in leptin sensitivity, thereby impacting weight and body fatness.

Another theory focuses specifically on the role of vitamin C as an antioxidant, maintaining that a deficiency of dietary antioxidant vitamins results in a state of

oxidative stress, causing an increase in the deposition of adipose tissue, particularly in the abdominal area. However, there is debate as to whether deficiencies of antioxidant vitamins such as vitamin C are the cause or the result of the condition of obesity. Some researchers hypothesize that the condition of obesity results in a state of inflammation in the body, causing rapidly depleted levels of antioxidant vitamins such as vitamin C. Regardless of the direction of causality, the low antioxidant status of many overweight and obese individuals is associated with a state of oxidative stress in the body. Oxidative stress is associated with increased risk for the development of chronic diseases such as heart disease, cancer, and type 2 diabetes that so often plague those with excess fat mass. Research has repeatedly shown that obese individuals have lower plasma concentrations of antioxidant vitamins such as vitamin C, vitamin E, and beta carotene, indicating that this theory merits further investigation to determine the physiological mechanism responsible for this relationship (2).

There has also been research examining the affect of vitamin C on plasma adiponectin levels as a potential mechanism of weight regulation. Adiponectin is a hormone that stimulates increased energy expenditure and helps to prevent insulin resistance. Plasma levels of adiponectin are typically decreased in a state of inflammation. In a randomized controlled trial by Johnston et. al., twenty sedentary obese subjects were randomly assigned to receive either a control or a vitamin C supplement. All subjects consumed a low-calorie, high-carbohydrate diet. After controlling for weight, there was no association between vitamin C and adiponectin as a result of the intervention. However, a statistically significant inverse relationship was shown between vitamin C status and BMI, waist circumference, and percent body fat after controlling for age and supplement use (4). This data indicates that while there was an inverse relationship between plasma

vitamin C and BMI, this effect was not due to a relationship between vitamin C concentration and adiponectin levels.

Supplementation and weight loss. There have been few randomized controlled trials in humans attempting to induce weight loss with the supplementation of vitamin C, although there was an animal study that yielded compelling results. In a randomized controlled trial by Campion et. al., overweight male Wistar rats were randomized to three different groups, one of which was fed a vitamin C supplemented diet. After 56 days on the supplemented diet, weight was significantly reduced in the Vitamin C supplemented group as compared to the other two groups regardless of caloric intake. The investigators attributed this effect to the antioxidant action of ascorbic acid, maintaining that it was able to alter the expression of genes affecting adiposity. Regardless of the physiological mechanism responsible for the observed effect, this study was able to show causality due to its experimental study design (54).

There are additional weight-related benefits of Vitamin C that exhibit its potential advantages for successful weight loss and weight maintenance. In a randomized controlled trial by Huck and colleagues, subjects were randomized to receive either a placebo or a 500 mg vitamin C supplement each day for four weeks while on a low-calorie, low vitamin C diet. While there was no significant difference in the amount of weight lost between groups, the subjects in the vitamin C group experienced a significant decrease in both heart rate and perception of fatigue while exercising than those in the control group. Due to the decrease in heart rate and perceived fatigue, the subjects were able to perform 10% more work after supplementation with vitamin C. The researchers attributed these findings to the fact that in a state of vitamin C deficiency there is little carnitine in the muscles, which causes fatigue. Additionally, vitamin C is essential for the synthesis of norepinephrine, which is a neurotransmitter that affects mood, heart

rate, and overall exercise performance. It is likely a combination of each of these components that contributed to the effects witnessed in the study (55). The data indicate that vitamin C supplementation could prove effective in other areas of weight loss and weight maintenance. These findings are important because obese individuals are often much more easily fatigued than normal weight individuals, which can prove detrimental to weight-loss efforts. The decreased perceived effort experienced during exercise in combination with the increased efficiency that were noted with vitamin C supplementation could serve to encourage obese and overweight individuals to stay on their exercise regimen, thus enabling them to continue to lose weight successfully. This effect in combination with a potential increase in fat oxidation could work to maximize weight loss and exercise adherence in those who typically struggle to lose weight.

Another important effect that vitamin C could have in overweight individuals is its antioxidant properties. The conditions of overweight and obesity tend to induce an inflammatory state in the body, often resulting in the onset of chronic disease. The antioxidant properties of vitamin C can help to lessen inflammation, which could in turn help to prevent or reduce the severity of such life-threatening chronic diseases as cancer, heart disease, and type II diabetes. Of course, as with everything relating to health, there is a genetic component that will determine the extent to which each individual will respond to a given dietary intervention (11). Considering each of these factors, it is clear that regular supplementation with vitamin C could potentially have a myriad of positive effects on overweight and obese patients even beyond weight loss and weight maintenance.

Vitamin C and Fat Oxidation

The role of carnitine. There is emerging evidence that the inverse relationship between vitamin C and weight could be attributed to the role that vitamin C plays in the synthesis of carnitine. Vitamin C serves as the reducing agent in two reactions that convert iron from the ferric to ferrous state, which are essential reactions in the biosynthesis of carnitine (5). In fact, muscle tissue exhibits a very high uptake of dietary vitamin C. This effect is likely attributed to the function of vitamin C as a cofactor in the synthesis of carnitine and collagen (56). Carnitine is a molecule that is required for the transport of long-chain fatty acids into cell mitochondria for the process of β-oxidation, or fat oxidation, the process by which long-chain fatty acids are metabolized for energy. In the absence of carnitine, long-chain fatty acids are unable to be transported from the cell cytosol into the mitochondria via the 'carnitine shuttle'. Carnitine is essential to the oxidation of long-chain fatty acids, and vitamin C is necessary for the synthesis of carnitine (5). Based on this premise, the vitamin C status of an individual should be directly related to fat oxidation.

The relationship between vitamin C and carnitine was further illustrated in a study by Johnston and colleagues in a depletion-repletion study of guinea pigs. Thirty-six male guinea pigs were depleted of vitamin C for a period of three weeks to the point that signs of scurvy were apparent. The guinea pigs were then fed a diet containing progressively stronger concentrations of vitamin C at 0.5 mg/100 g body weight, 2.0 mg/100 g body weight, with each of these repletion periods lasting for four weeks. Five guinea pigs were sacrificed at the end of each period for tissue analysis. At this time, the remaining guinea pigs were divided into groups and supplemented with either 6.0 mg/100 g body weight or 10.0 mg/100 g body weight for a final four-week period. At the end of the depletion period, acid-soluble carnitine in the muscle was depleted to levels lower than those

found in healthy guinea pigs. Additionally, after repletion with the 2.0, 6.0, and 10.0 mg doses of vitamin C, acid-soluble muscle carnitine increased, although not significantly (p=0.087) (57). This study is important because it shows that in a state of vitamin C depletion, muscle carnitine decreases as well. This is likely due to the fact that vitamin C is a cofactor in carnitine synthesis.

Research overview. The relationship between vitamin C and fat oxidation has recently become the basis for a hypothesis stating that this link could be a component in the pathogenesis of overweight and obesity, although very few studies have been conducted to test this hypothesis (8,9). Because of this physiological link, and the relationship that has been found between plasma vitamin C concentrations and body weight in a number of studies (2,4), this hypothesis merits further investigation. There have been two recent studies examining the relationship between vitamin C and fat oxidation that have concluded that there is a significant relationship between the measures of plasma vitamin C and fat oxidation (8,9).

Johnston and colleagues conducted a trial that supported this hypothesis. Initially, a group of seventy-eight non-smoking male and female participants ages 18-38 y were screened only for vitamin C status. It was found that 40% of the participants in this sample were deficient in plasma vitamin C, which is an unusually high prevalence. Out of this group of individuals, fifteen agreed to participate in an exercise study. The participants' fat oxidation was recorded as they walked on the treadmill for 60 minutes. Within this group, it was found that the subjects with adequate vitamin C had 25% higher fat oxidation than those with low levels of vitamin C. Upon completion of this trial, a very small group of eight of the subjects agreed to participate in a randomized controlled trial. These subjects were all given a placebo for the first four weeks of the trial in order to deplete vitamin C levels. In addition, they were given a list of foods high

in vitamin C that they were instructed to avoid consuming. At week five vitamin C levels were depleted, at which point the control group continued to take the placebo and the intervention group began taking 500 mg of vitamin C each day. In the intervention trial, subjects who took the 500 mg vitamin C supplement each day were found to oxidize fat at four times the rate of the vitamin C depleted control group ($p=0.011$). Additionally, an inverse relationship was found between vitamin C status and protein metabolism, indicating that the vitamin C depleted group tended to metabolize protein rather than fat (8). These results are quite significant, indicating that there is a need for future research utilizing a larger sample size in order to better illustrate a causal effect.

In a randomized controlled trial conducted by Ji and colleagues with one-year-old black sea bream, the fish were divided into 2 groups and fed either a casein-based diet or a casein-based diet fortified with 1,100 mg vitamin C for 60 days. The black sea bream in the vitamin C supplemented group showed a significant increase in acid-soluble carnitine. Additionally, those that consumed the diet high in vitamin C were found to experience increased fat oxidation. The findings of this study are especially important because they show a causal link between plasma vitamin C, carnitine, and fat oxidation. It is interesting to note that this method was tested in farmed fish in an effort to find a way to reduce the excess fat that these fish gain due to relative inactivity as compared to the wild species. Because they did find a significant correlation between vitamin C and fat metabolism, this could be considered as a relatively inexpensive supplement for farmed fish in the future (9). Although the results of the study supported the hypothesis, more studies need to be conducted in humans, as animal studies are not relatable to a human population.

The results of the existing studies examining the relationship between vitamin C and fat oxidation are very compelling, showing a promising link between vitamin C status

and fat oxidation. Further study is needed in this area utilizing large sample sizes of human research participants.

In summary, very few studies have been conducted examining the link between vitamin C status and fat oxidation. Determining the factors involved in increasing fat oxidation could result in a decrease in overall body mass, thereby reducing the prevalence of overweight and obesity. The studies that have been conducted in this area have been few and limited; therefore, more research is necessary in this area. There have been several studies that have shown a significant inverse relationship between plasma vitamin C concentration and weight. This relationship requires further investigation in order to determine causation.

Based on the research that has already been conducted, it appears that vitamin C can have a significant impact on fat oxidation during moderate exercise, thereby making weight loss easier for individuals on an exercise routine. Additionally, it could decrease the perceived effort expended during exercise, making it easier to adhere to an exercise plan. Based on findings from previous research, vitamin C could improve the weight loss efforts of overweight and obese individuals, as well as potentially improving their health. Vitamin C supplementation could potentially be a safe and affordable way to help achieve and maintain a healthier weight. This is not only because of the weight loss that could occur based on the increased efficiency of fat oxidation, but also because of the positive health effects that could be experienced because of its antioxidant action, possibly alleviating some of the damaging oxidative stress that may have been caused by the excess weight. It is clear that further research in this area could help to advance our understanding of the pathology of overweight and obesity, allowing us to find the safest

and most effective means of treatment for individuals attempting to achieve a healthier body weight.

Methods

Participants

Men and pre-menopausal women aged 18-50 were recruited from a campus population and social networking websites via electronic advertisement. Participants (n=84) included lacto-ovo vegetarians (n=27), vegans (n=28) and omnivores (n=29). All data was analyzed Qualifying participants had adhered to their respective diets for a minimum of 6 months, were not endurance trained athletes, had a BMI <30, had no known chronic diseases, and were free from all prescription medications other than oral contraceptives. All female participants experienced regular menstrual cycles, and had not been pregnant within the preceding six months. All participants provided written consent. This research was approved by the Institutional Review Board at Arizona State University prior to the start of the study. (see Appendix A)

Study Design

A cross-sectional design was employed in this study. Participant recruitment took place over a period of two months with the use of fliers, e-mail lists, and advertisement in online vegan and vegetarian groups. An online survey was used to determine participant inclusion. Those who met the criteria and chose to participate in the study reported to the laboratory for two separate 90-minute visits. Written consent was obtained from all participants prior to beginning the study. This study was part of a

larger study on fat oxidation in vegans and vegetarians; some data was collected for the purposes of the larger study that will not be discussed herein.

Study Protocol

Visit 1. Participants signed the informed consent, completed a health history form, and completed a physical activity questionnaire. A multiple pass 24-hour diet recall was conducted with each participant. Anthropometric measurements were obtained, including height, waist circumference, and body weight. Body fat percentage and lean body mass were obtained using DEXA. Prior to DEXA testing, all female participants were required to pass a urine pregnancy test. For the DEXA assessment, participants were positioned face up on a flat bed for 7 minutes to allow the DEXA scanner to pass over the entire body. All participants were required to wear metal-free clothing while under the DEXA scanner. Finally, participants were provided with 24-hour urine vessels and instructions on completing the urine collection prior to visit 2.

Visit 2. Participants arrived to the laboratory after a 12-hour overnight fast, with the completed 24-hour urine collection. Each participant was allowed to rest in a dark, quiet room for a 30-minute period before obtaining resting energy expenditure (REE). The stationary metabolic cart was employed to measure O2 consumption and CO2 production via indirect calorimetry in order to determine respiratory quotient (RQ) values for each participant (60). In order to collect these measurements, the resting participant was fitted with a facemask, which was connected to the metabolic cart with an inspired air hose and an expired air hose for the duration of the 30-minute period. Participants were asked to consume a typical diet and not to engage in moderate to intense physical activity during the 24-hour period preceding REE measurement. Upon completion of REE assessment, a venous blood sample was collected from each

participant in order to assess plasma vitamin C. A research nurse collected all samples using EDTA-treated vacutainers and immediately centrifuged each sample.

The RQ data used in this study are nonprotein RQ values. These were calculated for each participant using the UUN values obtained from the 24-hour urine collection. (see Appendix B)

Measurements

Height was measured using a mechanical stadiometer. Waist circumference was measured at the umbilicus and recorded in duplicate using a Gulick tape measure with constant tension in order to ensure accuracy.

Laboratory Analysis

Plasma was extracted from the fasting blood samples, mixed with an equal volume 10% TCA, centrifuged, and frozen at -80°C until vitamin C analysis. At the time of analysis, all samples were thawed and the 2,4-dinitrophenylhydrazine method was employed to determine ascorbic acid concentrations (58). (see Appendix C)

Statistical Analysis

Statistics were analyzed using IBM SPSS Statistical Software (Version 19, SPSS Inc., Chicago, IL). When necessary, data were log-transformed for normality, and examined for outliers and confounders. Grouped data was reported as mean ± SD, two-way ANOVA was used to examine subject characteristics between groups, and a partial Pearson's correlation was used to assess relationships between variables. Data was considered significant at $p \leq 0.05$.

Results

A total of 82 participants completed the study. However, due to missing RQ values, only 69 participants were included in the analysis of RQ, fat oxidation, and carbohydrate oxidation data. For all other data analysis, all 82 participants were included. Of these 82 participants, 27 were omnivores (8 men and 19 women), 27 were lacto-ovo vegetarians (6 men and 21 women), and 28 were vegans (10 men and 18 women). The overall mean age of participants was 31.0±8.5 years, mean body weight was 67.4±10.2 kg, and the mean plasma vitamin C concentration was 0.58±0.16 mg/dl. The overall mean BMI was 22.9±2.6 kg/m2, mean waist circumference was 80.1±9.9 cm, mean body fat percent was 27.8±6.4, and the mean visceral fat was 381.8±335.0 cm3. The overall mean RQ was 0.81±0.06, mean fat oxidation was 52.3±16.3 g, and mean carbohydrate oxidation was 32.5±17.7.

Assessment of participant characteristics of plasma vitamin C concentration revealed that 23 participants had inadequate plasma levels (<0.5 mg/dl). Inadequate levels of plasma vitamin C were found in 59.3% of participants in the omnivore group, 18.5% of the lacto-ovo vegetarian group, and 10.7% of the vegan group. Further, a correlation between vitamin C intake (mg/day) and plasma vitamin C (mg/dl) showed a significant positive correlation ($p=.041$, $r=.248$).

Subject characteristics as grouped by diet type and gender are displayed in Table 2. Two-way ANOVA analysis was employed, and the post-hoc test used was the least significant difference test. Two-way ANOVA analysis revealed that age, plasma vitamin C, BMI, and carbohydrate oxidation were similar in men and women. However, gender was found to affect weight ($p<.001$), waist circumference ($p=.002$), visceral fat ($p=.001$), body fat percent ($p<.001$), RQ ($p=.048$), and fat oxidation ($p=.028$). No significant interaction was found between gender and diet type.

Table 2. Descriptive Measures Grouped by Gender
(mean values, standard deviations)

	Males (n=24)	Females (n=58)	P-value (Gender)
Age	31.5±8.2	30.5±8.8	0.648
Weight (kg)	73.8±11.1	61.0±9.2	.000*
Vitamin C (mg/dl)	0.57±0.15	0.60±0.16	0.332
BMI	23.4±2.3	22.4±2.9	0.153
Waist Circumference (cm)	85.0±7.7	77.9±9.6	0.002*
Visceral Fat	546.5±430.6	217.0±239.4	0.001*
Body Fat Percent	22.7±6.3	32.8±6.5	.000*
RQ**	0.83±0.06	0.80±0.06	0.048*
Fat Oxidation**	47.3±16.3	57.3±16.3	0.028*
Carb Oxidation**	36.8±17.6	28.2±17.8	0.083

*p-values <0.05 represent significantly different results of two-way ANOVA
**69 participants were analyzed for measures of fat oxidation: 18 males, 51 females

A partial correlation was used to determine the relationships between plasma vitamin C and various measures of substrate utilization, controlling for group, gender, and BMI. Correlations between vitamin C and measures of substrate utilization are displayed in Table 3. No significant correlations were found between vitamin C and RQ, fat oxidation or carbohydrate oxidation. The same correlations were run on participants after omitting vegans, and no significant correlations were found. Additionally, correlations

were run separately on male and female participants, and no significant correlations were found with either gender group.

Table 3. Correlations between plasma vitamin C and measures of substrate utilization

		Vitamin C (mg/dl)	RQ	Fat Oxidation	Carb Oxidation
Vitamin C (mg/dl)	Correlation	1.000	-0.113	-0.139	0.095
	Significance**	.000*	0.364	0.265	0.446
RQ	Correlation	0.113	1.000	-0.92	0.99
	Significance**	0.364	.000*	.000*	.000*
Fat Oxidation	Correlation	-0.139	-0.92	1.000	-0.89
	Significance**	0.265	.000*	.000*	.000*
Carb Oxidation	Correlation	0.095	0.99	-0.89	1.000
	Significance**	0.446	.000*	.000*	.000*

*p-values ≤0.05 considered statistically significant
**2-tailed significance

A partial correlation was used to determine the relationships between plasma vitamin C and various measures of body composition, controlling for group, gender, and age. Correlations between vitamin C and measures of body composition are displayed in Table 4. A significant inverse correlation was found between plasma vitamin C and body fat percent: r=-.248, and p=.027. There was also a significant inverse relationship between plasma vitamin C and visceral fat: r=-.251, and p=.025. These data reveal that as plasma vitamin C decreases, there is an increase in both visceral fat and percent total body fat. No significant correlations were found between plasma vitamin C and weight, BMI, or waist circumference. All body composition correlations were also run on the

participants after omitting vegans, and no significant correlations were found. Additionally, correlations were run separately on male and female participants. The significance for plasma vitamin C and visceral fat was r=-.270 and p=0.044 for females, and r=-.272 and p=.221 for males . The significance for total body fat percent was r=-.241 and p=0.074 for females, and r=-.300 and p=.174 for males.

Table 4. Correlations between plasma vitamin C and measures of body composition

		Vitamin C (mg/dl)	Weight (kg)	BMI	Waist (cm)	Visceral Fat	Body Fat Percent
Vitamin C (mg/dl)	Correlation	1.000	-0.053	-0.153	-0.115	-0.251	-0.248
	Significance**	.000*	0.643	0.177	0.313	0.025*	0.027*
Weight (kg)	Correlation	-0.053	1.000	0.807	0.818	0.579	0.628
	Significance**	0.643	.000*	.000*	.000*	.000*	.000*
BMI	Correlation	-0.153	0.807	1.000	0.825	0.609	0.699
	Significance**	0.177	.000*	.000*	.000*	.000*	.000*
Waist (cm)	Correlation	-0.115	0.818	0.825	1.000	0.692	0.764
	Significance**	0.313	.000*	.000*	.000*	.000*	.000*
Visceral Fat	Correlation	-0.251	0.579	0.609	0.692	1.000	0.647
	Significance**	0.025*	.000*	.000*	.000*	.000*	.000*
Body Fat Percent	Correlation	-0.248	0.628	0.699	0.764	0.647	1.000
	Significance**	0.027*	.000*	.000*	.000*	.000*	.000*

*p-values ≤0.05 are considered statistically significant

**2-tailed significance

Figure 3 illustrates the relationship between plasma vitamin C and percent body fat for all participants. Figures 4 and 5 illustrate the relationship between vitamin C and visceral fat for all participants.

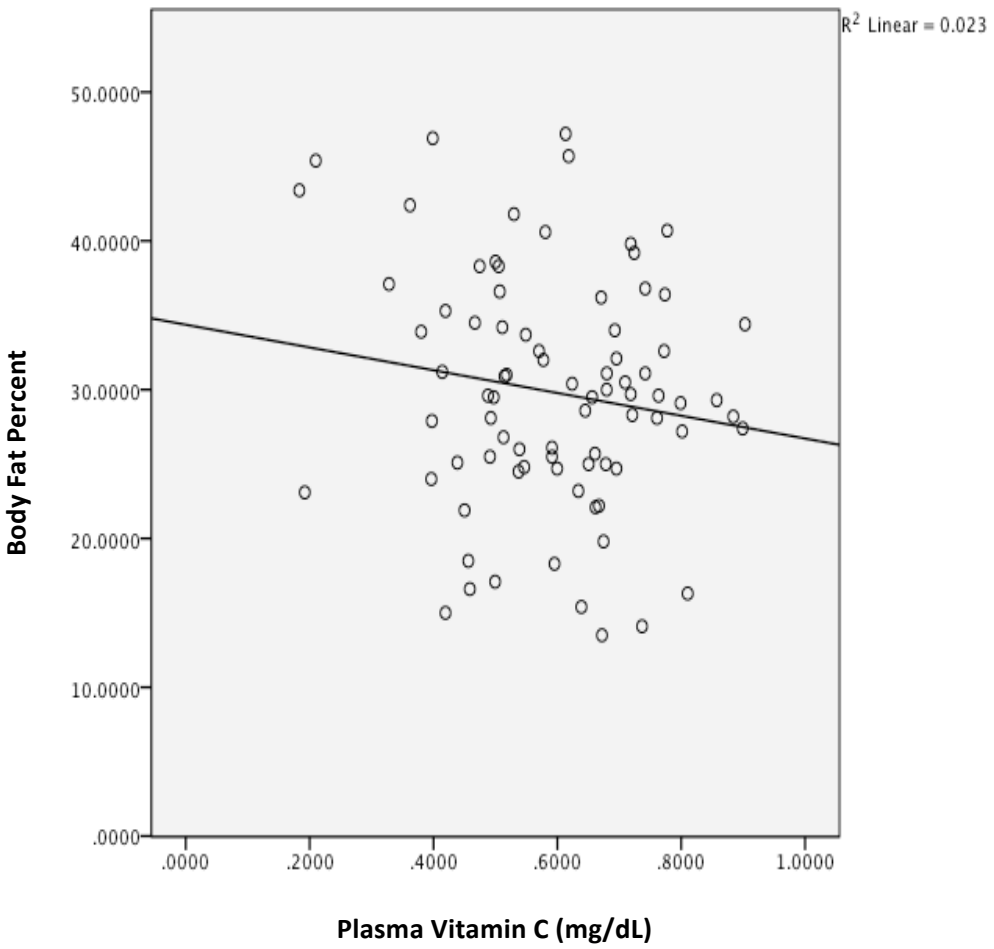


Figure 3. Simple Scatter Plot Depicting the Trend Between Body Fat Percent and Plasma Vitamin C (n=82)

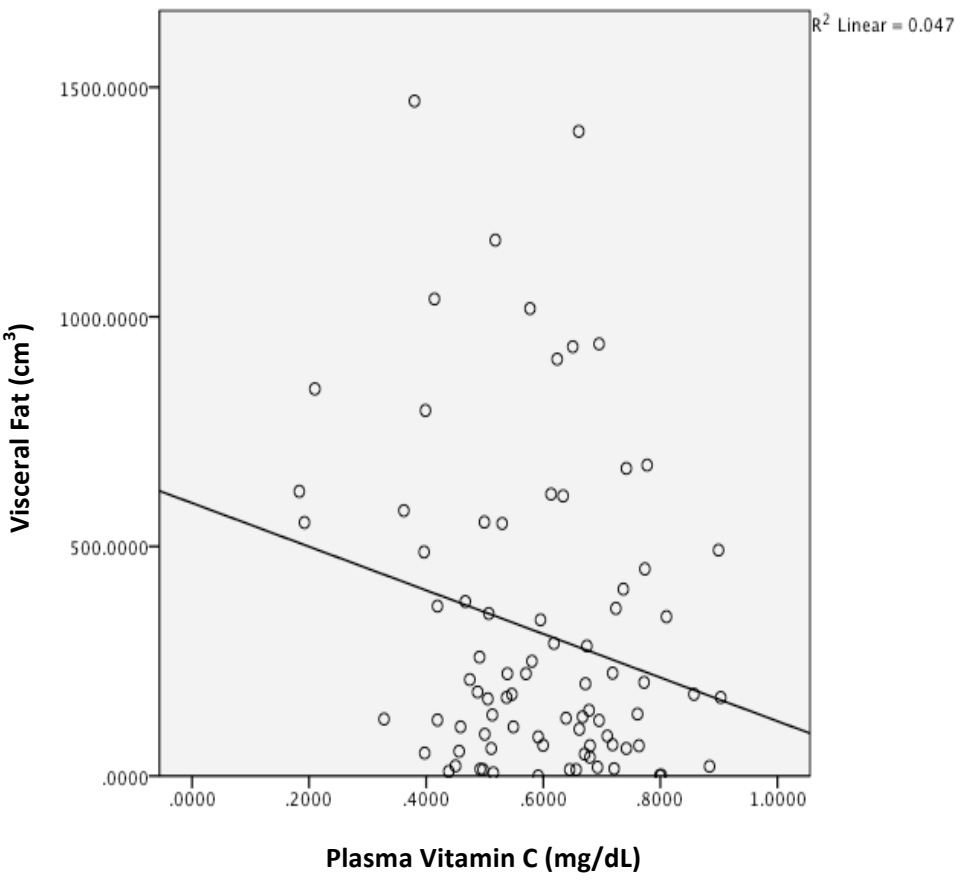


Figure 4. Simple Scatter Plot Depicting the Trend Between Visceral Fat and Plasma Vitamin C (n=82)

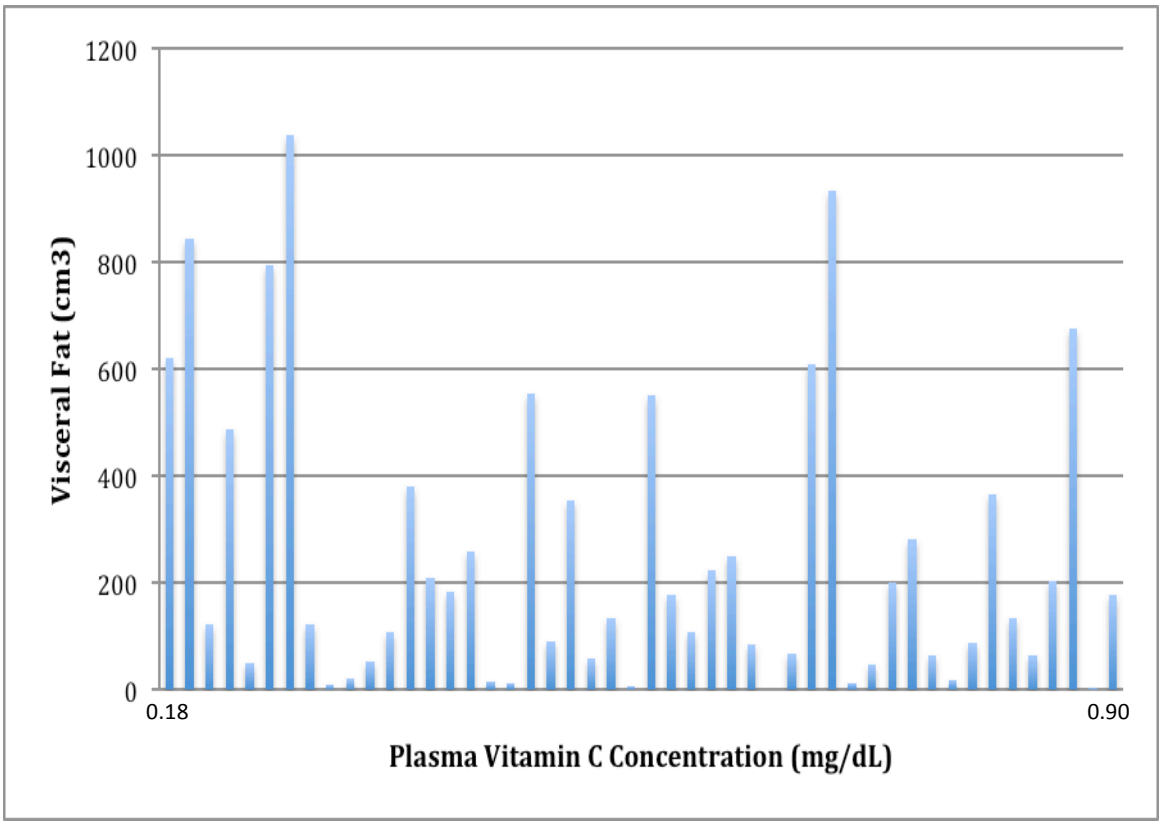


Figure 5. Bar Graph Depicting the Trend Between Visceral Fat and Plasma Vitamin C (n=82)

Discussion

There have been two previous studies documenting a significant association between plasma vitamin C concentration and fat oxidation, attributing the relationship to the role of vitamin C as a cofactor in carnitine synthesis (8,9). However, no significant association was found between vitamin C and fat oxidation in this study. This could be attributed to the fact that this study was a correlational trial assessing fat oxidation of individuals in a resting state. In contrast, the only previous study finding a relationship between vitamin C and fat oxidation in humans was an intervention trial conducted in

individuals during exercise (8). The dramatic difference in results between these studies indicates that vitamin C could play different roles in substrate utilization depending on the level of energy expenditure. There is a need for further intervention trials exploring whether there is a significant correlation between vitamin C and fat oxidation during vigorous physical activity and at rest, or whether there appears to be a difference in substrate utilization depending on the level of physical activity. Additionally, obesity has been associated with decreased fat oxidation in previous research (59). Therefore, obese individuals were excluded from this study. Further research is required to explore whether obesity significantly impacts the relationship between vitamin C and fat oxidation.

The potential role of vitamin C in carbohydrate metabolism deserves further attention. In a recent study by Castro and colleagues, a significant correlation was found between vitamin C and the metabolism of glucose and lactate in the brain. The cells of the brain are full of ascorbic acid transporters SVCT2 and GLUT1 and 3. Because of this, the brain retains a high concentration of vitamin C even in a state of deficiency. This indicates that vitamin C plays an especially important role in the brain. The study found that vitamin C had a high concentration in neuronal cells and astrocytes, and had the ability to switch off glucose metabolism in favor of lactate metabolism in order to maintain a high antioxidant capacity in the brains (60). Because vitamin C could potentially play such an important role in carbohydrate metabolism in the brain, further study is necessary in this area.

A number of studies have found a significant inverse relationship between plasma vitamin C concentration and body fat percent (4,40,51). A significant inverse association was found between vitamin C and body fat in this research as well, thus supporting the research hypothesis and the findings of previous research. This association could be

attributed to an increase in fat oxidation during periods of physical activity among individuals with higher plasma concentrations of vitamin C. Individuals with higher plasma vitamin C would be able to synthesize adequate carnitine to facilitate increased fat oxidation during physical activity (8).

The most unexpected finding in this research was the significant inverse association between plasma vitamin C and visceral fat. This association has not been reported in any other research. Visceral fat has been shown repeatedly to correlate with an increased risk of chronic disease (40,41,61). In a cross-sectional study of 113 adults by Sironi and colleagues, there were significant associations between visceral fat and risk factors for both heart disease and diabetes. Visceral fat was significantly associated with reduced insulin sensitivity ($p=.002$), increased blood pressure ($p=.04$), and hypertriglyceridemia ($p<.02$). Overall, there was a significantly higher accumulation of visceral fat in individuals with impaired glucose tolerance and type 2 diabetes, and in individuals with three or more risk factors for the metabolic syndrome (61). Because such strong associations have been shown between visceral fat and markers for chronic disease risk, the mechanisms for the accumulation of visceral fat should be explored further.

It is important to consider gender as a significant factor in the relationship between plasma vitamin C and both visceral fat and body fat percent. The 2-way ANOVA results revealed that there were significant differences in visceral fat and body fat percent between males and females. Additionally, there were changes in the strength and significance of the correlations between vitamin C, body fat percent, and visceral fat when they were run separately for males and females. In fact, the only correlation that remained significant after separating by gender was between plasma vitamin C and visceral fat in females. However, the strength of the correlations between vitamin C and both visceral fat and body fat percent were higher for males as compared to females.

This is likely due to the much smaller sample size of males (n=24) as compared to females (n=58). If the sample size of males had been larger, the correlations between vitamin C and both visceral fat and body fat percent may have been more significant among males than females.

There is a need for further research exploring the relationship between vitamin C and body fat, especially visceral fat. The cause of this relationship remains unclear because this study failed to show a significant relationship between vitamin C status and fat oxidation in a rested state. However, previous research has shown a relationship between vitamin C and fat oxidation in a physically active state, therefore this relationship cannot be disregarded (8,9). Additionally, the possibility of a difference in the physiological role of vitamin C in varying states of physical activity should be considered. This research showed that vitamin C did not have a significant correlation with fat oxidation while the participants were at rest. Therefore, the involvement of vitamin C in substrate utilization may be dependent on whether an individual is at rest or engaging in physical activity. Further research is needed in order to explain these very different results.

In conclusion, the inverse association that was found between vitamin C and visceral fat in this research should be further explored in order to determine the physiological mechanisms involved as well as the direction of causality. It is unclear whether increased visceral fat could contribute to more rapid depletion of vitamin C due to increased inflammation, or whether a lower concentration of plasma vitamin C contributes to an increase in the accumulation of visceral fat. Alternately, the inverse relationship that has been found between vitamin C and leptin resistance could be a mechanism behind the increased deposition of fat among individuals with inadequate vitamin C. Further research is needed exploring a potential link between leptin, vitamin

C, and visceral fat. The potential differences between genders should be considered in all future studies due to the significant difference in body composition between males and females. Additionally, further research is required in order to clarify whether the metabolic function of vitamin C is dependent on an individual's level of energy expenditure.

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APPENDIX A

INSTITUTIONAL REVIEW BOARD APPROVAL

APPROVAL:CONTINUATION

Carol Johnston
SNHP - Nutrition
602/827-2265
CAROL.JOHNSTON@asu.edu

Dear Carol Johnston:

On 11/5/2013 the ASU IRB reviewed the following protocol:

Type of Review:	Continuing Review
Title:	
Investigator:	Carol Johnston
IRB ID:	1211008557
Category of review:	(8)(a) Long-term follow-up, (8)(c) Data analysis
Funding:	None
Grant Title:	None
Grant ID:	None
Documents Reviewed:	None

The IRB approved the protocol from 11/5/2013 to 11/4/2014 inclusive. Three weeks before 11/4/2014 you are to submit a completed "FORM: Continuing Review (HRP-212)" and required attachments to request continuing approval or closure.

If continuing review approval is not granted before the expiration date of 11/4/2014 approval of this protocol expires on that date. When consent is appropriate, you must use final, watermarked versions available under the "Documents" tab in ERA-IRB.

In conducting this protocol you are required to follow the requirements listed in the INVESTIGATOR MANUAL (HRP-103).

Sincerely,

IRB Administrator

APPENDIX B

DETERMINING SUBSTRATE UTILIZATION USING INDIRECT CALORIMETRY DATA

RQ = CO_2 produced ÷ O_2 consumed

RQ for carbohydrate:

$$C_6H_{12}O_6 + 6\ O_2 \text{ è } 6\ CO_2 + 6\ H_2O$$

$$RQ = 6\ CO_2 \div 6\ O_2$$

$$=1.00$$

RQ for Fat:

$$C_{16}H_{32}O_2 + 23\ O_2 \text{ è } 16\ CO_2 + 16\ H_2O$$

$$RQ = 16\ CO_2 \div 23\ O_2$$

$$=0.696$$

RQ for Protein:

$$C_{72}H_{112}N_2O_{22}S + 77\ O_2 \text{ è } 63\ CO_2$$

$$RQ = 63\ CO2 \div 77\ O2$$

$$=0.818$$

Determining Nonprotein RQ:

1 g N Metabolized = 6.0 L O2 consumed, and 4.8 L CO2 produced
Multiply by g N excreted to determine amounts consumed/produced by protein catabolism

Total O2 consumed – amount consumed by protein catabolism = Nonprotein O2
Total CO2 produced – amount produced by protein catabolism = Nonprotein CO2

Nonprotein CO2 ÷ Nonprotein O2 = Nonprotein RQ

Thermal Equivalents of Oxygen for the Nonprotein RQ, Including Percentage Kilocalories and Grams Derived From Carbohydrate and Fat (57)

RQ	kcal/liter O_2	Percentage kcal derived from		Grams/Liter O_2	
		Carbohydrate	Fat	Carbohydrate	Fat
0.70	4.686	0.0	100	0.0	0.496
0.71	4.690	1.1	98.9	0.012	0.491
0.72	4.702	4.8	95.2	0.051	0.476
0.73	4.714	8.4	91.6	0.090	0.460
0.74	4.727	12.0	88.0	0.130	0.444
0.75	4.739	15.6	84.4	0.170	0.428
0.76	4.750	19.2	80.8	0.211	0.412
0.77	4.764	22.8	77.2	0.250	0.396
0.78	4.776	26.3	73.7	0.290	0.380
0.79	4.778	29.9	70.1	0.330	0.363
0.80	4.801	33.4	66.6	0.371	0.347
0.81	4.813	36.9	63.1	0.413	0.330
0.82	4.825	40.3	59.7	0.454	0.313
0.83	4.838	43.8	56.2	0.496	0.297
0.84	4.850	47.2	52.8	0.537	0.280
0.85	4.862	50.7	49.3	0.579	0.263
0.86	4.875	54.1	45.9	0.621	0.247
0.87	4.887	57.5	42.5	0.663	0.230
0.88	4.899	60.8	39.2	0.705	0.213
0.89	4.911	64.2	35.8	0.749	0.195
0.90	4.924	67.5	32.5	0.791	0.178
0.91	4.936	70.8	29.2	0.834	0.160
0.92	4.948	74.1	25.9	0.877	0.143
0.93	4.961	77.4	22.6	0.921	0.125
0.94	4.973	80.7	19.3	0.964	0.108
0.95	4.985	84.0	16.0	1.008	0.090
0.96	4.998	87.2	12.8	1.052	0.072
0.97	5.010	90.4	9.60	1.097	0.054
0.98	5.022	93.6	6.4	1.142	0.036
0.99	5.035	96.8	3.2	1.186	0.018
1.00	5.047	100.0	0.0	1.231	0.0

APPENDIX C

VITAMIN C ASSAY

PROCEEDURES AND CALCULATIONS FOR PLASMA ASCORBIC ACID ASSAY

SOLUTIONS

		Weigh	Add up to	
		TCA	**dH20**	
1	**5% TCA***	5 gms	100ml	*TCA: Trichloroacetic Acid
2	**10% TCA**	10 gms	100ml	

		Measure	Slowly Add	**** ADD ACID TO WATER**
		dH20	**36N H2SO4**	** (use 0.75ml 65% H2SO4 per sample,
3	**9N H2SO4**	300 ml	100 ml	calculate how much needed and mix
4	**65% H2SO4 ****	35 ml	65 ml	larger volume if necessary)

		Weigh	Weigh	Weigh	Slowly add
		Thiourea	**CuSO4-5H2O**	**2,4 dinitrophenylhydrazine**	**9N H2SO4**
5	**DTC *****	0.4 gms	0.05 gms	3 gms	100 ml

*** Use only 100µl per sample so decrease amount depending on how many samples you run

		Weigh	Add up to	
		Ascorbate	**5% TCA**	
6	**Stock Solution**	5 mg	25 ml	Separate in to 1 ml aliquots and freeze at -43° C

		Pipette	Add up to	
		Stock Soln	**5% TCA**	
7	**Working Solution**	500µl	5 ml	Use graduated test tubes to measure

Sample Processing

Draw blood into 7ml EDTA tube. Mix well and ice immediately. Spin at 2800rpm x 10 min. Mix 2.5ml plasma with 2.5mls cold 10% TCA and vortex x 10 sec. Spin immediately at 3500g x 20 minutes at 0° C. Aliquot supernatant into 3 aliquots ~ 1.5 ml each. Freeze immediately at -80° C.

Collect 5ml urine in plain tube. Add 5ml 10%TCA+0.2gms Norit (activated charcoal)/ml urine. Spin immediately at 3500g x 20 minutes at 0° C. Aliquot supernatant into 3 aliquots ~ 2 ml each. Freeze immediately at -80° C.

Prepare Standards

	REAGENTS IN TUBE			*** CONCENTRATION**	
Tube #	**µl Work Soln**	**µl 5%TCA**	**µl DTC**	**µg AA/0.5ml**	**AA mg/dl**
1	0	500	100	0	0
2	100	400	100	2	0.4
3	200	300	100	4	0.8
4	300	200	100	6	1.2
5	400	100	100	8	1.6
6	500	0	100	10	2
SAMPLE	500	0	100	sample	sample

Assay Procedure

1. Prepare standards as described above
2. Incubate tubes for 3 hrs at 37° C.
3. Add 0.75ml (750µl) ICE COLD 65% H2SO4 to all tubes. Vortex for a count of 5 seconds after each addition to mix.
4. Let stand at room temperature for 30 minutes
5. Read at 520nm in spectrophotometer. Use tube #1 as the blank.
6. Using Excel, produce standard curve using AA mg/dl concentration as X axis and absorbance as Y axis. Produce a linear trendline and show equation. Obtain AA concentrations in mg/dl by solving for y=mx+b.

Volume calculator:

		Blood samples
Standards	6	urine samples
Number of samples in duplicate	0	
65% H2So4 requried	4.5	mls
DTC required	0.6	mls

*** NOTE REGARDING CALCULATION OF AA IN mg/dl**

Factor of 0.2 used to convert AA µg/0.5ml to mg/dl. This factor takes into account that only 0.5ml of sample is used and then converts µg/ml to mg/dl

stock Soln: 0.2mg/ml

Working Solution: 0.2mg/ml

Tube	Work Soln	Conc	convert to µg/ml **x 2 =**	convert to mg/dl **x0.1**
1	….			
2	0.1 ml	2µg/0.5ml	4µg/ml	0.4mg/dl
3	0.2 ml	4µg/0.5ml	8µg/ml	0.8mg/dl
4	0.3 ml	6µg/0.5ml	12µg/ml	1.2mg/dl
5	0.4 ml	8µg/0.5ml	16µg/ml	1.6mg/dl
6	0.5 ml	10µg/0.5ml	20µg/ml	2.0mg/dl

OR multiply Conc x 0.2 to convert µg/0.5ml to mg/dl