Targeted Metabolomics Reveals the Effect of Nitrate Supplementation on Vascular Function

by

Jeffrey Patterson

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Graduate Supervisory Committee:

Haiwei Gu, Chair
Carol Johnston
Karen Sweazea

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ABSTRACT

In the United States, two-thirds of adults are considered hypertensive or prehypertensive. In addition, chronic illness, such as hypertension, cardiovascular disease, and type II diabetes, results in $3.5 trillion in annual healthcare cost and is the primary cause of disability and death. As a result, many individuals seek cheaper and simpler alternatives to combat their conditions. In this exploratory analysis, a study assessing nitrate intake and its effects on vascular function in 39 young adult males was investigated for underlying metabolic variations through a liquid chromatography – mass spectrometry-based large-scale targeted metabolomics approach. A two-way repeated measures ANOVA was used, and 18 significant metabolites were discovered across the time, treatment, and time & treatment groups, including prostaglandin E_2 ($p<0.001$), stearic acid ($p=0.002$), caprylic acid ($p=0.016$), pentadecanoic acid ($p=0.027$), and heptadecanoic acid ($p=0.005$). In addition, log-transformed principal component analysis and orthogonal partial least squares – discriminant analysis models demonstrated distinct separation among the treatment, control, and time variables. Moreover, pathway and enrichment analyses validated the effect of nitrate intake on the metabolite sets and its possible function in fatty acid oxidation. This better understanding of altered metabolic pathways may help explicate the benefits of nitrate on vascular function and reveal any unknown mechanisms of its supplementation.

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CHAPTER 1

INTRODUCTION

In 2013, one in every three US adults had been diagnosed with hypertension, while another one-third was considered to have prehypertension ("Hypertension among adults in the United States: National Health and Nutrition Examination Survey, 2011-2012 - PubMed," n.d.). High blood pressure costs the United States $48.6 billion per year and results in more than 1,100 deaths each day ("Underlying Cause of Death 1999-2018," n.d.). Largely due to healthcare costs, only half of the hypertensive population has their condition controlled (Farley, Dalal, Mostashari, & Frieden, 2010). However, recent studies have shown that blood pressure can be reduced through dietary intake. Nitrate, commonly found in green, leafy vegetables, is a precursor of nitric oxide (NO), a vasodilator tasked with blood pressure maintenance (Versari, Daghini, Virdis, Ghiadoni, & Taddei, 2009).

The vascular benefits of nitrate intake were first proposed when reductions in blood pressure were observed several hours after consuming beetroot juice, a high nitrate containing food (Webb et al., 2008). Over the ensuing decade, numerous researchers have investigated beetroot juice supplementation and nitrate-rich diets for potential benefits in endothelial function. Its effects in vasodilation and exercise performance have been extensively tested in both healthy subjects and in the presence of vascular disease (Berry et al., 2015; Kenjale et al., 2011; McMahon, Leveritt, & Pavey, 2017). While its mechanism in improved cognitive function of older adults has yielded mixed results (Gilchrist et al., 2014; Kelly et al., 2013), it has demonstrated improved postprandial

endothelial function following a high-fat meal in overweight and slightly obese men (Joris & Mensink, 2013).

In 2013, a systematic review of sixteen randomized crossover trials displayed a strong correlation between nitrate supplementation and a reduction in systolic and diastolic blood pressure (Siervo, Lara, Ogbonmwan, & Mathers, 2013). While other meta-analyses have demonstrated similar results in older populations, differing study designs have yielded conflicting results (Stanaway, Rutherfurd-Markwick, Page, & Ali, 2017). It has been postulated that the method of nitrate intake may influence its expected benefits. A randomized crossover trial concluded that increased plasma nitrate levels were observed following a high-nitrate supplement, but not with a high-nitrate diet in normotensive adults (Miller et al., 2012). The method and quantity of intake, as well as, their effects on the metabolome warrant further consideration.

As an emerging science, metabolomics offers a physiological report of cellular metabolism. The quantification and analyzing of intermediates and end products in metabolic pathways enable it to be a powerful tool in research. Utilizing altered metabolites, or metabolic signatures, biomarkers of altered states can be identified. To date, only one study has used the advantages of metabolomics to investigate nitrate supplementation on vascular function. A single metabolic pathway among every vascular measure was identified in response to nitrate intake, while more than one-hundred metabolites were significantly associated with more than eleven pathways. The study also documented nearly sixty percent of its compounds as unidentified due to an untargeted methodology (De Van et al., 2016). Untargeted metabolomics utilizes a broad strategy to analyze all of the analytes in a provided sample to discover novel target compounds. An

inherent disadvantage is that it is unable to identify many smaller compounds. With a targeted approach, defined groups of metabolites are identified and quantitated with the use of internal standards, such as ^{13}C Lactate and ^{13}C Glutamic acid. The analytes can then be assessed for their associations with multiple biochemical pathways and their influence in altered physiological states (Roberts, Souza, Gerszten, & Clish, 2012). With only one previous metabolomics study, a large gap in understanding still exists regarding nitrate metabolites and improved vascular function. Further metabolomics research may help identify unknown metabolic pathways and explicate any variations in findings with differing nitrate studies.

The intent of this research is to use a targeted metabolomics methodology to investigate potential metabolite signatures of nitrate supplementation and its effects on vascular function. A recent randomized, placebo-controlled, parallel arm study design demonstrated improved vascular function with a beetroot juice supplement in normotensive, young adult men in the Phoenix area (Sweazea, Johnston, Miller, & Gumpricht, 2018). The aim of this study is to perform an exploratory analysis on the previous trial to examine any effects of nitrate supplementation on the metabolome.

H: Liquid nitrate supplementation that resulted in improved blood pressure will be significantly correlated with altered metabolic pathways in normotensive, young adult males in the Phoenix area.

Definitions:

- **Vascular function**: Blood pressure and flow-mediated dilation measurements

- **Normotensive**: 140/90 mm Hg > blood pressure ≥ 100/65 mm Hg
- **Baseline**: Prior to starting treatment or placebo supplementation

Delimitations:

- Phoenix area
- Healthy, young adult male participants

Limitations**:**

- Convenience sample
- Study designs had not considered this analysis
- Proprietary blend of nitrate sources
- Fourteen-day intervention period

CHAPTER 2

THE REVIEW OF LITERATURE

Overview

Within the last ten years, nearly one-third of the entire US population has been diagnosed with hypertension, with an additional third being considered prehypertensive ("Hypertension among adults in the United States: National Health and Nutrition Examination Survey, 2011-2012 - PubMed," n.d.). With it being one of the most prevalent chronic illnesses, it costs the United States almost $50 billion annually ("Underlying Cause of Death 1999-2018," n.d.). With blood pressure levels at or exceeding 130/80 mm Hg, hypertension plays a significant inflammatory role in several comorbidities, such as cardiovascular disease, metabolic syndrome, and cognitive decline (Whelton et al., 2018). Typically originating from poor dietary intake and environmental factors, elevated blood pressure has a profound impact on vascular function. Despite its high incidence, hypertension still remains one of the most preventable chronic illnesses. Recent studies have investigated various ways to supplement the Western diet with lower cost, healthy alternatives.

The aim of this review of literature is to examine five aspects pertaining to nitrate intake and its effect on vascular function. First, to explain the role of the nitric oxide pathways, their substrates, and vascular function within the body. Furthermore, research current studies investigating nitrate supplementation through various metabolic conditions, exercise routines, and cognition. Finally, demonstrate the advantages of metabolomics and statistical analyses, while carefully examining the only metabolomics analyses of nitrate intake studies.

The Nitric Oxide Pathways

The Nitrate-Nitrite-NO Pathway

The endogenous L-Arginine-nitric oxide pathway and dietary intake are the two significant sources of nitric oxide (NO) within the body. Exogenous sources of nitrate require a reduction to nitrite, which commonly occurs via bacteria throughout the mouth and gastrointestinal tract. Within the bacteria, are nitrate reductases (NAR), which are reducing enzymes. Due to the dependence upon oral bacteria, studies that have not controlled for antibacterial mouthwashes have shown reduced plasma nitrite levels (Lundberg & Weitzberg, 2010). While much less effective, mammalian enzymes, such as xanthine oxidoreductase (XO), are also able to carry out the reduction (Lundberg, Weitzberg, & Gladwin, 2008). The further reduction of nitrite to NO may be catalyzed by the molybdopterin enzyme, XO, and in differing conditions, such as hypoxia and/or acidosis. Through single electron transfer reactions, NO can be produced independent of oxygen via deoxygenated hemoglobin, myoglobin, neuroglobin, as well as, in lowered pH conditions (Vanderpool & Gladwin, 2015).

The L-Arginine-NOS Pathway

As previously mentioned, the L-Arginine-NOS pathway is a significant source of NO within body tissues. The nitric oxide synthase (NOS) enzymes are tasked with the formation of NO, and citrulline, from L-Arginine and are comprised of neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). Found throughout various cell types, the mechanisms behind NO production differ between the NOS enzymes. The endothelial and neuronal NOS enzymes are intracellularly expressed in response to calcium levels and/or stressors. Conversely, iNOS is not dependent upon calcium levels

and can bind tighter to calmodulin than nNOS and eNOS. Therefore, NO production from iNOS lasts considerably longer and is typically higher than that of the other NOS enzymes. Though its levels are quite low, iNOS can be expressed through transcription. The overall conversion of NO results in vasodilation, antiplatelet aggregation, and neurotransmission (Burke et al., 2013).

Regulating the Production of NO

NO can be regulated through a variety of processes, such as production location, enzyme activity, gene expression, and NOS inhibitors. The localization of the three NOS enzymes with their targeted proteins, while also having very little overlap with each other, is a central component of the period and location that NO is manufactured. In addition, eNOS has been shown to be regulated by protein kinase B (Akt), while nNOS and iNOS can be mediated through phosphorylation. However, it appears that iNOS is largely controlled through transcriptional channels. As an arginine analogue and NOS inhibitor, asymmetrical dimethylarginine (ADMA) competes with arginine for binding with NOS and, subsequently, reduces NO production (Luiking, Engelen, & Deutz, 2010).

Biological Effects of NO

NO and its pathways have critical biological effects within the body, such as vasodilation, blood pressure regulation, energy metabolism, inflammatory response, immune defense, and neurotransmission. As a signaling molecule, NO has been shown to regulate glucose, amino acid, and fatty acid metabolism. The uptake of glucose, fatty acid oxidation, and lipolysis in multiple tissues, such as skeletal muscle, heart, adipose, and liver, may be stimulated by physiological levels of NO. Additionally, NO levels have shown to lessen glucose, glycogen, and fat formation in targeted tissues. As a result, the

reduction of NO production is thought to cause fat accumulation and hyperlipidemia, while arginine intake has induced lipolysis in diabetic rats. Multiple cGMP pathways, increased blood flow to insulin-sensitive tissues, NO-induced phosphorylation, and gene expression are thought to be the underlying mechanisms of its metabolic capacities (Jobgen, Fried, Fu, Meininger, & Wu, 2006). The NO produced by the NOS enzymes within the L-Arginine-NOS Pathway have shown to have their own unique biological effects as well.

The nNOS enzyme can be found within the cytosol of nervous tissue and skeletal muscle. Within the central nervous system (CNS), its derived NO regulates blood pressure via smooth muscle relaxation and synaptic plasticity, which is long-term inhibition and potentiation. In addition, nNOS acts as an atypical neurotransmitter within the peripheral nervous system (PNS) that can control vasodilation, peristalsis, and penile erection. The membrane attached eNOS enzyme is found within the endothelium and its produced NO is tasked with vasodilation, vasoprotection from platelet aggregation, and atherosclerosis protection through gene expression. A significant component of cardiovascular disease risk results from the uncoupling of eNOS, endothelial dysfunction, and/or oxidative stress. Also typically positioned within the cytosol, the iNOS enzyme is found within the cardiovascular and immune system. The iNOS enzyme is stimulated by cytokines and inflammatory diseases, and its produced NO regulates numerous inflammatory states. Additionally, it is a primary regulator of vasodilation and decreased blood pressure in the presence of septic shock (Förstermann & Sessa, 2012).

The L-Arginine Paradox

The paradox is a phenomena that depicts the rise in NO production after exogenous arginine intake, despite the already saturated intracellular concentrations of arginine (Bode-Böger, Scalera, & Ignarro, 2007). Numerous studies have found conflicted findings with regards to the importance of exogenous L-arginine on the production of NO. The synthesis of NO has shown to be comprised of fifty-four percent of endogenous L-arginine sources, while only 2.7-5.7% is derived from dietary intake (Mariotti et al., 2013). Such a large discrepancy in endogenous and exogenous sources warrants further analysis to entirely understand the NO pathways.

A past review of NO production and regulation in health and disease provided an interpretation for the L-Arginine Paradox (Luiking et al., 2010). A previous study had examined NO and vascular insulin resistance and discovered an important cofactor, (6R)-5,6,7,8-tetrahydrobiopterin (BH_4), for NO synthesis from endogenous arginine. It was determined that obesity and type II diabetes induced insulin resistance can be hindered by modulating the L-arginine-NO pathway with BH_4 and arginine supplementation (Wu & Meininger, 2009). However, in a current 2020 systemic review, it was concluded that ADMA and the arginine/ADMA ratio is the most accurate predictor of NO bioavailability. The supplementation of arginine increases its availability, which will stabilize the arginine/ADMA ratio. The excess arginine then competes with ADMA for binding with eNOS (Gambardella et al., 2020). More recent studies have attempted to examine the effects of L-arginine intake, while also considering ADMA concentration, and its subsequent NO production.

In a randomized, double-blind, placebo-controlled trial, the effects of L-arginine supplementation in seventeen healthy, young adult males was analyzed for NO

production, while plasma levels of ADMA and symmetric dimethylarginine (SDMA) were assessed. As an endogenous NOS inhibitor, increased levels of plasma ADMA have been associated with endothelial and vascular dysfunction (Tsikas, Bollenbach, Hanff, & Kayacelebi, 2018)("Asymmetric dimethylarginine (ADMA): an endogenous inhibitor of nitric oxide synthase and a novel cardiovascular risk molecule - PubMed," n.d.). While not disturbing NOS activity, SDMA has shown to compete with L-arginine (Tsikas, Böger, Sandmann, Bode-Böger, & Frölich, 2000). It was found that ADMA and SDMA levels were unaffected, and NOS activity yielded no significant findings with L-arginine consumption (Alvares, Conte-Junior, Silva, & Paschoalin, 2012).

In a cross-sectional study of 2771 young and older adult men and women, the dietary intake of L-arginine and its correlation with serum nitrate + nitrite (NOx) was examined. A 168-food frequency questionnaire was employed to calculate intake, and linear regression models were used to contrast demographics, anthropometrics, and health status. The mean nitrate intake (4.43 ± 2.56 g/day) was determined to be similar to most other populations, such as US Caucasian, African-American, and Hispanic adults. A significant positive association was found in both genders, with a stronger correlation in women. Additionally, middle-aged and older adults demonstrated a higher association of increased NOx. When stratified for BMI and vascular disease, non-hypertensive, overweight, and obese participants had a greater indication. Strong correlations in middle-aged to older adults and overweight to obese subjects can be attributed to increased serum ADMA levels. However, all findings were only significant in the absence of hypertension (Mirmiran, Bahadoran, Ghasemi, & Azizi, 2016). With a greater understanding of all of the components of nitrate, nitrite, and arginine metabolism and

their effects on NO production, it is important to examine the efficacy of nitrate supplementation on various vascular functions.

Nitrate & Metabolic Conditions

Healthy Subjects

As one of the pioneering nitrate studies, Webb (2008) examined its effects on cardiovascular disease in healthy volunteers. The trial utilized a randomized, crossover design that administered 500 mL of beetroot juice to 14 healthy subjects. Blood pressure (BP) measurements were recorded at fifteen-minute intervals from 1 hour pre- to 3 hours post-treatment, hourly until hour six post-treatment, and at 24 hours. After 3 hours of supplementation, plasma nitrite concentrations had increased, and BP had reduced by 10.4/8 mm Hg. Flow-mediated dilation (FMD) was also assessed in ten individuals via brachial artery diameter (BAD) two hours after treatment. It was found that the FMD response was significantly reduced by nearly 60% ($p<0.001$), and dietary nitrate had prevented against ischemia/reperfusion (I/R) injury. This study would eventually lead to several of the subsequent studies investigating the effects of nitrate supplementation on vascular function (Webb et al., 2008).

A randomized, placebo-controlled, crossover trial was employed to examine the effect of nitrate-rich food on vascular function. Healthy post-menopausal women (52.6 ± 5.7y) with normal to mildly elevated blood pressure were recruited in the Phoenix Metropolitan area. The post-menopausal group was selected to potentially control for the release of progesterone and its influence on vascular reactivity in a younger population. The participants ($n = 10$) were randomly assigned to a high-nitrate intake (HNI) group or a low-nitrate control (LNC) group. Subjects were asked to consume a high-nitrate

vegetable salad (HNI) or a low-nitrate vegetable medley (LNC) twice each day for ten consecutive days. A washout period of two to three weeks was then observed, followed by reassignment into the opposing group. Peripheral and central-aortic blood pressures and FMD were recorded at baseline, prior to treatment, and following treatment. It was found that nitrate-rich salad intake significantly increased plasma nitrates/nitrites concentration and improved FMD, while peripheral and central-aortic blood pressure demonstrated no association with nitrate intake (Mayra, Johnston, & Sweazea, 2019).

Several studies have associated diets rich in green leafy vegetables with a decreased risk of cardiovascular disease and stroke incidence. A randomized, crossover trial looked to measure the effects of a diet containing high-nitrate vegetables on blood pressure and plasma nitrate concentrations. Nineteen healthy, young adult women either ingested or avoided high-nitrate vegetables for one week and were measured prior to and after each intervention. Plasma nitrate concentrations (+36.6 μmol/L; $p<0.05$) were significantly improved in the high-nitrate diet with no change in the control diet. Resting systolic blood pressure (-4 ± 6 mm Hg; $p<0.05$) was also reduced in the high-nitrate group, while the control group observed no change. Consuming a diet of high-nitrate vegetables was able to produce similar benefits in vascular function to previously conducted trials of liquid nitrate supplementation in healthy women (Ashworth, Mitchell, Blackwell, Vanhatalo, & Jones, 2015).

Over the past decade, the vascular effects of beetroot supplementation have been extensively researched. Due to its emphasis, several varying study designs have derived different conclusions. Researchers had performed a systematic review and meta-analysis to elucidate several factors regarding beetroot juice supplementation and its effects on

systolic and diastolic blood pressure. Randomized clinical control trials conducted between 2009 and 2017 were investigated, and twenty-two studies, 47 intervention and 43 control groups, were selected. It was determined that both systolic (-3.55 mm Hg) and diastolic (-1.32 mm Hg) blood pressure were significantly lower in the beetroot supplementation group. It was also noted that chronic ingestion, fourteen days or greater, had the largest mean difference (-5.11 mm Hg) in systolic blood pressure (SBP). Additionally, higher doses (500 mg) were more effective than low or moderate (70-140 mg) doses at reducing SBP (-4.78 mm Hg and -2.37 mm Hg, respectively) (Bahadoran, Mirmiran, Kabir, Azizi, & Ghasemi, 2017).

Several of the previously conducted trials have examined the effects of nitrate intake as a supplement, such as beetroot juice, sodium nitrite capsule, and a gel. However, very few studies have investigated the source of nitrate in a beverage as a potential variable for benefits in vascular function. A semi-randomized, crossover trial examined the effects of four nitrate (12.9 mmol) containing beverages (sodium nitrate, concentrated beetroot juice, a rocket salad beverage, and a spinach beverage) in healthy, normotensive young adults. Eleven men and seven women had their plasma nitrate levels and blood pressure measured at baseline and 150 minutes and 300 minutes post-treatment. All beverages produced similar peak plasma nitrate levels but differed in systolic blood pressure response. At 150 min post-treatment, a reduction was observed in the beetroot juice (-5 ± 2 mm Hg; $p<0.001$) and rocket salad beverage (-6 ± 2 mm Hg; $p=0.007$) groups. Systolic blood pressure was significantly decreased at 300 minutes post-treatment in the spinach beverage group (-7 ± 3 mm Hg; $p<0.001$). Sodium nitrate supplementation did not exhibit any effects on systolic blood pressure. Diastolic blood

pressure was significantly lower ($p<0.05$) at 150 min for all groups and remained reduced in the rocket salad ($p=0.045$) and spinach beverage ($p<0.001$) groups at 300 min. Dietary intake of nitrate-rich vegetables demonstrated the ability to improve plasma nitrate concentrations and blood pressure in normotensive individuals, while sodium nitrite supplementation was less effective (Jonvik et al., 2016).

To further examine the effects of diet on nitrate supplementation, a randomized, four period, crossover, placebo-controlled study measured the effects of a three-day control diet compared to a nitrate-rich diet in the presence and absence of a nitrate supplement (beetroot juice). Eight healthy, normotensive older men and women (72.5 ± 4.7 years, 5 men and 3 women) were randomly assigned to one of the possible eight arrangements of intervention. It was determined that plasma nitrate levels of the nitrate-rich diet did not differ from the control diet, which conflicts with several previous studies. In addition, the nitrate-rich diet did not have an effect on plasma levels when combined with the beetroot juice supplement. All four dietary interventions had no outcome on systolic or diastolic blood pressure. Although, the nitrate supplement was able to significantly increase plasma nitrate levels for the remaining day. It was stated that older individuals suffering from chronic conditions, such as peripheral artery disease, may still benefit from nitrate supplementation. While factors like demographics and health status may clarify the lack of vascular results, the study design may have also been influential. The nitrate concentration of the nitrate-rich diet was evenly allocated throughout the day instead of a bolus feeding, which may explain the unaffected plasma levels. The study should have administered the same nitrate content in one feeding, similar to the beetroot juice. In addition, the plasma levels following supplementation

were lower than previous findings. The supplement was given with a meal, which may have delayed absorption. The beetroot juice could have been ingested in between meals to improve absorption (Miller et al., 2012).

After ingestion of a mixed meal, endothelial function becomes diminished during the postprandial state, and the reduced bioavailability of NO is thought to be related (Westphal et al., 2006). The promotion of reactive oxygen species, chylomicron remnants, and free fatty acids are believed to lessen NO availability (Nappo et al., 2002). A study investigated if nitrate supplementation could improve endothelial function in a postprandial state. A randomized, placebo-controlled, crossover trial measured the effects of beetroot juice (140 mL) with a high fat meal (56.6 g) and its effects on flow-mediated dilation (2 h post-meal) in twenty healthy overweight and slightly obese (BMI: 28-35 kg/m^2) men (61 ± 7 years). The supplement was ingested within ten minutes of meal consumption. Postprandial plasma nitrate concentrations were statistically significant at all time periods ($p<0.001$) and highest at 1, 2, and 4 h. The increase in postprandial flow-mediated dilation was also significant ($p=0.03$). Peripheral and central augmentation indices of the arterial waveform were significantly reduced following meals ($p<0.01$), as well as, lowered diastolic blood pressure ($p<0.01$). A single dose of beetroot juice was shown to ameliorate postprandial endothelial dysfunction potentially by increasing NO bioavailability (Joris & Mensink, 2013). The previous studies have accounted for various populations and methods of delivery. It is also prudent to consider the outcomes in a population with already elevated blood pressure.

Hypertension

Hypertension is a serious risk factor for incidence of stroke and other cardiometabolic disorders. Moreover, pregnant females with hypertension are at a significantly greater risk of adverse outcomes, such as pre-eclampsia, restricted fetal growth, and preterm birth. A randomized, double-blind, placebo-controlled, parallel arm trial examined the effects of daily nitrate intake (70 mL beetroot juice) in forty hypertensive pregnant women. The subjects were randomized and had their plasma and salivary samples, blood pressure, cardiovascular function, and uteroplacental blood flow measured at baseline and 3 h and 8 days post-treatment. Plasma and salivary ($p<0.001$) nitrate levels were significantly increased in the beetroot juice group and displayed a large variation between subjects. While there was no significant reduction in blood pressure, a significant correlation between reduced diastolic blood pressure and plasma nitrate concentration ($p=0.0042$) was recorded. 97% of the subjects found nitrate supplementation acceptable for daily intake. Differences in the oral microbiome between participants may explain the varied results (Ormesher et al., 2018).

Many studies have examined the varied effects of nitrate supplementation in individuals with hypertension. However, a limited amount of studies have measured the potential benefits of a daily, high dose intake beyond four weeks. A randomized, double-blind, placebo-controlled, parallel-arm study examined the effects of beetroot juice on clinical, ambulatory, and home blood pressure, as well as, arterial stiffness (pulse wave velocity) and endothelial function (flow-mediated dilation). Treated ($n = 34$) and untreated ($n = 34$) hypertensive subjects were stratified and randomized to consume a 250 ml bottle of nitrate-rich or nitrate-depleted beetroot juice each morning for four weeks. Subjects were tasked with documenting daily blood pressure at home and returning to the

clinic one day prior to completion of the intervention. The ambulatory blood pressure (ABP) was then measured for 24 hours and clinical blood pressure, arterial stiffness, and endothelial function were recorded. All three blood pressure measurements, ABP (-7.7/5.2 mm Hg, $p<0.001$), clinical (-7.7/2.4 mm Hg, $p<0.05$), and at home (-8.1/3.8 mm Hg, $p<0.01$), showed significant reductions. In addition, arterial stiffness ($p<0.01$) and endothelial function ($p<0.001$) demonstrated significant improvements. The study was well tolerated and displayed the effectiveness of chronic, high dose nitrate intake (Kapil, Khambata, Robertson, Caulfield, & Ahluwalia, 2015).

Due to the numerous trials investigating the effects of nitrate supplementation over the past ten years, its results have been largely mixed regarding hypertension. A randomized, double-blind, placebo-controlled, crossover study design aimed to determine its influence in a treated, but uncontrolled hypertensive population. Twenty overweight (BMI = 30.7 kg/m^2) individuals were administered a beetroot juice drink (12.9 mmol/L nitrate ~ 230 mL) or placebo (0.5 mmol/L) once each day for seven consecutive days. Following a washout period, the participants then switched groups. It was found that supplementation significantly reduced 24 h ambulatory systolic and diastolic blood pressure (-8 mm Hg; $p=0.012$, and -4 mm Hg; $p=0.018$, respectively) and increased plasma nitrate levels ($p=0.0004$). Dietary nitrate intake demonstrated no negative interactions with hypertension medication and provided improvements in vascular function in overweight individuals with uncontrolled hypertension (Conor P. Kerley, Dolan, James, & Cormican, 2018).

The benefits of a nitrate-rich diet have been widely reported among several differing populations, but its interactions with vasoactive medications are not well

understood. A parallel arm trial investigated beetroot juice supplementation (12.9 mmol/L nitrate ~ 230 mL) and its effects on 24 h ambulatory blood pressure (BP), arterial stiffness, and fasting blood levels. Eleven individuals with controlled BP and eight with uncontrolled BP were monitored for fourteen days. It was found that plasma nitrate was significantly increased in both groups, but only significant reductions in diastolic blood pressure (-6 ± 4.8 mm Hg, p=0.03), arterial stiffness (-0.08 ± 0.03, p=0.05), and LDL cholesterol (-0.36 ±0.42 mmol/L, p=0.046) were observed in the uncontrolled group. Nitrate supplementation had anti-hypertensive properties but not in individuals with controlled hypertension (C. P. Kerley, Dolan, & Cormican, 2017). After examining the effects of nitrate in the hypertensive population, it is important to consider its use in the presence of cardiovascular disease.

Cardiovascular Disease

A current systemic review and meta-analysis examined thirty-four randomized, parallel and crossover trials to evaluate the ability of nitrate/nitrite to prevent and/or treat risk factors of cardiovascular disease. In this diverse review, there were a total of 813 subjects, fifty-one percent male, and a mean age from young to older adult (20 - 72.5 y/o). The analysis considered blood pressure as the primary outcome, while the secondary outcomes were endothelial function, arterial stiffness, platelet aggregation, and lipid profiles. Endothelial function was measured via FMD, arterial stiffness was assessed with PWV, and lipid profiles included total cholesterol, triglycerides (TGs), high-density lipoproteins (HDLs), and low-density lipoproteins (LDLs). It was determined that resting systolic (-4.80 mm Hg, p<0.0001) and diastolic (-1.74 mm Hg, p=0.001) blood pressure were significantly reduced, while endothelial function displayed significant improvement

(FMD: 0.59%, $p<0.0001$). Additionally, both arterial stiffness (PWV: -0.23 m/s, $p<0.0001$) and platelet aggregation (-18.9%, $p<0.0001$) were significantly reduced. It was concluded that nitrate consumption is beneficial in an acute setting, but long-term supplementation studies are needed (Jackson, Patterson, MacDonald-Wicks, Oldmeadow, & McEvoy, 2018).

In a 15-year longitudinal study, 5324 women were prospectively assessed for the correlation between daily nitrate intake and cardiovascular disease (CVD) incidence. The study defined cardiovascular complications as hypertension, heart disease, stroke, and thrombosis. With a mean age of 52.4 years, only healthy participants at baseline were included. For nitrate consumption, the study employed generalized estimating equations with 95% confidence intervals among four groups. There was a 27% and 24% reduced risk of CVD incidence with the participants who reported vegetable nitrate intake above 64.4 mg/day and total nitrate intake above 78.2 mg/day, respectively. Furthermore, there was a significant inverse association with CVD incidence and vegetable nitrate intake. Additionally, a significant association was also found for higher nitrate intake and higher BMI. Despite many reporting a higher BMI, the results still exhibited a marked reduction in CVD risk. The study does have clear limitations, such as a reliance on food frequency questionnaires and self-reporting incidence, therefore, all results are observational data, which do not indicate causality. Conversely, it is one of the few longitudinal studies that accounts for daily nitrate intake, and with the large number of participants, the results should not be discounted (Jackson et al., 2019).

Plasma $_{L}$-arginine has been shown to be diminished in individuals with hypercholesterolemia (Jeserich, Münzel, Just, & Drexler, 1992). The anti-inflammatory

role of NO and dysfunction of the L-arginine/NOs pathway have been associated with adverse effects in flow-mediated dilation in individuals with risks for cardiovascular disease. A randomized, double-blind, placebo-controlled parallel trial examined the effects of beetroot juice supplementation (250mL) on vascular and platelet function in subjects with hypercholesterolemia for six weeks. Sixty-seven nondiabetic participants (24 male and 43 female) with high cholesterol (>6.0 mmol/L) or a QRISK2 score above 15% were administered one nitrate supplement or placebo each day. Brachial flow-mediated vasodilation (FMD), pulse wave analysis, and blood and saliva samples were taken each visit. A 24% increase in FMD was measured in the treatment group ($p<0.001$), while a small improvement in aortic pulse wave velocity (PWV) was recorded ($p=0.06$). The treatment group displayed significant reduction in platelet aggregation compared to the control subjects ($p=0.004$). In addition, the beetroot juice supplement was correlated with increases in *Neisseria flavescens* and *Rothia mucilaginosa*, which are species of the salivary microbiome and associated with denitrification ($p<0.001$). It was concluded that nitrate supplementation promotes anti-atherosclerotic effects through improved vascular function and oral bacteria composition (Velmurugan et al., 2016). With the role of NO in energy metabolism, especially stimulating glucose uptake, it is necessary to consider its role in conditions concerning insulin resistance and secretion.

Type II Diabetes

While insulin resistance has demonstrated a reduced capacity for NO synthesis, a trial investigated sodium nitrite supplementation on insulin secretion and glucose tolerance in type II diabetic obese rats. Four groups (control, control + nitrite, diabetes, and diabetes + nitrite) were used to examine 50mg/L of sodium nitrite + water for eight

weeks. The control and diabetic groups had significantly improved insulin secretion and islet insulin content (27% and 39%, respectively) with supplementation and 16.7mM glucose. Serum interleukin-1β, a β-cell damaging inflammatory cytokine, was significantly decreased (p=0.001) and GLUT4 was significantly improved in the diabetic group. Prolonged nitrite supplementation demonstrated no association with HbA_{1c} levels, but did improve inflammatory markers, insulin secretion, and glucose and insulin tolerance (Gheibi et al., 2017).

A randomized, double-blind, placebo-controlled, crossover design study explored if nitrate intake may reduce blood pressure, insulin sensitivity, and improve endothelial dysfunction in type II diabetic individuals. Over a two-week period, twenty-seven participants (18 male and 9 female), aged (67.2 ± 4.9 y), were administered beetroot juice (7.5 mmols nitrate – 250mL). The primary measures were 24-hour ambulatory blood pressure monitoring (ABP), flow-mediated dilation (FMD), and insulin sensitivity via a hyperinsulinemic isoglycemic clamp (HIC). It was determined that after two weeks of supplementation, ABP remained unchanged. Plasma nitrate and nitrite levels were increased, but endothelial function and insulin sensitivity were also uninfluenced (Gilchrist et al., 2013). After considering a large portion of chronic metabolic conditions, nitrate supplementation should be assessed for its ergogenic effectiveness.

Nitrate & Exercise Performance

There are several important factors to consider when examining the helpfulness of nitrate intake during exercise, such as improved endurance and reduced fatigue, reduced muscle pain and soreness, and perception of effort. In a recent, randomized, double-blind, crossover design, twelve athletes consumed beetroot juice or a placebo for five

continuous days before completing a time-to-exhaustion test. The male participants were considered recreationally active and asked to consume a 6.5 mmol/70 mL nitrate drink or placebo (0.04 mmol/70 mL) 2 h prior to laboratory visits. The participants who exceeded their time to exhaustion by at least 8% after nitrate supplementation were asked to complete a ten-day washout period, followed by another five days of supplementation. The analysis of the intervention and control times were used to find the difference in time-matched trials. Maximal voluntary torque (MVT) and quadriceps twitch torque (PS100) were measured before and after testing to assess muscle fatigue. The perception of muscle and leg pain were assessed as perceptual data. On average, it was found that nitrate supplementation resulted in an increase of 3.5 minutes ($p=0.01$) of time to exhaustion per trial. Additionally, MVT and PS100 were both significantly lower ($p=0.002$ and $p=0.001$, respectively). The study concluded that nitrate intake for five continuous days prior to exercise resulted in improved time to exhaustion, lowered muscle fatigue, and a perception of reduced effort muscle pain (Husmann, Bruhn, Mittlmeier, Zschorlich, & Behrens, 2019).

A systemic review and meta-analysis aimed to investigate the effects of dietary supplementation on endurance exercise performance in healthy adults. Seventy-six randomized, placebo-controlled, crossover trials measuring exercise performance beyond thirty seconds were included. The review included 581 participants (494 males and 87 females) aged 16.7 to 64 years with fifty-nine, four, and thirteen trials containing only men, only women, and both genders, respectively. The mean sample size was 10.8 ± 4. Univariate meta-regression models were utilized to evaluate moderator variables, such as study design, nitrate dose and type, exercise method, fitness, and plasma nitrate level.

Twenty-nine trials demonstrated statistically significant results ($p<0.05$), while sixty-one trials showed enhanced performance. Only one study resulted in a decrease in performance. Twenty of the twenty-two studies examining time to exhaustion showed a mean improvement, with sixteen being significant. Additionally, twenty-seven of thirty-eight trials displayed enhanced time trails and fourteen of eighteen improved graded-exercise test results, with seven and five being statistically significant, respectively. It was concluded that moderator variables did not have a significant impact on the analysis. Chronic consumption (15 days) of a nitrate supplement was better associated with improved performance than an acute dose (2.5 h). The level of fitness did not appear to influence the efficacy of supplementation. However, one study revealed greater aerobic enhancement in sedentary and moderately trained subjects, but not in elite athletes (Porcelli et al., 2015). Overall, dietary nitrate supplementation has shown to be beneficial for endurance exercise capacity (McMahon et al., 2017).

The majority of previous exercise studies have examined both the endurance and short distance capabilities of nitrate intake. In a 2020 systematic review, the use of nitrate supplementation was examined during resistance training. Four articles within the previous five years met the inclusion criteria of nitrate supplementation, weightlifting exercise, and a parallel arm study design. Forty-nine trained male participants and variety of factors were assessed, such as muscular power improvement, muscular endurance, and contractile velocity. The participants were administered 70 mL of beetroot juice (~6.4 mmol/L) or a placebo 1-2 hours prior to exercise. They were asked to complete a bench press with both free weights and a Smith machine and box squats with the same Smith machine. It was determined that upper body performance (contractile power and velocity

and repetitions to failure) was aided by nitrate intake in two of the trials. Another trial found that lower body repetitions to failure performance was also improved. Further studies are needed to determine if nitrate supplementation in resistance training is effective as aerobic exercise (San Juan et al., 2020).

While there have numerous documented successful trials with exercise and nitrate supplementation, some researchers wonder if it is better served as an ergogenic aid in healthy individuals or cardiovascular disease (CVD) patients. With many differing study designs, such as delivery amounts and methods, time of delivery, smaller sample sizes, and varying levels of fitness and exercise tests, the ergogenic benefits are still slightly unclear. Due to the more promising results in the clinical setting, this review examines if supplementation is more effective in reestablishing exercise tolerance and quality of life in patients with CVD than as an ergogenic aid for healthy individuals. Many CVD patients present with poor oxygen transport and a reduced exercise tolerance resulting from endothelial dysfunction, reduced platelet aggregation and tissue perfusion, mitochondrial dysfunction, and increased oxidative stress. The incidence and development chronic heart failure (CHF) have been associated with diminished NO availability and endothelial dysfunction (Marti et al., 2012). Patients with CHF also exhibit a reduction of nearly 53% cardiac output during maximal exercise (Dhakal et al., 2015). In a crossover design, twenty patients (69 ± 7 y/o) with heart failure with preserved ejection fraction (HFpEF), which is the improper filling of the left ventricle (diastole) resulting in less volume ejected, were administered a single dose of beetroot juice (6.1mmol/L nitrate), as well as, a week of daily doses. The participants were asked to cycle until a submaximal exhaustion output (75%). It was found that aerobic endurance

was significantly improved at 24% ($p=0.02$) following the seven-day routine, while there was no change in performance after acute intake. In addition, plasma nitrate/nitrite levels increased significantly and resting systolic blood pressure was also significantly reduced. The majority of the cohort studies referenced in the review demonstrate that improving exercise capacity with nitrate intake may help ameliorate the morbidity and mortality rate of CVD (Eggebeen et al., 2016).

In a randomized, crossover, repeated measures study, nitrate intake and exercise were analyzed for their influence on peripheral artery disease (PAD). The condition is summarized as possessing atherosclerotic occlusions that do not allow oxygenated blood to the peripheral tissues, such as the legs, arms, stomach, and head. Eight subjects (4 male and 4 female), aged 67 ± 13 years, were asked to ingest 500 mL of beetroot juice (9 mmol/L nitrate ~ 81mg) three hours prior to a maximal cardiopulmonary exercise (CPX) on a treadmill. Prior to ingesting the control or intervention, blood pressure was measured, and blood was withdrawn. Blood pressure was recorded after every other stage and two minutes after completion, while blood was withdrawn directly following and ten minutes after completion. It was concluded that there was no difference in plasma nitrate levels between the groups at baseline. Also, plasma nitrate levels had significantly increased before exercise ($p\leq0.01$) in comparison with the placebo. After CPX testing, the subjects had shown the ability to walk 18% longer prior to the onset of pain ($p\leq0.01$), while demonstrating a 17% longer peak walking time ($p\leq0.05$). At rest and throughout CPX testing, diastolic blood pressure was significantly lower in the intervention group ($p\leq0.05$). In addition, gastrocnemius muscle oxygenation was significantly improved in the nitrate group ($p\leq0.01$) (Kenjale et al., 2011).

It has been suggested that an effective therapy for hypertensive individuals can be aerobic exercise training. Researchers aimed to examine if nitrate supplementation and aerobic training would create a synergistic effect on controlled hypertension. A pilot study examined an aerobic training regimen, in conjunction with beetroot supplementation, in patients with hypertension (65 ± 5 y). The randomized, placebo-controlled, double-blinded, parallel arm trial investigated the effects of 500 mg of nitrate daily in twenty-six sedentary individuals with systolic blood pressure between 130-160 mm Hg. Participants taking more than two hypertensive medications and/or medications interfering with nitrate metabolism were excluded. It was determined that the nitrate and placebo groups both showed a significant increase in time to exhaustion and arterial compliance ($p<0.05$). In addition, the nitrate group demonstrated a significant reduction in supine resting systolic blood pressure at follow-up when compared with baseline measurements (Shaltout et al., 2017). With metabolic conditions and various scenarios with elevated heart rates being studied, the potential benefit of nitrate on cognitive function individuals warrants exploration.

Nitrate & Cognitive Function

With the success that many studies have shown regarding vascular improvement, some researchers have explored if nitrate supplementation may improve cognition. In a systemic review, 12 randomized, controlled, crossover trials were selected to discern the potential benefits of nitrate intake in an older population. The studies also investigated potential improvements in physiological, cognitive, cardiovascular, cerebrovascular, and metabolic health. The nitrate dose (350-700 mg/day) and the time of delivery varied by study design. Five studies examined the benefits of acute consumption (2-4 h before

testing), while six trials looked at chronic consumption (2.5-14 days of nitrate intake). A single study considered both acute and chronic consumption (1.5-2 h before testing and 7 days of nitrate loading, respectively). In older adults, both acute and chronic supplementation showed significant improvement in physiological performance, such as time to exhaustion in submaximal cycling exercise ($p=0.031$ and $p=0.02$, respectively). Ten of the studies measured cardiovascular health in older adults and five found significant reductions in systolic blood pressure, while four of those five also found a significant decrease in diastolic blood pressure (Berry et al., 2015; Kemmner et al., 2017; Kenjale et al., 2011). Utilizing magnetic resonance imaging, one study did evaluate its effects on cerebrovascular health. It was found that frontal cortex perfusion had significantly increased ($p<0.005$) after two days of a high nitrate diet (Presley et al., 2011). After fourteen days of supplementation (7.5 mmol/400 mg nitrate), there was a significant increase in simple reaction time (Gilchrist et al., 2014). However, there were no significant variances in information processing, attention, or memory. Despite improved vascular function in certain areas of the brain, cognition seemed fairly unaffected (Stanaway et al., 2017).

At a relatively high altitude, NO availability is diminished, which can lead to physiological and cognitive decline. In this randomized, placebo controlled study, ten healthy males (23 ± 3 y) were given a 140 mL nitrate supplement (~12.5 mmol/L nitrate) and tested twice at moderate and very-high (~14% O_2; ~3000m and ~11.7% O_2; ~4300m, respectively) simulated altitudes for NO bioavailability, exercise performance, and cognitive and physiological function. The participants ingested the supplement two hours prior to testing and completed a forty-five-minute walk and 3 km time-trial (TT) with a

10 kg backpack, simulating hiking in altitude. Resting plasma levels were significantly higher following nitrate intake than the control group ($p=0.001$). During exercise, peripheral oxygen saturation (PO_2) was significantly increased ($p=0.005$) at each altitude. The TT performance only indicated a 3.8% and 4.2% at the different altitudes, respectively. Cognitive function assessments, such as an attention switching task, spatial span task, and rapid visual information processing task (RVP), were not significant in relation to the placebo group ($p\geq0.141$). The RVP, which tests sustained attention with false alarming images for ten minutes, displayed the greatest influence from nitrate supplementation ($p=0.056$). At moderate and very high simulated altitudes, only plasma nitrate levels and PO_2 were significantly affected (Shannon et al., 2017).

A randomized, double-blind, crossover study investigated the outcomes of acute nitrate intake on cognitive and muscle function, blood pressure, and oxygen uptake kinetics in older adults. Over a six-week period, twelve subjects (60 - 70 y) were administered beetroot juice (70 ml/day, ~9.6 mmol/day) or a placebo twice daily for three days. Resting blood pressure and plasma samples were collected before and after ingestion. Cognitive function was measured through a variety of tests, such as serial subtractions (counting backward in 3's or 7's), rapid visual information processing (RVP), and number recall. Plasma nitrate levels were significantly increased with supplementation and resting systolic and diastolic blood pressure (-5 mm Hg, $p<0.05$ and -3 mm Hg, $p<0.05$, respectively) were reduced significantly. All other measurements, including cognition, oxygen cost of exercise, and metabolic response to low-interval exercise, remained unaffected (Kelly et al., 2013). After examining nitrate supplementation with numerous conditions and scenarios, it is now important to explore

the possibilities of metabolomics, statistics, and how they can be employed to better understand subject data.

Metabolomics & Statistics

Still a rapidly emerging science, metabolomics is a robust method used to quantify metabolic intermediates. The physiological depiction of complex metabolic pathways can then be determined through statistical analysis. The current state of metabolomics research utilizes numerous targeting methods and equipment, such as liquid chromatography-mass spectroscopy (LC-MS), gas chromatography-mass spectroscopy (GC-MS), and proton nuclear magnetic resonance (^{1}H NMR). Due to its powerful selectivity and superb quantitative abilities, LC-MS is largely considered the gold standard in metabolomics. While the equipment is vital in detection, the method is equally important. A targeted approach allows for a finite range to identify a small number of known metabolites. A major disadvantage is its reduced capability of detecting unknown metabolites, which is the benefit of a global profiling approach. Within the last few years, pioneering methods, such as globally optimized targeted (GOT)-MS have been developed to blend the specificity of targeted metabolomics with increased metabolome analysis (Gu, Zhang, Zhu, & Raftery, 2015). In addition, stable isotope resolved metabolomics (SIRM) has enabled the tracing of labeled atoms throughout complex metabolic pathways (Higashi, Fan, Lorkiewicz, Moseley, & Lane, 2014).

To date, only one study has used a metabolomics approach to investigate the effects of nitrate supplementation on vascular function. Two concentrations of an oral sodium nitrate capsule, 40 or 80 mg, were administered twice daily to middle-aged and older adults for ten weeks in a randomized, double-blind, placebo-controlled, parallel-

arm study. The subjects were men (50-65 years) and post-menopausal women (66-79 years) without the presence of cardiovascular and metabolic diseases. Baseline metrics of brachial artery flow-mediated dilation (FMD), carotid artery elasticity, aortic pulse wave velocity, plasma nitrate, and blood pressure were measured for significant alterations in endothelial function. Metabolites were quantified with liquid chromatography-mass spectroscopy (LC-MS, 6210 ESI-TOF; Agilent Technologies). In addition, the differences between means were evaluated with a one-way ANOVA ($p<0.05$), and the differences between placebo and sodium nitrite treatments was analyzed with a mixed-model ANOVA. Altered metabolites were examined for significance with a paired t-test ($p<0.05$) and placed in linear regression models (De Van et al., 2016).

While, plasma nitrite levels remained elevated after twelve hours at ten weeks, brachial artery blood pressure did not demonstrate improvement. FMD was increased in both groups and significantly altered metabolites were also detected. Lysophosphatidylethanolamine (LysoPE) and diacylglycerol (28:2) were increased, while *N,N*-dimethyl-$_{L}$-valine and 5-aminoimidazole-4-carboxamide-1-1-β_{D}-ribofuranosyl 5'-monophosphate were decreased in the lower dose group. Lysophosphatidylcholine (LysoPC) was decreased in the 160 mg/day group, and $_{L}$-glutamine and phosphatidylcholine were elevated. Overall, three metabolic pathways, benzene, fatty acyl, and glycerophospholipid metabolism, were affected with improvement in FMD. A reduced β-stiffness index suggested a relaxing effect of nitrite in carotid artery stiffness and was significantly associated with an increase in phosphatidylethanolamine. Baseline concentrations of 73 metabolites across eleven pathways, azoline, carboxylic acid, fatty acid, fatty acyl, glycerolipid, glycerophospholipid, isoprenoid, *N*-acrylamide, nucleoside,

sphingolipid, and steroid metabolism, were significantly associated with the β-stiffness measure. Each dose of sodium nitrite supplementation was well-tolerated by the study participants and exhibited no signs of symptomatic hypotension (De Van et al., 2016).

While the benefits of nitrate supplementation on endothelial function are well documented, a metabolomics, and comprehensive -omics, methodology was used to investigate its role in the browning of white adipose tissue (WAT) to treat metabolic syndrome. It has been shown that cyclic guanosine monophosphate (cGMP) possesses the capability of browning WAT (Mitschke et al., 2013). The objective was to examine how increased nitrate intake may be positively associated with increases of cGMP via the nitrate-nitrite-NO pathway. It was determined that browning was heightened during hypoxic conditions, such as those correlated with WAT in obese individuals. As previously mentioned, the nitrate-nitrite-NO pathway can be altered in hypoxia. The expression of xanthine oxidoreductase (XO), which reduces nitrate to NO, is enhanced and tasked with adipocyte homeostasis. LC-MS and GC-MS were used to examine the effect of nitrate on β-oxidation. Short chain fatty acids, myristate and laurate, and their subsequent TCA cycle intermediates, citrate, succinate, and malate, were elevated due to fatty acid β-oxidation. Nitrate demonstrated the ability to alter the expression of thermogenic genes and develop a phenotype similar to brown adipocytes in WAT (Roberts et al., 2015).

Nearly as vital as mass spectroscopy methods and equipment, proper statistical analysis is essential to metabolomics. Various models and tests can be integrated to identify significant metabolites, but overly fitted data can lack validation. A principal component analysis (PCA) is used to identify the data with the most variance and

demonstrates patterns through an orthogonal transformation. By reducing dimensions, a small set of data can be utilized to correlate variables from larger data. Univariate analysis, such as a two samples t-test, examines the mean difference of two groups for every metabolite to determine significance. A partial least squares-discriminant analysis (PLS-DA) demonstrates the maximum amount of variance from X and Y matrices through the regression of X against Y. As a classification model, PLS-DA denotes metabolite peak intensities as an X matrix and class labels for a Y matrix. The model possesses the ability to use maximum variance to further separate the targeted groups beyond PCA (Xi, Gu, Baniasadi, & Raftery, 2014).

As a comprehensive, web-based -omics tool, MetaboAnalyst offers complex integrative analysis for metabolomics data. The software offers 12 different modules within four classes, including exploratory statistics and biomarker, power, and time-series analyses. The metabolic pathway and enrichment analyses functions accentuate altered metabolisms and upregulated metabolite sets for significant metabolites, while pathway activation via mass spectroscopy peaks utilizes a predictive algorithm. Additionally, dimensional reduction and maximized variation models, such as principal component analysis (PCA) and predictive models like partial least squares – discriminant analysis (PLS-DA) can be easily generated. The software uses data from updated knowledge sources, i.e. metabolite sets and pathways, and the Human Metabolome Database (HMDB) (Chong et al., 2018a). MetaboAnalyst is a great tool for mapping and assessing significant metabolites, while generating univariate and multivariate figures.

Conclusion

In this review of literature, the nitrate-nitrite-NO and L-arginine-NOS pathways were explored. The biological functions of NO and its NOS substrates, as well as, the L-arginine paradox were further explained. In addition, several study designs were investigated, including the effects on metabolic conditions, exercise capacity, and cognitive ability. The only metabolomics study examining nitrate intake on vascular function was also dissected for any applicable parallels to this analysis. Finally, pertinent metabolomics studies and statistical measures were examined due to their importance in this paper.

CHAPTER 3

METHODS

The study design is an exploratory analysis of a previous study examining the effects of nitrate supplementation on vascular function. The study was approved by the Institutional Review Board at Arizona State University (Appendix A). The subjects provided written consent to participate in the trial examining the effects of nitrate supplementation on blood pressure and blood vessel function.

Nitrate-rich Fruit and Vegetable Juice Supplementation (FVS)

The details of this study have been reported (Sweazea et al., 2018). A randomized, placebo-controlled, double-blinded, parallel arm trial design was used to determine if nitrate-rich juice supplementation improved vascular function, mood, and sleep. Healthy normotensive men (18-40y) were recruited from a college campus and the surrounding community in the Phoenix area via flyers, listservs and website announcements (Appendix C). The volunteers were then asked to complete health history questionnaire prior to selection (Appendix D). Subjects (n = 39) were stratified by age, height, weight, BMI, athletic status, and randomly assigned to a nitrate-rich supplement (FVS) or a nitrate-depleted juice control group (PRU). Participants consumed one bottle each morning for fourteen consecutive days. Blood pressure and flow-mediated dilation (FMD) were measured, and plasma was collected at baseline, week 1, and week 2. It was determined that supplementation significantly increased plasma nitrates/nitrites and decreased diastolic blood pressure. However, increased plasma NO was not significantly associated with FMD, mood, and sleep. (Sweazea et al., 2018).

Power Analysis

According to the only metabolomics study examining nitrate and vascular function noting an effect size of 0.74 and more than 2,000 outcome measures, a paired samples t-test would require 49 subjects per group for an actual power of 0.952. A repeated measures MANOVA examining between factors and within factors would require 10 subjects per group with an actual power of 0.982 and 14 per group with an actual power of 0.976, respectively (De Van et al., 2016).

Power calculations with an eighty percent probability to detect treatment difference at a 0.05 significance level were used to determine the sample size of 10 per group for a crossover trial based upon an FMD mean of 7.2% and 0.3% standard deviation (Rodriguez-Mateos et al., 2013). In addition, it was determined that a sample size of 17 per group was required for a parallel arm trial to detect a treatment effect of FMD at 2% with a 2% standard deviation (Dalli et al., 2002). Power calculations with the same probability and significance indicated that 6 subjects were necessary to detect a treatment effect of -5 mmHg with a 1.4 mmHg standard deviation on systolic blood pressure for a crossover study (Appendix F) (Bahra, Kapil, Pearl, Ghosh, & Ahluwalia, 2012).

Reagents

LC-MS grade acetonitrile (ACN), methanol (MeOH), acetic acid (AcOH), and ammonium acetate were obtained from Fisher Scientific (Pittsburgh, PA). Standard compounds (purity >95-99%) analogous to measured metabolites were obtained from

Fisher Scientific (Pittsburgh, PA) and Sigma-Aldrich (Saint Louis, MO). Internal standards (purity >99%) were purchased from Cambridge Isotope Laboratories (Tewksbury, MA, UK).

Sample Preparation

The sample preparation protocol was produced from previous studies (Beckonert, 2007; Dunn, 2011). Frozen samples were thawed overnight under 4ºC, and 50 µL of each plasma sample was placed in a 2 mL Eppendorf vial. The addition of 550 µL of methanol containing $^{13}C_3$-lactate and $^{13}C_5$-Glutamic acid was performed for protein precipitation and metabolite extraction. It was vortexed for 2 min and stored at -20°C for 30 min. The mixture was then sonicated in an ice bath for 10 min and centrifuged at 14,000 RPM for 10 min at 4°C. The supernatant (150 µL) was obtained and transferred into a new Eppendorf vial, which was then dried with a Vacufuge Plus evaporator. It was reconstituted with 150 µL of 40% PBS/60% ACN and centrifuged for 10 min at 14,000 RPM at 4°C. 100 µL of supernatant was than placed into 2 mL glass vial. A plasma mixture of each study participant was pooled for quality control (QC) purposes and was analyzed every 10 subjects.

Liquid Chromatography and Mass Spectrometry Conditions

The targeted LC-MS/MS method used here was modeled after already developed protocols and was used in a growing number of studies (Jove, 2017; More, 2018; Zhu, 2014; Carroll, n.d.; Gu, 2015, 2016). LC-MS/MS experiments were performed on an Agilent Technologies 6490 Triple-Quad (QQQ) LC/MS system (Santa Clara, CA). Each

sample was injected twice, 4 µL and 10 µL for analysis using positive and negative ionization modes, respectively. Chromatographic separation was performed on an Agilent Technologies 1290 Infinity II column (*2.5 µm, 2.1 x 150 mm*) (Santa Clara, CA) at 40 °C. The flow rate was 0.3 mL/min. The mobile phase was composed of Solvents A (10 mM ammonium acetate + 10 mM ammonium hydroxide in 95% H_2O/ 5% ACN) and B (10 mM ammonium acetate + 10 mM ammonium hydroxide in 95% ACN/ 5% H_2O). The same LC gradient conditions were utilized for both positive and negative ionization modes. After an initial 1 min isocratic elution of 90% Solvent B, the percentage of Solvent B decreased linearly to 40% at t=11 min. The percentage of B then remained the same (40%) for 4 min (t=15 min). The percentage of B increased back to 90% for the next injection. Metabolite identities were confirmed by spiking mixtures of standard compounds. Extracted MRM peaks were integrated using Agilent MassHunter software (Santa Clara, CA).

Data Processing and Statistical Analysis

After exporting from Agilent MassHunter software, data were log10-transformed to approximate normality, and general linear models (GLMs) were used for comparison of metabolite levels between the groups. Statistical analysis was performed with SPSS 22.0 (SPSS Inc.; Chicago, IL). Principal component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA) were implemented with log10- transformed, auto scaled data to construct models. The models were developed using MetaboAnalyst 4.0 software package (Montreal, Quebec, Canada). Pathway and enrichment analyses were

performed and conceptualized with the MetaboAnalyst 4.0 software package (Montreal, Quebec, Canada).

CHAPTER 4

RESULTS

Subject Characteristics and Demographics

Of the forty-five available male subjects, plasma samples for thirty-nine subjects underwent a metabolomics analysis. Any missing samples during the study protocol resulted in the omission of the subject for this final analysis. The subjects were asked to meet for an initial screening and complete a health history questionnaire. Plasma samples were then collected at baseline, after one week of supplementation, and after two weeks of supplementation. As seen in Table 1, the subjects were randomized into two equivalent groups, Treatment (n=19) and Control (n=20). The mean ages for the subjects in the Treatment and Control groups were 24.2 ± 3.8 and 24.4 ± 4.5 years, respectively. The mean BMI at screening for the Treatment and Control groups was also comparable each other (25.1 ± 3.6 and 25.9 ± 3.5, respectfully).

Table 1. Subject characteristics and demographics

	Treatment	**Control**
Subjects (n=39)	19	20
Age (years)	24.2 ± 3.8	24.4 ± 4.5
BMI (Kg/m^2)	25.1 ± 3.6	25.9 ± 3.5

Data are expressed as mean ± standard deviation

Two-way Repeated Measures ANOVA

Utilizing a two-way repeated measures ANOVA, forty-one compounds with a coefficient of variance (CV) less than 20% of the subjects' metabolome were analyzed for significance with treatment and time of plasma collection, baseline, week 1, and week 2. As illustrated in Table 2, benzoic acid was the only significant metabolite for time

(p<0.001), treatment (p=0.047), and time & treatment (p=0.016). Nonadecanoic acid was the only other metabolite to test significant for time & treatment (p=0.013). Assessing for only metabolite changes over time, several compounds had shown similar significance, including benzoic acid, prostaglandin E_2 (PE_2), 4-methyl-2-oxopentanoic acid, valeric acid, 3-methyl-2-oxovaleric acid, maleic acid, and citraconic acid (p<0.001). Stearic acid, nonadecanoic acid, and palmitic acid all displayed a high level of significance (p=0.002, p=0.002, and p=0.003, respectively) for time collection. Additional metabolites, heptadecanoic acid, hexanoic acid, and 3-phenyllactic acid (p=0.005, p=0.007, and p=0.007, respectively), were also significant.

Table 2. Two-way repeated measures ANOVA assessing time, treatment, and the metabolome.

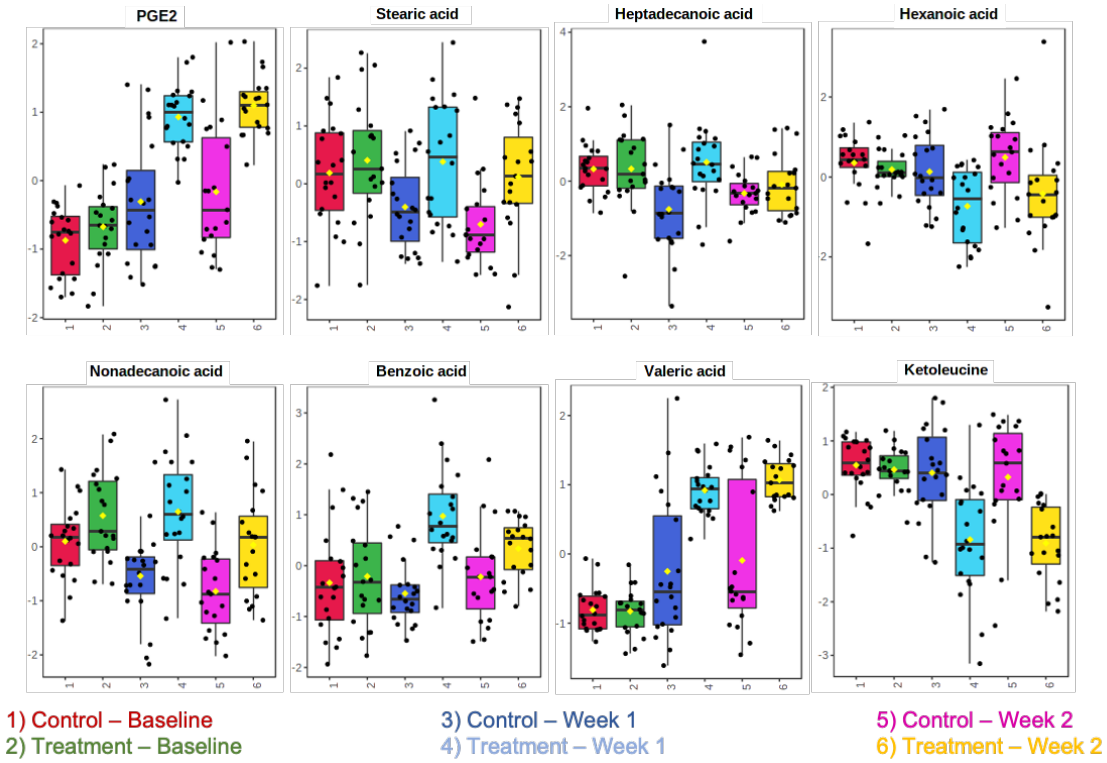
Time	
Metabolites	***P*-Value**
Benzoic acid	<0.001
Protocatechuic acid	0.026
Nonadecanoic acid	0.002
Caprylic acid	0.016
Heptadecanoic acid	0.005
Prostaglandin E_2	<0.001
Stearic acid	0.002
3-Phenyllactic acid	0.007
Hexanoic acid	0.007
Ketoleucine	<0.001
Valeric acid	<0.001
Pregnenolone sulfate	0.024
3-Methyl-2-oxovaleric acid	<0.001
Palmitic acid	0.003
Maleic acid	<0.001
Citraconic acid	<0.001

Treatment	
Metabolites	***P*-Value**
Benzoic acid	0.047

Time & Treatment	
Metabolites	***P*-Value**
Benzoic acid	0.016
Nonadecanoic acid	0.013

Data are considered significant at p<0.05.

Figure 1: Boxplots of significant metabolites of Control at Baseline and Treatment at Baseline, Week 1, and Week 2.



Independent Samples t-Test

Employing an independent samples t-test, the subjects' metabolome was analyzed for significance with treatment and time of plasma collection. The Treatment and Control groups were assessed at Baseline, Week 1, and Week 2. Depicted in Table 3, it was found that after the first week of treatment, phenylpyruvic acid was significant (p=0.048) in comparison with the control group. After two weeks of supplementation, phenylpyruvic acid remained significant (p=0.048) and pentadecanoic acid was also discovered to be significant (p=0.027).

Table 3. Independent samples t-test comparing Treatment and Control groups.

Week 1 Treatment vs Control			Week 2 Treatment vs Control	
Metabolite	***P*-Value**		**Metabolites**	***P*-Value**
Phenylpyruvic acid	0.048		Pentadecanoic acid	0.027
			Phenylpyruvic acid	0.048

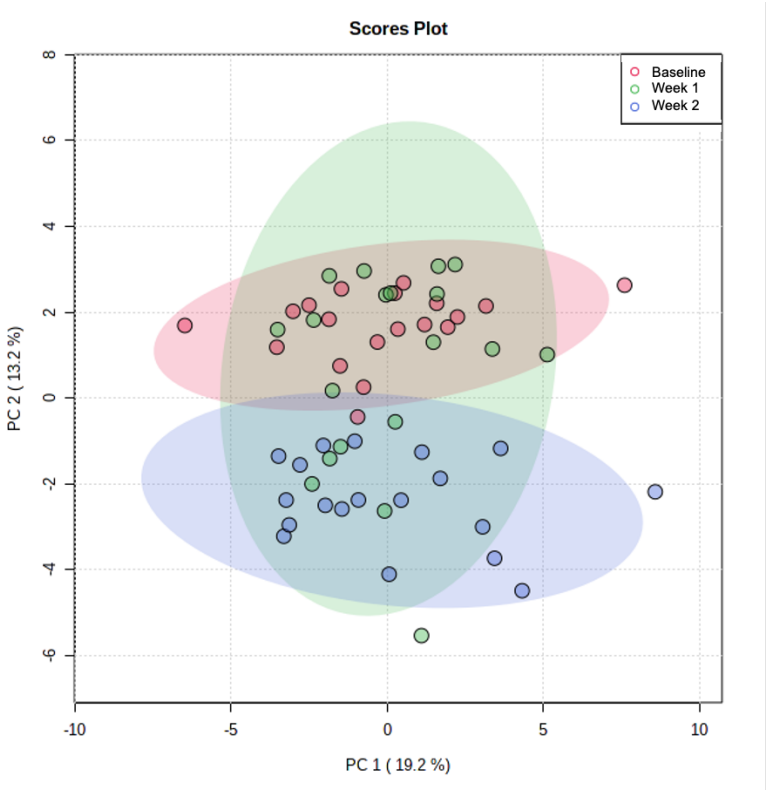
Data are considered significant at $p<0.05$.

Principal Components Analysis (PCA) &

Partial Least Squares – Discriminant Analysis (PLS-DA)

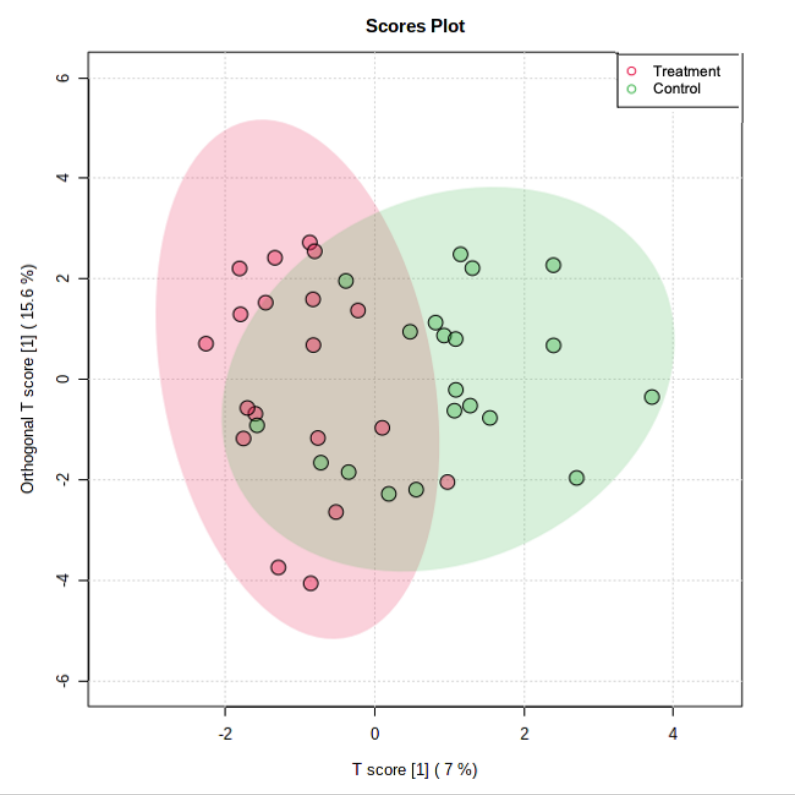
A principal component analysis (PCA) was generated to better understand the impact of nitrate supplementation. In Figure 2, a strong, observable pattern occurred in the treatment group across Baseline, Week 1, and Week 2. At Baseline, there was tight clustering among the group. After Week 1, a trend emerged as nearly half of the group migrated toward Week 2, while also remaining clustered with Baseline. In the Week 2 group, there was tight clustering and a clear separation from the Baseline group.

Figure 2: Principal Component Analysis (PCA) of the Treatment group at Baseline, Week 1, and Week 2.



In Figure 3, an orthogonal partial least squares – discriminant analysis (OPLS-DA) model was generated to better assess the Treatment and Control groups after Week 2 of nitrate supplementation. While some overlap between the two groups is observed, there is an identifiable trend. The Control group is plotted centrally, while the Treatment group has a tighter cluster and is off-center in comparison with the Control.

Figure 3: Orthogonal Partial Least Squares – Discriminant Analysis (OPLS-DA) of the Treatment and Control groups at Week 2.



Pathway & Enrichment Analyses

In order to examine potentially altered metabolic pathways with supplementing nitrate, a pathway analysis was produced to investigate the metabolomes of the Treatment and Control groups. The analysis in Figure 4 and Table 5 did not reveal any significant pathways, but it did demonstrate the large impact of phenylalanine metabolism. Three metabolites were found within this pathway, including phenylpyruvate, phenylacetic acid, and 2-hydroxyphenylacetate. Cysteine and methionine metabolism, as well as serine as a metabolite, were not significant, but exhibited a minor pathway impact.

Figure 4: Log-transformed Pathway Analysis of the Treatment and Control group metabolomes after Week 2. Data is plotted as significance [$-\log_{10}(P)$] versus pathway impact.

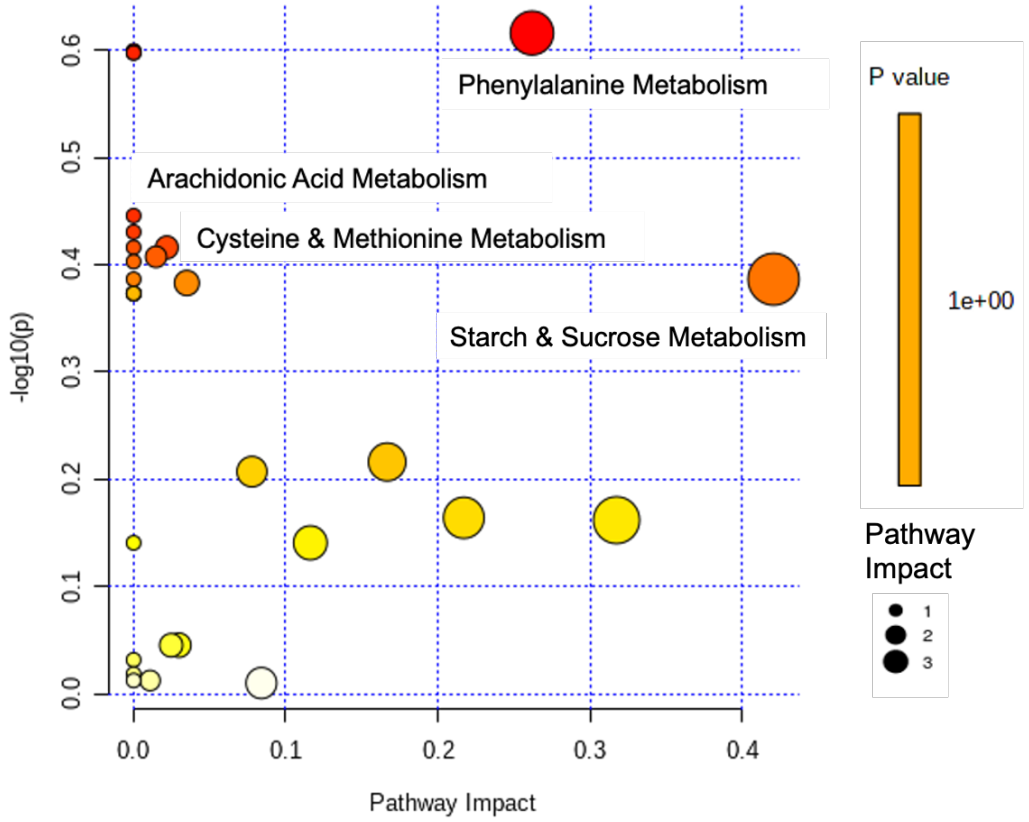


Table 4: Pathway Analysis of the Treatment and Control group metabolomes after Week 2.

Pathway Name	Metabolites	P value	Impact
Phenylalanine metabolism	3	0.2422	0.2619
Arachidonic acid metabolism	1	0.25163	0
Fatty acid elongation	1	0.25269	0
Fatty acid degradation	1	0.25269	0
Biosynthesis of unsaturated fatty acids	2	0.35848	0
Amino sugar and nucleotide sugar metabolism	2	0.37123	0
Cysteine and methionine metabolism	1	0.38371	0.02184

Data are considered significant at $p<0.05$.

An enrichment analysis was conducted to explore differing metabolite sets and enzymes after two weeks of nitrate supplementation. As depicted in Figures 5 & 6 and Table 5, several pathways were all found to be similarly significant, including fatty acid-CoA ligase, fatty acid transport via diffusion, ß-oxidation of long chain fatty acids, carnitine fatty-acyl transferase, methylmalonyl-CoA mutase, and propionyl-CoA carboxylase (p=0.016). The compounds, pentadecanoic acid and heptadecanoic acid, were the common metabolites among the sets.

Figure 5: Enrichment Analysis of the Treatment and Control group metabolomes after Week 2.

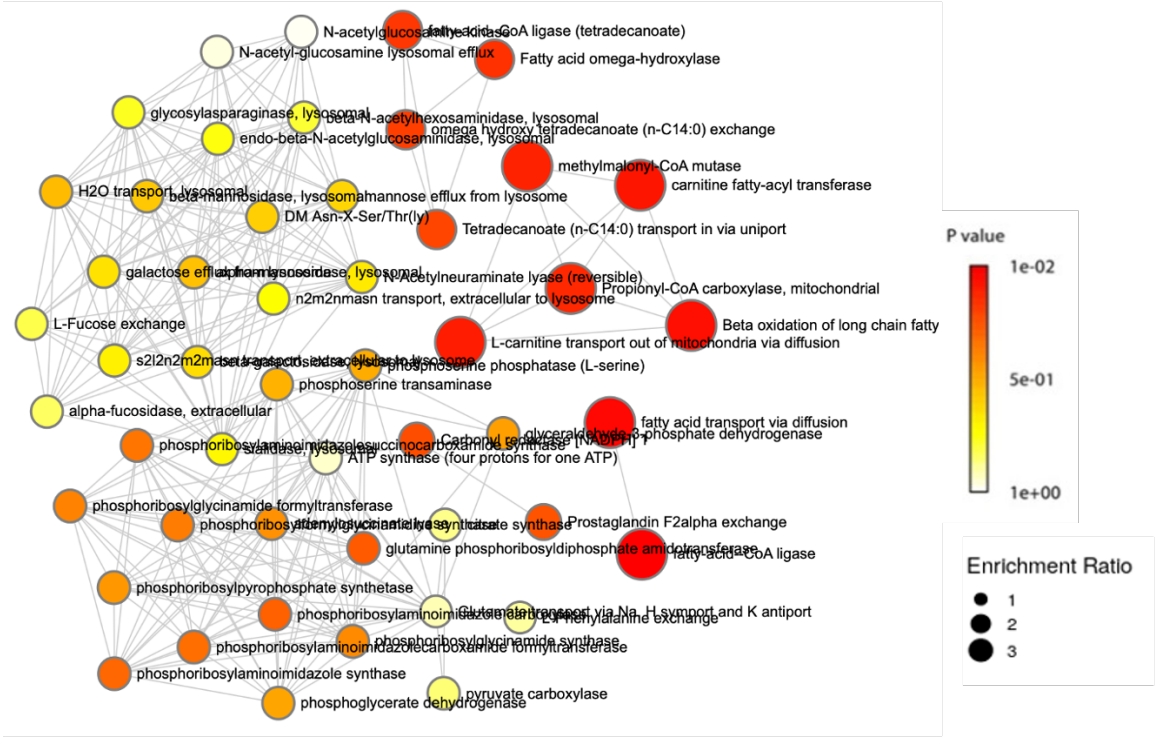


Figure 6: Metabolite sets Enrichment Overview of the Treatment and Control group metabolomes after Week 2.

Table 5: Metabolite sets Enrichment Overview of the Treatment and Control group metabolomes after Week 2.

Metabolite Set	Total	Metabolites	P Value
Fatty-acid-CoA ligase	24	2	0.016319
Fatty acid transport via diffusion	22	2	0.016319
ß-oxidation of long chain fatty acid (odd chain)	2	2	0.016319
Carnitine Fatty-acyl transferase	2	2	0.016319
L-carnitine transport out of mitochondria via diffusion	7	2	0.016319
Methylmalonyl-CoA mutase	8	2	0.016319
Propionyl-CoA carboxylase, mitochondrial	8	2	0.016319

Data are considered significant at $p<0.05$.

A pathway analysis was generated to study any metabolic pathways that may be significant in the first and second week of supplementing nitrate. The analysis in Figure 7 and Table 6 did not reveal any significant pathways, but it did demonstrate a substantial impact on starch and sucrose metabolism (p=0.09) and galactose metabolism (p=0.11). Glucose was identified within starch and sucrose metabolism, while mannose and glucose were found within galactose metabolism.

Figure 7: Log-transformed Pathway Analysis of the Week 1 and Week 2 Treatment group metabolomes. Data is plotted as significance [$-\log_{10}(P)$] versus pathway impact.

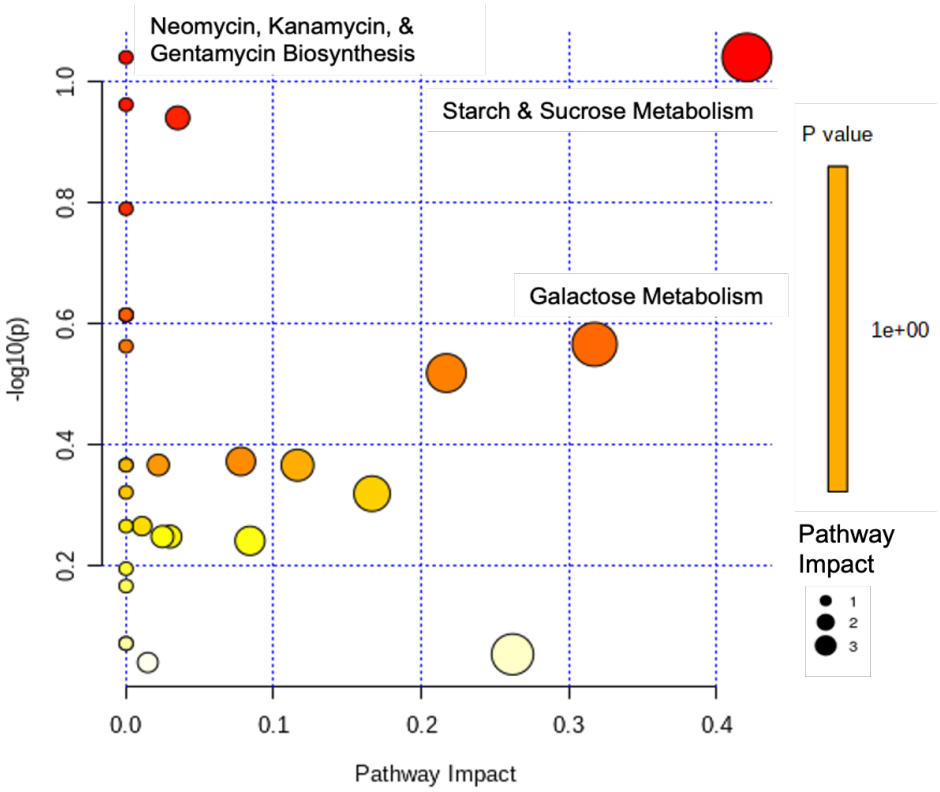


Table 6: Pathway Analysis of the Week 1 and Week 2 Treatment group metabolomes

Pathway Name	Metabolites	P value	Impact
Starch and sucrose metabolism	1	0.09123	0.4207
Neomycin, kanamycin and gentamicin biosynthesis	1	0.09123	0
Amino sugar and nucleotide sugar metabolism	2	0.1092	0
Galactose metabolism	2	0.11483	0.0349

Data are considered significant at $p<0.05$.

In order to investigate the differing metabolite sets and enzymes of the Treatment group after Week 1 and Week 2 of nitrate supplementation, an enrichment analysis was employed. As Figures 8 & 9 and Table 7 show, no significant pathways were discovered between the treatments ($p=0.058$). The analysis did reveal several similar pathways to the Week 2 Treatment and Control Group analysis, such as fatty acid-CoA ligase, fatty acid transport via diffusion, ß-oxidation of long chain fatty acids, carnitine fatty-acyl transferase, methylmalonyl-CoA mutase, and propionyl-CoA carboxylase.

Figure 8: Enrichment Analysis of the Week 1 and Week 2 Treatment group metabolomes.

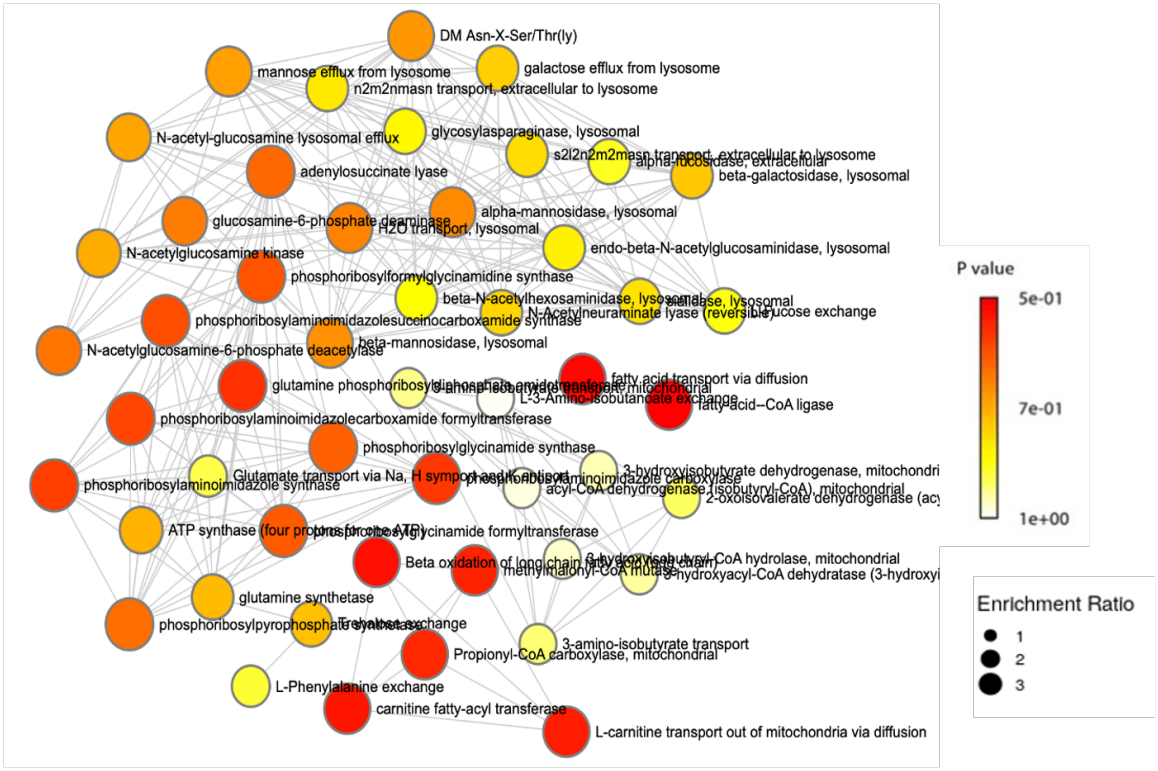


Figure 9: Metabolite sets Enrichment Overview of the Week 1 and Week 2 Treatment group metabolomes

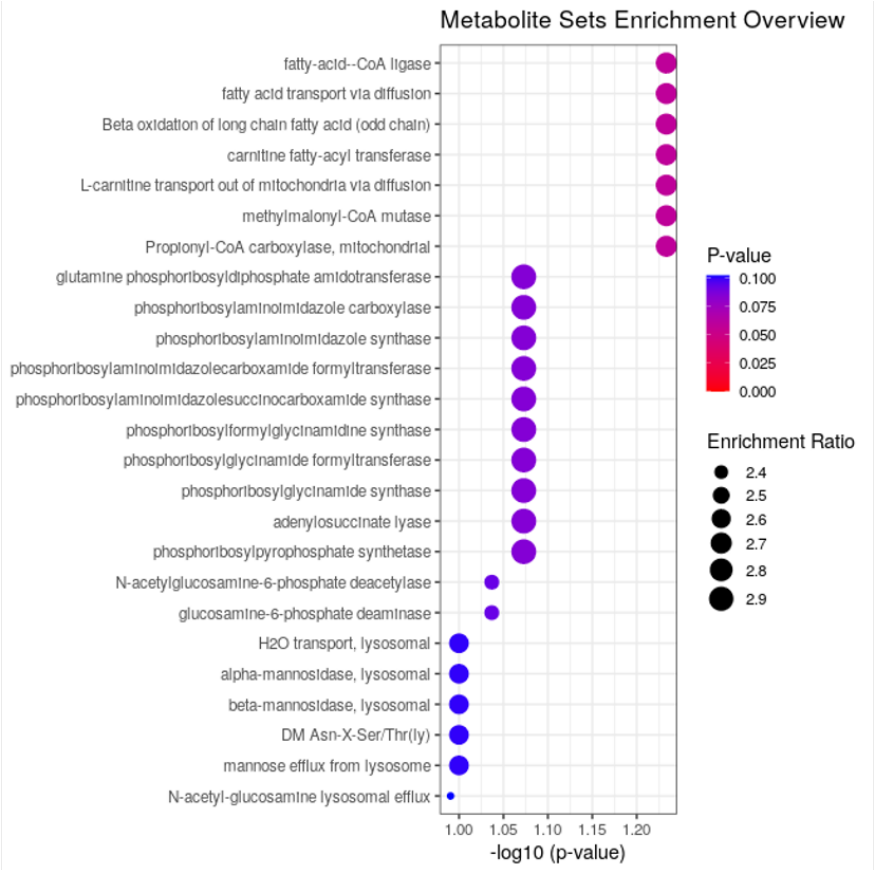


Table 7: Metabolite sets Enrichment Overview of the Week 1 and Week 2 Treatment group metabolomes

Metabolite Set	Total	Metabolites	P Value
Fatty-acid-CoA ligase	24	2	0.05843
Fatty acid transport via diffusion	22	2	0.05843
ß-oxidation of long chain fatty acid (odd chain)	2	2	0.05843
Carnitine Fatty-acyl transferase	2	2	0.05843
L-carnitine transport out of mitochondria via diffusion	7	2	0.05843
Methylmalonyl-CoA mutase	8	2	0.05843
Propionyl-CoA carboxylase, mitochondrial	8	2	0.05843

Data are considered significant at $p<0.05$.

CHAPTER 5

DISCUSSION

Chronic metabolic conditions, such as hypertension, cardiovascular disease, and type II diabetes, are largely caused through poor diet and daily habits, which often translates as some of the most preventable illnesses. With the Western diet being such a large contributing factor, many Americans find themselves afflicted by one of these conditions. Nearly sixty percent of US adults suffer from a chronic illness, while forty percent suffer from at least two or more conditions. As the leading cause of disability and death in the US, chronic disease results in $3.5 trillion in healthcare costs annually ("Chronic Diseases in America | CDC," n.d.). As a result, many would like to seek less expensive and simpler alternatives to treat their illness. Within the past twelve years, the effects of nitrate intake have been extensively researched as a potential therapy for various metabolic conditions (Kapil et al., 2015; Webb et al., 2008). While varying study designs have yielded varying results, many trials have found success in improving vascular function (Ashworth et al., 2015; Mayra et al., 2019). A recent study investigated the effectiveness of a nitrate-rich, beetroot juice supplement (FVS) on blood pressure, flow-mediated dilation (FMD), plasma nitrate/nitrite levels, and sleep quality and mood over a two week treatment period (Sweazea et al., 2018).

In this exploratory analysis, a metabolomics approach was applied to the previous study to potentially uncover significant metabolites, metabolic pathways, and their relationships that facilitate the vascular benefits of nitrate supplementation. The subjects were randomized into Treatment and Control groups and were asked to consume beetroot juice (240-280 mg nitrate/60 ml) or a placebo prune juice (<0.6 mg nitrate) each morning

for fourteen consecutive days. In order to assess this effect, a two-way repeated measures ANOVA was used to reveal significant metabolites across the time, treatment, and time & treatment groups. It was found that benzoic acid was the only significant metabolite across all three experimental factors ($p<0.001$, $p=0.047$, and $p=0.016$, respectively). Benzoic acid is mostly known as an antimicrobial agent and is commonly included in food and beverages as a preservative (Grumezescu & Holban, 2019). With the Western diet being saturated with food preservatives and the experimental design including a beverage supplement, it is not surprising that benzoic acid was increased in the subjects and found significant. In addition, nonadecanoic acid, a long chain saturated fatty acid found in vegetable oils, was the only other significant metabolite ($p=0.013$) in the time & treatment group. While not known as a compound associated with vascular function, it has been shown as an antiproliferative agent and cancer growth inhibitor (Gao et al., 2012).

Across the three time points, Baseline, Week 1, and Week 2, sixteen metabolites were identified as significant and several have been associated with vascular function. As an eicosanoid and hormone-like lipid, prostaglandin E_2 (PE_2) significantly increased each week ($p<0.001$). PE_2 modulates smooth muscle tone and is produced by endothelial cells. It has the ability to both positively and negatively affect blood pressure. Dependent upon the location of release, PE_2 can elevate local blood pressure, while also inducing a hypotensive effect systemically (Yang & Du, 2012). Saturated fatty acids are mostly considered harmful and enhancers of atherosclerosis risk and LDL cholesterol, with the exception of stearic acid. Commonly found in animal and vegetable fats, stearic acid has been linked to improved blood pressure, cardiovascular function, and protective effects

from cancer (Hunter, Zhang, Kris-Etherton, & Childs, 2010; Senyilmaz-Tiebe et al., 2018). Throughout the trial, stearic acid ($p=0.002$) demonstrated a significant decline and rise after Week 1 and Week 2 of supplementation, respectively. An additional anti-atherosclerotic compound, caprylic acid, was deemed to be significantly elevated ($p=0.016$). The medium-chain triglyceride was shown to suppress inflammatory cytokines, improve lipid panels, and reduce body fat and atherosclerosis in ApoE-deficient mice (Zhang et al., 2019). Protocatechuic acid (PChA), a phenolic acid commonly found in grapes and olives, significantly increased as the trial progressed as well ($p=0.026$). PChA has been shown to possess certain pharmacological properties, such as a robust antioxidant, anti-inflammatory, antihyperglycemic, antimicrobial, and anticancer agent (Semaming, Pannengpetch, Chattipakorn, & Chattipakorn, 2015). Many of the remaining metabolites, including valeric acid, maleic acid, and citraconic acid, are not considered metabolically active and, instead, are commonly used as food additives and/or preservatives. (Hou, Lu, Lin, Lin, & Tsai, 2016). This analysis identified potential contributors to vascular function improvement, while also differentiating biologically inactive metabolites from nitrate supplementation.

The Treatment and Control groups were better understood after conducting Week 1 and Week 2 independent samples t-tests. After one week of daily nitrate and placebo intake, plasma phenylpyruvic acid levels were significantly elevated in the Treatment group ($p=0.048$). Phenylpyruvic acid (PPA) is a byproduct of phenylalanine metabolism and may have a critical role in phenylketonuria, a condition resulting from the inability to metabolize phenylalanine, which may lead to mental impairment. It has been shown that PPA significantly reduces the activity of glucose-6-phosphate dehydrogenase, which is

vital for antioxidant defense and causes impaired NADPH production (Rosa et al., 2012). After two weeks of supplementation, PPA was similarly significant, while pentadecanoic acid also displayed significantly increased concentrations ($p=0.027$). Odd-chain saturated fatty acids (OCFAs), such as pentadecanoic acid and heptadecanoic acid ($p=0.005$, Table 2), can be found in small amounts in plants, fish, and dairy. Due to the fact that OCFAs cannot be made endogenously and their critical role in repairing mitochondrial function and reducing inflammation, dyslipidemia, fibrosis, and anemia, some researchers are beginning to consider OCFAs as a possible essential fatty acid (Venn-Watson, Lumpkin, & Dennis, 2020).

In order to better visualize the relationship between the Treatment group and its three time points, Baseline, Week 1, and Week 2, a principal component analysis (PCA) was performed. By reducing the dimensionality of a large dataset and maximizing the variance, the PCA allows for observable trends in the data (Jolliffe & Cadima, 2016). In Figure 2, there is a clear pattern regarding the three time points. At Baseline, there was distinct, tight clustering of units. After Week 1 of supplementation, nearly half of the values were unaffected, while the rest displayed a similar trend to the final time point and encompassed both groupings. After the second week, the third grouping resembled the tight clustering of the Baseline group but also clearly separated from it.

An orthogonal partial least squares – discriminant analysis (OPLS-DA) model was also generated to explore any correlation between the Treatment and Control groups after two weeks of nitrate intake. An OPLS-DA is a discrimination and descriptive modeling tool that possesses the ability to identify variable discrepancies in a multivariate dataset (Worley & Powers, 2013). In Figure 3, much of the control group does not

demonstrate an identifiable trend, except a nonspecific clustering. After two weeks of supplementing, the Treatment group displays a more concentrated grouping and evidence of an intervention. After examining the relationships of the two groups and the Treatment group throughout the trial via OPLS-DA and PCA modeling, it is clear that nitrate supplementation was having a potentially positive effect on the metabolome.

Pathway and enrichment analyses were performed to uncover any potentially significant metabolic pathways in the Treatment and Control groups. Pathway analyses use pathway-based metabolite sets and databases to analyze for the significance and impact of various metabolisms among two groups. The identification of altered metabolic pathways can discover many unknown differences between two metabolome groups and explain their potential effect. Similarly, an enrichment analysis examines the alterations in metabolite concentrations and identifies over-represented metabolites. The impact of these enriched metabolites can then be observed within a metabolic network (Chong et al., 2018).

A pathway analysis investigating the Treatment and Control groups at Week 2 did not reveal any significant pathways but did clarify the impact of a few significant metabolites (Figure 4 and Table 4). Phenylalanine metabolism had the largest impact (I=0.26) on the metabolome due to the significance of PPA (p=0.048), as well as, phenylacetic acid and 2-hydroxyphenylacetate. Interestingly, arachidonic acid (AA) metabolism was discovered. AA is a central mediator of the inflammatory response and catalyzes into various prostaglandins, including PE_2 ($p<0.001$). As one of the most abundant prostaglandins, PE_2 has numerous biological functions, but most notably, it is a pro- and anti-inflammatory agent and regulator of blood pressure (Ricciotti & Fitzgerald,

2011). While the pathway did not contain sufficient metabolites to be considered significant, the upregulation of PE_2 warrants further investigation.

An enrichment analysis was employed to evaluate metabolite sets for over-represented compounds that may be due to the difference in the Treatment and Control groups after two weeks. As illustrated by Figures 5 & 6, and Table 5, several sets were found to be equally significant, including fatty-acid-CoA ligase, fatty acid transport via diffusion, ß-oxidation of long chain fatty acids (LCFAs) (odd chain), carnitine fatty-acyl transferase, methylmalonyl-CoA mutase, etc ($p=0.016$). As expected, many of the significant metabolite sets involved fatty acid metabolism and transport due to the number of significant fatty acid metabolites, including the OCFAs, heptadecanoic acid ($p=0.005$) and pentadecanoic acid ($p=0.027$), which were found within each set. The quantity of fatty acid metabolites should not be that surprising. Previous studies have begun to show that nitrate supplementation may actually enhance fatty acid oxidation (FAO). Nitrate intake has been associated with expressed FAO enzymes via NO-cGMP PPAR mechanisms, elevated muscle carnitine, and lowered malonyl-CoA levels resulting in ß-oxidation of LCFAs and improved mitochondrial respiration (Ashmore et al., 2015).

In the only other nitrate intake metabolomics analysis, fatty acids, acyls, and their conjugates were also predicted to produce a positive vascular response to supplementation, which included brachial artery FMD, ß-stiffness index, and carotid artery cross-sectional compliance. The predictive linear-regression model was developed based upon significant metabolites, a lack of influence by dosage concentrations, and subjects demonstrating a one, eight, and fifteen percent improvement in FMD, carotid-artery cross-sectional compliance, and ß-stiffness, respectively. (De Van et al., 2016).

Secondary pathway and enrichment analyses were generated to assess the Treatment group after Week 1 and Week 2. While the pathway analysis did not discover a significant pathway, simple sugar breakdown was accentuated, such as starch and sugar metabolism and galactose metabolism ($p=0.09$ and $p=0.11$, respectively). Despite lacking significance, starch and sugar metabolism, and glucose as the metabolite, did have a relatively large impact between the two metabolomes A recent study did find that chronic nitrate intake (two months) did result in improved carbohydrate metabolism, including improved glucose tolerance, insulin resistance, and reduced inflammation (Gheibi, Jeddi, Carlström, Gholami, & Ghasemi, 2018). In addition, the enrichment analysis of the two time points yielded consistent but less significant metabolite sets and OCFAs as the Treatment and Control groups. Each analysis aptly demonstrated the metabolic differences of one and two weeks of supplementation, as well as, the variations in the Treatment and Control groups.

When considering the only previous metabolomics study on nitrate supplementation, this analysis drew many parallels while also potentially offering new insights. The prior study employed a randomized, double blind, placebo-controlled trial with sodium nitrite capsules (80 or 160 mg/day nitrite) in older adults over a ten-week period. The study was able to predict classes of metabolites that would yield improved vascular function, such as benzene and its substituents for FMD and fatty acids/acyls and glycerophospholipids for ß-stiffness index and carotid artery cross-sectional compliance (De Van et al., 2016). Despite the robust results, a large quantity of potential metabolites was unmentioned and/or unidentified, while multivariate, pathway, and enrichment analyses were not performed. In this current analysis, eighteen significant metabolites

were identified after nitrate supplementation and assessed through PCA and OPLS-DA modeling, as well as, investigated through pathway and enrichment analyses.

The initial study was able to show that daily nitrate intake (240-280 mg/60 mL nitrate) for a fourteen-day period led to a significant increase in plasma nitrate/nitrite concentrations and a decrease in diastolic blood pressure, while also slightly improving systolic blood pressure and FMD (Sweazea et al., 2018). It was the aim of this research to build upon the original study design, explore the metabolome for significant changes during supplementation, and interpret those findings. All discovered significant metabolites were investigated for their biological function and any potential association with vascular function. After completing multivariate analyses, the efficacy of nitrate intake was then arranged into observable models. In addition, pathway and enrichment analyses identified possible metabolic alterations with nitrate supplementation.

Upon completion of this analysis and review of previous studies, there are still numerous unidentified compounds associated with vascular function. Of the approximate 160 metabolites found to affect FMD, ß-stiffness, and carotid cross-sectional compliance in De Van et al. (2016), nearly sixty percent were unknown. The greatest identified molecular classes across the measurements were glycerophospholipids (25%) and the aforementioned fatty acids/acyls (~15%). The next step in research is to employ a study design that combines the strengths of both analyses. A randomized, double blind, crossover study investigating daily sodium nitrate intake (~200 mg/capsule) in young adult males over a five-week period (Treatment/Control – 2 weeks + Washout – 1 week) should be emphasized. In addition to the LC-MS method conducted for this analysis, a lipidomics approach is necessary to account for the glycerophospholipid compounds.

Furthermore, the vascular measurements in the previous metabolomics study and the statistical analysis of this paper would best compliment the study design.

Despite the findings, this exploratory analysis did contain limitations. The original study design had not considered this analysis. The nitrate supplement was a proprietary blend of nitrate-rich fruits and vegetables comprised of a variety of compounds. The intervention of the study was a fourteen-day period and did not account for long-term, chronic intake.

Conclusion

In this exploratory analysis, a previous study assessing nitrate intake and its influences on vascular function in young adult males was investigated for underlying metabolic shifts through a LC-MS/MS-based large-scale targeted metabolomics approach. Eighteen significant metabolites were discovered across the time, treatment, and time & treatment groups. With a significant improvement in plasma nitrate/nitrite levels and diastolic blood pressure, several metabolites were found to be anti-inflammatory and anti-atherosclerotic mediators. Additionally, PCA and OPLS-DA modeling demonstrated clear separation among the time, Treatment, and Control groups. Moreover, pathway and enrichment analyses confirmed the effects of nitrate intake on varying metabolite sets and its possible role in fatty acid oxidation. A better understanding of altered metabolic pathways may help explain the benefits of nitrate on vascular function and reveal any unknown mechanisms of its supplementation.

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APPENDIX A

IRB APPROVAL

APPROVAL: EXPEDITED REVIEW

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

Dear Carol Johnston:

On 8/18/2015 the ASU IRB reviewed the following protocol:

Type of Review:	Initial Study
Title:	Evaluation of the protective effects of a beet juice energy drink on vascular health
Investigator:	Carol Johnston
IRB ID:	STUDY00002962
Category of review:	(2)(a) Blood samples from healthy, non-pregnant adults, (4) Noninvasive procedures, (7)(b) Social science methods, (7)(a) Behavioral research
Funding:	Name: Isagenix, Funding Source ID: in process
Grant Title:	
Grant ID:	
Documents Reviewed:	• diet recall, Category: Measures (Survey questions/Interview questions /interview guides/focus group questions); • Health history questionnaire, Category: Screening forms; • flyer and script, Category: Recruitment Materials; • Mood assessment, Category: Measures (Survey questions/Interview questions /interview guides/focus group questions); • Isagenix proposal, Category: Grant application; • food list, Category: Resource list; • protocol, Category: IRB Protocol; • consent, Category: Consent Form; • meta analysis demonstrating safety , Category: Drug Attachment; • sleep assessment, Category: Measures (Survey questions/Interview questions /interview guides/focus group questions); • screener, Category: Screening forms;

The IRB approved the protocol from 8/18/2015 to 8/17/2016 inclusive. Three weeks before 8/17/2016 you are to submit a completed Continuing Review application and required attachments to request continuing approval or closure.

If continuing review approval is not granted before the expiration date of 8/17/2016 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

cc:

Karen Sweazea
Brendan Miller

APPENDIX B

HUMAN CONSENT FORM

CONSENT FORM

Title of research study: Energy drinks and vascular health

Investigators: Drs. Karen Sweazea and Carol Johnston, ASU Nutrition Professors

Why am I being invited to take part in a research study?

We invite you to take part in this research study because you are a healthy male between the ages of 18 and 40 years with normal blood pressure and you are willing to drink juice-based energy drinks daily for 2 weeks.

Why is this research being done?

The purpose of this research is to determine if juice supplementation is associated with significant reductions in blood pressure and significant improvements in blood vessel function.

How long will the research last?

We expect individuals to spend less than 3 weeks participating in the proposed activities.

How many people will be studied?

We expect 51 people will participate in this research study.

What happens if I say yes, I want to be in this research?

You will be a participant in this study for approximately 3 weeks. Your participation includes four visits to the research laboratory: visit 1 – consenting and health history screening, blood pressure measurement, and discussion of study instructions; visits 2 and 3 – to measure blood pressure and blood vessel function, to collect information on body weight, mood, and sleep, to collect a blood sample, and to dispense the juice supplement; and visit 4 – to perform final measurements (repeating the same measurements as described above). For the blood sampling, you will need to fast overnight for 12 hours (no food or beverage with the exception of water). Approximately 2 tablespoons of blood will be collected by a research nurse at each of these visits. The length of visit 1 is about 20 minutes; visits 2, 3 and 4 will take about 45 minutes each. In addition to receiving instructions at visit 1, you will be given a compliance calendar to remind you of the study procedures and to check off when the juice supplements are taken. The study procedures include: restricting certain vegetables from your diet throughout the 2-week study period (see chart). Also, you will be asked to abstain from all dietary supplements and antiseptic mouthwash for 3 days prior to testing (e.g., visits 2, 3, and 4); caffeine for 12 hours prior to testing; exercise for 12 hours prior to testing. At visit 2, you will be randomly assigned (by coin toss) to receive the active juice or a placebo juice; you are to consume 2 oz of the juice supplement each morning for 2 weeks (you will be asked to consume the drink before brushing your teeth in the morning). You will not know which juice group you are in, nor will the investigator. During the research process, you will interact with the research team, consisting of the investigator, a registered nurse, and a lab technician. The contact information of the investigators will be provided to you. All measurements, as well as the processing of the blood sample, will be done at the Arizona Biomedical Collaborative laboratory (ABC) on the ASU downtown campus in Phoenix. The research trial is expected to last from August 2015-December 2015.

What happens if I say yes, but I change my mind later?

You can leave the research at any time it will not be held against you. If you decide to leave the research, there will be no adverse consequences. If you decide to leave the research, contact the investigator so that the investigator can appropriately exclude you from the trial.

Is there any way being in this study could be bad for me?

The juice supplements may turn your urine and feces a red/brown color, a consequence of pigments in the

Please turn over ⟶

juice. This is not harmful, but may surprise you. Many trials have been conducted by numerous investigators using the juice supplement with no reports of major adverse effects. You may feel temporarily nauseous or faint during the blood draw. Blood draws are performed by a registered nurse or a trained phlebotomist who are experienced in handling these issues. The measurement of blood vessel function entails placing a blood pressure cuff on your arm and pumping air into the cuff to cut off blood circulation. You will feel pressure on your arm for 5 minutes. In other research, participants ranked the pain associated with this procedure a '2' on a 10-point scale. A trained sonographer (ultrasound technician) will perform this procedure. Taking part in this research study may lead to added costs and time commitments. Driving to the ASU nutrition laboratory in Phoenix will entail gas costs, and you may need to pay the meter for curbside parking (costs should be ≤ $1.50).

Will being in this study help me any way?

We cannot promise any benefits to you or others from your taking part in this research. However, your participation will help the scientists advance knowledge regarding juice supplementation for improving cardiovascular health.

What happens to the information collected for the research?

Efforts will be made to limit the use and disclosure of your personal information, including research study data, to people who have a need to review this information. We cannot promise complete secrecy. Organizations that may inspect and copy your information include the Institutional Review Board at Arizona State University and other representatives of this organization. All data and blood samples collected during this study will be identified only by a number assigned to you and stored in a secure setting in the ABC laboratory.

What else do I need to know?

If you agree to participate in the study, written consent does not waive any of your legal rights. However, no funds have been set aside to compensate you in the event of injury. You will receive a $30 at visits 2, 3 and 4 ($90 total).

Who can I talk to?

If you have questions, concerns, or complaints, or think the research has hurt you, talk to the research team at Arizona State University. You may email or call the investigators [Carol.Johnston@asu.edu (602)827-2265 or Brendan.Miller@asu.edu (480)580-0099] to express any concerns.

This research has been reviewed and approved by the Bioscience Institutional Review Board at ASU. You may talk to them at (480) 965-6788 or research.integrity@asu.edu if:

- Your questions, concerns, or complaints are not being answered by the research team.
- You cannot reach the research team.
- You want to talk to someone besides the research team.
- You have questions about your rights as a research participant.
- You want to get information or provide input about this research.

Your signature documents your permission to take part in this research.

Signature of participant	Date
Printed name of participant	Email or phone#
Signature of person obtaining consent	Date

APPENDIX C

RECRUITMENT FLYER

Juice Energy Drink Trial

THE NUTRITION PROGRAM AT ASU IS RECRUITING HEALTHY, NON-SMOKING ADULTS (18-40 y of age) – BOTH SEDENTARY INDIVIDUALS AND ATHLETES. THIS STUDY WILL EXAMINE WHETHER A CONCENTRATED 'JUICE SHOT' IMPROVES CARDIOVASCULAR HEALTH

Participation will include:

- Enrolling in a 2-week trial with daily juice ingestion and 4 visits to the Nutrition Laboratories at the downtown Phoenix campus (5th and Van Buren Streets)
- Providing 3 blood samples and having vascular health measured at one week intervals, recording diet, health history, sleep, and mood states
- Maintaining usual diet and activity patterns
- Incentives will be provided during the study, totaling $90 for 2-week participation

INTERESTED?? Please visit our recruitment site:
https://www.surveymonkey.com/r/JuiceStudyatASU

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- Incentives will be provided during the study, totaling $90 for 2-week participation

INTERESTED?? Please visit our recruitment site:
https://www.surveymonkey.com/r/JuiceStudyatASU

Adults Needed for ASU Juice Trial

The ASU Nutrition Program is recruiting healthy non-smoking adults (18-40 years of age) for a research trial. This 2-week trial will examine whether a natural juice concentrate ('shot') improves cardiovascular health such as blood pressure. If you are willing to consume a juice shot daily (2 ounces; 30 calories) and provide 3 blood samples at 1-week intervals along with other testing, you may be interested in this trial. Incentives will be provided during the study, totaling $90 for 2-week participation.

For more information or to apply for the study, please visit our recruitment site:
https://www.surveymonkey.com/r/JuiceStudyatASU

APPENDIX D

HEALTH HISTORY QUESTIONNAIRE

Health History Questionnaire

ID#_____

Age:_____

Gender (please circle): Female Male

Smoked cigarettes in the past year?

(please circle): Yes No

To be completed by investigator

Height:_____ft. _____in.

Weight:_____lbs.

Waist:_____in.

1. Are you taking any medications regularly? (including aspirin, steroids, thyroid meds, etc.) Y N

If yes, what medications and how often?

_____

_____

2. Do you currently take supplements? (vitamins, minerals, herbs, etc.) Y N

If yes, what supplements and how often?

_____

_____

3. Has a doctor ever told you that you have any of the following conditions?

Heart disease?	Y N	Thyroid problems?	Y N
Kidney disease?	Y N	Cancer?	Y N
Liver disease?	Y N	High blood pressure?	Y N
Type 2 Diabetes?	Y N		

Food Allergy? Y N (if yes, what foods?)_____

If you have other chronic conditions, please list: _____

4. Are you willing to drink a concentrated juice 'shot' each morning before brushing your teeth for 2 weeks? Y N

5. Have you ever fainted at a blood draw? Y N

Are you willing to participate in a blood draw? Y N

Have you donated blood in the past 8 weeks? Y N

6. Will you be willing to consume an oral supplement 4 times per day for 12 weeks? Y N

Please turn over ⟶

7. Would you be able to restrict certain vegetables from your diet (aside from that pertaining to the study) for 2 weeks? [see chart] Y N

8. Do you follow a specific diet? (weight loss/gain, vegetarian, low-fat, etc.) Y N

If yes, please explain _____

9. Are you willing to travel to the ASU Downtown Phoenix campus on 4 separate occasions? Y N

10. Are you able to follow this pre-visit protocol for visits 2, 3, and 4: abstain from dietary supplements for 3 days, abstain from caffeine for 12 hours, abstain from heavy exercise for 12 hours prior to testing, and fast overnight (12 h)? Y N

11. Will you be able to maintain your typical diet, lifestyle, and activities during the trial? Y N

12. Over a 7 day period, how many times do you engage in any regular activity long enough to work up a sweat (e.g., heart beats rapidly)? _____

How many times per week do you exercise moderately?_____

Please circle the total time you spend in each category for an average week.

Light activities such as: slow walking, golf, easy swimming, gardening, etc.

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

Moderate activities such as: moderate walking, cycling, swimming, weight lifting, etc.

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

Vigorous activities such as: fast walking, jogging, cycling, heavy/intense weight lifting, etc.

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

13. Are you currently training to compete in a sport? Y N

If yes, please explain your training schedule:

_____

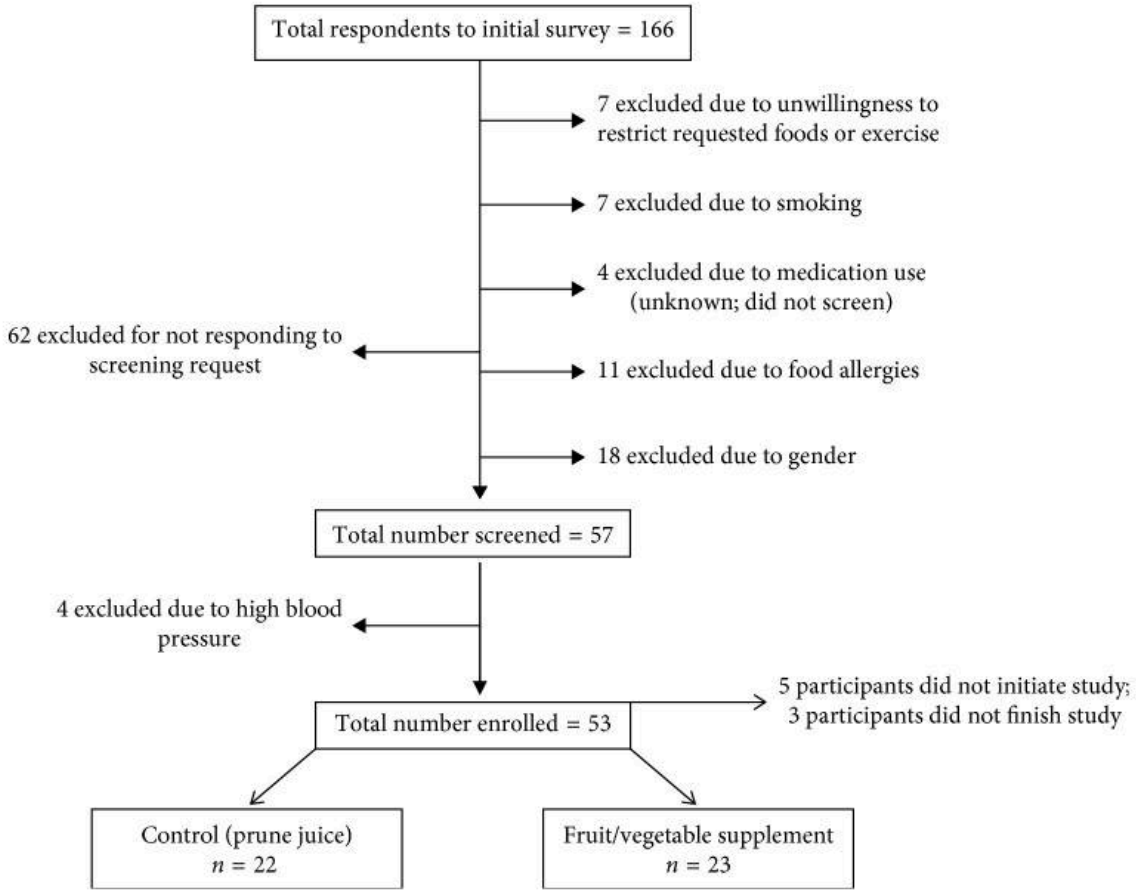
14. Please describe any other medical conditions or situations that may affect your ability to participate in this research trial (i.e., infections, travel, work deadlines, etc.).

_____

_____

APPENDIX E

STUDY DESIGN FLOW CHART


Total respondents to initial survey = 166
7 excluded due to unwillingness to restrict requested foods or exercise
7 excluded due to smoking
4 excluded due to medication use (unknown; did not screen)
62 excluded for not responding to screening request
11 excluded due to food allergies
18 excluded due to gender
Total number screened = 57
4 excluded due to high blood pressure
5 participants did not initiate study; 3 participants did not finish study
Total number enrolled = 53
Control (prune juice) n = 22
Fruit/vegetable supplement n = 23

APPENDIX F

SAMPLE SIZE CHART

DeVan: Effect size of 0.74, > 2,000 Outcome Measures

- Independent Samples T-test – 49 per Group, 0.952 Actual Power
- MANOVA
  - Between Factors – 10 per Group, 0.982 Actual Power
  - Within Factors – 14 per Group, 0.976 Actual Power

Author	Year	Change in Vascular Function ±SD	n Per Group	Calculated n Per Group	Age Range	Subject Health Status
Rodriguez-Mateos, et al	2013	7.2% ± 0.3%	10	10	23 ± 3	Healthy, Flow-mediated Dilation
Dalli, et al	2002	2% ± 2%	40	17	58.5 ± 7.8	Healthy, CVD risk, Flow-mediated Dilation
Bahra, et al	2012	-5 mmHg ± 1.4 mmHg	14	6	27.9 ± 1.8	Healthy/ Systolic Blood Pressure