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The Effects of Dietary Nitrate and Vitamin C on Endothelial Function and Oxidative Stress Biomarkers

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THE EFFECTS OF DIETARY NITRATE AND VITAMIN C ON ENDOTHELIAL FUNCTION AND OXIDATIVE STRESS BIOMARKERS

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By
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2020

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ABSTRACT OF DISSERTATION

THE EFFECTS OF DIETARY NITRATE AND VITAMIN C ON ENDOTHELIAL FUNCTION
AND
OXIDATIVE STRESS BIOMARKERS

Background: Endothelial dysfunction is associated with a decline in the vasodilator nitric oxide (NO) and presents alongside other cardiovascular risk factors in the early stages of atherosclerosis. Independently, inorganic nitrate and vitamin C supplementation have shown promise in providing some restoration of NO bioavailability, reduced oxidative stress, and improvements in endothelial function. Some preliminary evidence has suggested that vitamin C may augment the reduction of nitrite to further liberate NO and improve cardiovascular health. However, co-supplementation has not been adequately studied. Our objective was to compare the effect of inorganic nitrate in the form of concentrated beetroot juice (CBJ) with and without vitamin C on changes in clinical and blood biomarkers of endothelial dysfunction.

Methods: Older adults between 50-70 years with low density lipoprotein (LDL) >130 mg/dL and RHI score less than 2 were enrolled in a crossover randomized -double blinded study. Two 4-week intervention periods were separated by a 2-week washout period. Subjects were assigned randomly to start with either CBJ with 1000 mg of vitamin C (NC) or CBJ with a matched vitamin C placebo (N), then switched to the alternate treatment. Vascular testing was measured by EndoPAT to assess reactive hyperemia index (RHI) before and after each interventional period. Fasting blood samples were also collected for serum lipid parameters, plasma vitamin C, plasma oxidized LDL, and plasma NO metabolites to compare the differences between treatments.

Results: Of the 23 enrolled subjects, 18 completed all study visits (7 male, 11 females; age 53-70 years). No significant differences were observed in RHI change ($p>0.05$) between the two treatments. Some subjects showed a greater improvement in RHI after nitrate and vitamin C who entered the study with a lower baseline RHI score than those with mean baseline RHI score >1.67

(RHI: 1.23 vs 1.75; p=0.02). Changes in plasma nitrate and nitrite were significantly different between treatments (p<0.01 and p=0.03 for nitrate and nitrite, respectively). NC produced a greater reduction in the ratio of oxLDL/NOx (p=0.02). There were significant differences between treatment groups for LDL cholesterol (p= 0.049), total cholesterol (p=0.05), and triglycerides (p=0.03) with lower concentrations observed following the NC intervention. HDL was not influenced by dietary nitrate supplementation individually nor in combination with vitamin C.

Conclusions : Co-supplementation of CBJ and vitamin C was effective at enriching the NO pool and reducing a marker of oxidative stress suggesting a potential to improve endothelial function, and reduce the progression of atherosclerotic disease.

Keywords: Dietary nitrate, Vitamin C, Endothelial Function, Nitric Oxide, Oxidized Low-density-Lipoprotein, Lipid Profile.

Reem Othman Basaqr

(Name of Student)

02/04/2020

Date

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Chapter 1: INTRODUCTION

1.1 Background

Cardiovascular diseases (CVDs) have contributed to a rampant proportion of mortality in the United States [1, 2] resulting in more than 859,000 deaths per year. CVD causes a huge economic burden to US health care system costing $199 billion a year and causing $131 billion in lost productivity from premature death. Coronary heart disease (CHD) is the most common cause of death attributable to CVD in the United States, killing over 366,800 people annually. In the U.S, the estimated annual incidence of heart attack is 720,000 with an average age of 65.5 years for males and 72 years for females [3]. Hypercholesterolemia is one of the main cardiovascular risk factors that contribute to increased risk of atherosclerosis (AS), coronary mortality, and morbidity [3, 4] (see Table 1-1). In 1963, Keys and coworkers conducted a study of 12763 men aged 40-59 years from 7 countries and found that high cholesterol levels were associated with the prevalence of CHD [5-9]. A report from the American Heart Association (AHA) estimated that approximately 100 million U.S. adults have total cholesterol levels of 200mg/dL or greater. Of this number, 31 million have levels greater than 240mg/dL [10]. Research has clearly suggested that the elevation of plasma oxidative stress and high total cholesterol concentrations in hypercholesterolemia potentiate impairment in the endothelial function that represents early as a key first step toward AS development [11, 12]. Impairment of endothelial function is an important mechanism for cardiovascular (CV) risk [13]. Early studies demonstrated a positive relationship between endothelial dysfunction (ED) and cardiovascular risk factors [14-17]. Endothelial dysfunction (ED) is commonly observed in a number of chronic diseases such as diabetes mellitus, metabolic syndrome, hypertension, and dyslipidemia [18]. Numerous studies have documented that improving the functional integrity of the endothelium is inversely associated with CV risk events, including AS [19, 20]. Thus, as impaired endothelial function is a reversible and important surrogate for AS, early recognition and intervention may have therapeutic and prognostic value in vulnerable populations to slow the onset or progression of AS, and reduce the incidence of cardiovascular risk events [21]. Therefore, it is imperative to identify novel approaches to restore endothelial function and reduce the risk of major CVD events. Some studies have looked at using nutritional interventions to improve/restore endothelial function. Compelling evidence of modulating vascular function with dietary interventions are emerging and deserve greater attention as a viable option on modulating key markers of cardiovascular health [22]. For instance, the cardio-protective effects of green leafy vegetables are attributed to the high nitrate content and the presence of other active components including vitamin C, polyphenols, potassium, or carotenoids

[23]. Diet-derived inorganic nitrate may have impact on CVD prevention through the enhancement of vascular health by increasing blood flow, dilating arteries, and inhibiting platelet aggregation and leukocyte adhesion [22-24]. Questions have been raised whether the addition of vitamin C supplement complement the biological benefits of dietary nitrate by improving vasodilation through facilitating the conversion of salivary-derived nitrite to nitric oxide (NO), and diminishing oxidative damage [25, 26]. Therefore, the primary goal of this dissertation is to evaluate the additive effect of dietary nitrate combined with vitamin C on endothelial function and oxidative stress markers in patients at risk of CVD.

Table 1-1-1. Modifiable and non-modifiable risk factors for atherosclerotic CVD

Modifiable Risk factors	**Non-Modifiable Risk factors**
Diabetes mellitus	Age
Hypertension	Gender
Dyslipidemia	Family history
Obesity	Genetics
Tobacco smoking	Ethnicity
Poor diet	
Physical inactivity	

1.2 Atherosclerosis

Atherosclerosis is defined as a complex inflammatory disease that occurs primarily in the arterial intima at susceptible arterial sites in the coronary arteries [27]. Atherosclerosis develops progressively through accumulation of cholesterol- rich lipids, mainly low-density lipoprotein cholesterol (LDL) and an inflammatory response. These changes occur along a life-long continuum that begins with the development of early fatty streak during childhood or adolescence in response to endothelial injury, followed by fibrous plaque in late adolescence and early adulthood (15-30 years), advancing to an atheroma that occurs in older adults aged ≥55 years resulting in increased chances of a life-threatening thrombosis [28]. Therefore, the clinical manifestation of AS is silent in most people until later in life. The three most important factors in atherosclerotic lesion are endothelial dysfunction, alterations in lipid metabolism, and inflammation [28].

1.2.1 Pathophysiology of atherosclerosis

The first key stage in atherogenesis as proposed in "Response to Injury Theory" is dysfunctional alterations in the endothelium. Endothelial dysfunction induced by number of irritants including physical injury or stress as a result of direct trauma, turbulent blood flow, or circulation of toxins (reactive oxygen species (ROS)) from the presence of one or more cardiovascular risk factors for example; tobacco smoking, hyperlipidemia, hyperglycemia,

hypertension, or obesity [11, 29]. The second stage is fatty streak development in the context of local ED within the intima (sub-endothelium) where the underlined proteoglycan becomes exposed and permits an increase of LDL infiltration, deposition, and retention within the sub endothelial layer. This ultimately results in increased susceptibility of endothelial surface lipids to oxidative modification in the arterial wall that plays an important role in the activation of endothelial cells [27]. Consequently, recruitment of leukocytes and adhesion of monocytes to the activated endothelial layer occur [30]. Monocytes differentiate into macrophages that uptake oxidized low-density lipoprotein (oxLDL) by their scavenging receptors such as (LOX-1) to form foam cells. These foam cells which undergo inflammatory processes, secrete proinflammatory substances such as interleukin 1 (IL-1), and tumor necrosis factors (TNF) and growth factors [27, 31]. Subsequently, vascular smooth muscle cell proliferate and migrate to the endothelium. Thereby promoting plaque formation by synthesis of extracellular matrix such as collagen, elastin, and proteoglycan to form the fibrous cap [32] [31]. The final step is plaque rupture or thrombosis in which all the thrombogenic plaque materials get exposed and leads to blood clot formation producing blockage of arterial blood flow (myocardial infarction) [30].

In clinical settings, the estimation of risk for atherosclerotic CVD (ASCVD) depends on identifying and quantifying risk factors. However, this clinical tool may be not enough to assess individual's risk [33]. Evidence in literature suggest that an impairment of endothelial function acts as an independent predictor for CV events [34-36], and the pathologic changes of hypercholesterolemia in CV system are initiated when endothelial erosions appear [33].

1.3 Endothelium

Endothelium is a monolayer of cells that lines the inner layer of blood vessels forming an interface between the vascular wall and blood lumen [37]. The healthy endothelium maintains vascular homeostasis through optimal response to physical and chemical signals such as blood pressure (BP), shear stress, and hormonal stimuli. It produces of a wide range of vasoactive substances participating in modulation of coagulation, inflammatory reactions, as well as regulation of vascular tone that relax or constrict blood vessels [37]. Some of the vasodilatory substances released by endothelium include NO, prostacyclin I2 (PGI2), different endothelium-derived hyperpolarizing factors and protein C/protein S [19, 29, 37, 38]. Therefore, maintaining the integrity of endothelial function is important for overall vascular health [39, 40].

Under pathological conditions, such as the presence of CVRFs (hypercholesterolemia), the endothelium releases various vasoconstrictive, pro-inflammatory, pro-coagulant substances include endothelin-1, Ang II, thromboxane A2, platelet activating factor, and ROS. The production of the aforementioned substances shifts the balance toward over production of pro-oxidant, pro-

inflammatory, and pro-thrombotic effects that assist with platelet aggregation [38, 41, 42]. This imbalance between vasodilators and vasoconstrictors is an essential factor for the impairment of endothelial function that is further aggravated by oxidative stress [12]. Overall, an impairment of endothelial function is the early key event in the pathogenesis of AS which can independently predict CV events [43, 44]. Several mechanisms have been identified underlying ED in arteries predisposed to AS. These mechanisms can be summarized into two types, which either impairs NO production or increases NO inactivation/degradation as a result of oxidative stress [37, 45].

Importantly, pathophysiologic changes in endothelium can be detected before structural changes in the vascular wall, and can be partially repaired [37, 46]. Multiple studies have documented that improving the functional integrity of the endothelium is inversely associated with CV risk events, including AS [19, 20]. Thus, as endothelial function is reversible at every stage of AS and acts as an important surrogate for AS [33], early recognition and intervention may have therapeutic and prognostic values in vulnerable populations to slow the onset or progression of AS, and reduce the incidence of CV risk events [21]. These data suggest targeting endothelial function and correcting the true cause of malfunction (reduction of NO) by identifying appropriate interventions in patients at risk of CVD is a great of concern.

1.4 Factors known to modulate endothelial function

1.4.1 Nitric oxide

In 1980, Furchgott and Zawadzki first discovered the significance of NO as a predominant endothelium-derived relaxing-factor for CV health [47]. NO is recognized as a master signaling gaseous molecule that is involved in many physiological process of human organ systems including circulatory system[48]. The traditional pathway to synthesize NO in endothelium is from the amino acid L- arginine by the enzyme called endogenous nitric oxide synthase (eNOS) [47]. The activity of this enzyme depends on the presence of oxygen molecules as well as tetrahydrobiopterin (BH_4), flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN), the intracellular concentration of calcium ions, and the co-substrate nicotinamide-adenine dinucleotide phosphate hydrogen (NADPH) [18].

In response to vascular stimuli such as shear stress, bradykinin, acetylcholine, or histamine, NO synthesis and release are triggered by increasing the intracellular concentration of calcium that further binds with calmodulin to form calcium calmodulin complex. This complex detaches eNOS from its binding with Caveolin to facilitate the activation of eNOS in order to catalyze NO synthesis [49]. Eventually, this gaseous molecule vasodilates locally by diffusing into vascular smooth muscle cells to activate soluble guanylate cyclase (sGC) that catalyzes the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (GMP), and thereby causes vascular

smooth muscle relaxation [37, 50] . Vasodilation effect results in improved blood flow, and delivery of oxygen and nutrients to a variety organs and tissues [22]. Aside from vasodilatory potential, NO has anti-thrombotic and anti-proliferative roles which have been supported by studies of animal models of AS [49, 51]. Moreover, studies have been shown that NO has antioxidant properties, limits lipid peroxidation and mitochondrial ROS generation [52]. These data suggest that due to the strong vascular effects induced by NO, interest in maintaining circulatory NO pool remains a priority for future research.

A decrease of NO bioavailability has been suggested as a cause for endothelial phenotypical changes [49], and has been considered as a hallmark of ED associated AS [18]. Evidence has demonstrated that lacking of eNOS induces hyperlipidemia, hypertension, and insulin resistance [51]. Several mechanisms have been identified underlying ED in arteries predisposed to AS. These mechanisms can be summarized into two types, which either impairs NO production or increases NO inactivation/degradation as a result of oxidative stress [37, 45]. There are several factors that can modulate NO synthesis and degradation including the eNOS mRNA and protein expression and the amounts of eNOS inhibitor called asymmetric dimethylarginine (ADMA) [51], eNOS uncoupling , and overproduction of ROS, mainly superoxide radicals [45]. The eNOS uncoupling resulted from either the depletion of the substrate (L-arginine) or the oxidative modification of the cofactor (BH_4) resulting in superoxide anion production rather than NO formation [34].

1.4.2 Oxidative stress

Oxidative stress has a causal role in altering vascular function and tone [34, 49]. Oxidative stress defined as a state in which excess production of ROS (free radicals) disturb its balance with antioxidants defenses [53]. Free radicals, are highly reactive, consist of oxygen-containing molecules with an odd number of electrons, which allow them to react easily with other molecules and consequently cause tissue and cell damage [54].

One of the major sources of ROS production in atherosclerosis is NADPH oxidase (NOX). These oxidative enzymes use NADPH as an electron donor to generate endothelial oxidative superoxide leading to degradation of either NO or it cofactor BH_4 before leaving the endothelium [49]. Other sources of ROS have been identified within vascular tissues are xanthine oxidase, uncoupled eNOS, mitochondrial respiratory chain, and lipoxygenase. ROS include 1) free radical such as superoxide anion (O_{2-}), hydroxyl radical (OH), and 2) non-free radicals such as; hydrogen peroxide (H2O2), hypochlorous acid (HOCl), and peroxynitrite ($ONOO_-$) [45, 54]. To estimate the degree of oxidative stress, levels of circulating oxidative stress biomarkers can be detected by

measuring stable products produced by ROS such as serum lipid hydroperoxides, plasma malonaldialdehyde, plasma or urinary output of F2-isoprostanes, or plasma oxLDL [47, 50, 55].

The antioxidant scavengers in human subjects and animal models with AS are classified into enzymatic and non-enzymatic sources. The enzymatic sources are superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase, while the non-enzymatic sources such as vitamins C, vitamin E and beta-carotene, which present with very high levels in plant based sources including fruits, vegetables, and whole grains [56]. SOD converts superoxide to hydrogen peroxide (H_2O_2), which then can be detoxified to water (H_2O) and oxygen via either GPx or catalase [45, 57]. The capabilities of antioxidants defenses are attenuated with the excess production of ROS [58]. The findings from antioxidant vitamins interventions for cardiovascular health are controversial and more detail will be discussed in vitamin C section, pages 22&23.

1.4.3 Health status

As mentioned previously, the integrity of vascular health is affected by the presence of traditional risk factors of CVD such as aging, hypercholesterolemia, smoking, and diabetes. This dissertation primarily discusses the effect of aging and hypercholesterolemia on endothelial function. Aging is an independent risk factor of ED [18]. Vascular health of older individuals is a relevant clinical concern due to a structural and functional changes in the vascular system associated with aging. It is associated with functional abnormality of NOS expression/activity and cGMP-mediated vascular relaxation for multiple mechanisms [51, 59]. Furthermore, overproduction of oxidative stress and oxidized LDL are the main causative factors in aging leading to a decline in NO synthesis and bioavailability, further induces abnormality in endothelial function [60]. In healthy men greater than 40, the endothelial dependent vasodilation has been shown to decrease by 0.21% annually [61]. Other reports supporting that endothelium-dependent vasodilation declines significantly with age, have demonstrated a loss of greater than 50% of NO in adults after the age of 60 [62, 63]. Therefore, these phenomena highlight the importance for evaluating interventions targeting the NO pathway or providing an alternative source of NO to slow the progression of NO insufficiency in older individuals at risk of CVD [64, 65].

a. Hypercholesterolemia

The association between impaired endothelium dependent vasodilation and elevated LDL levels are well documented [37]. Early studies in animal models of hypercholesterolemia have examined the link between hypercholesterolemia and a reduction in endothelium dependent vasodilation in large arteries [66-68]. These findings were confirmed by human clinical studies that assessed endothelial function in healthy and hypercholesterolemic subjects and demonstrated an

impairment of NO release with hypercholesterolemia subjects compared to normocholesterolemic controls [69, 70].

Numerous observations supporting the view that high cholesterol levels in the blood stream, particularly LDL (often referred to as bad cholesterol) induces an increase of vascular oxidant load of ROS, particularly superoxide anion free radicals in the vascular wall therefore resulting in changes of endothelial permeability and induce an increase in LDL retention into the arterial wall [71-74]. In fact, Ang II concentration is increased during hypercholesterolemia and other atherosclerotic diseases, which in turn stimulate ROS derived from NOX and xanthine oxidase activation within endothelial cells and smooth muscle cells [75]. Over production of superoxide anions consume NO to produce potent form of reactive nitrogen species (RNS) called peroxynitrite ($OONO^-$), consequently, generates oxidized LDL. Increased ($OONO^-$) may in turn uncouple eNOS, inactivate NO, and inhibit sCG activity [76]. This reaction is faster than the reaction between superoxide radical and SOD, which may explain the failure of SOD administration to improve vascular health in hypercholesterolemia [77]. Since loss of NO generation and oxidative stress in AS are ultimately related to vascular health, identifying effective pharmacological or lifestyle interventions that augment the cumulative NO pool and induce a further reduction in oxidative stress biomarkers may provide a novel strategy to prevent and treat ED associated cardiovascular risk factors.

There are extensive studies in literature documenting that ED as measured by FMD is reversible by modifying risk factors [78-80]. Researchers have investigated whether improvement of endothelial NO-dependent vasodilation was correlated with cholesterol lowering. Data have suggested that lowering cholesterol even if the levels are within the normal range could have a beneficial impact on improving endothelial function and NO production and release [42]. Experimental studies have shown that ED in a primate model of AS are reversible after removal of the LDL [37, 81]. In addition, when cholesterol lowering treatments are removed, it has been observed that ED returns quickly [37].

Some investigators confirmed that cholesterol level reductions resulting from dietary modification improved endothelium dependent vasodilation. For example, Fuentes et al, (2001) observed a recovery of ED in hypercholesterolemic men after lowering cholesterol levels with diet high in fruits and vegetables for 28 days [82]. As ED is treatable/reversible with lifestyle modifications, much attention of cardiovascular research has focused on the nutrition arena because dietary intervention seems to be feasible and low-cost way to manage patients at risk of CVD [22]. Therefore, this thesis focused on investigating a novel dietary supplement combination that may have a beneficial effect in cholesterol profile and endothelial function in hypercholesterolemia. To

date, there is limited knowledge about the effects of inorganic nitrate in hypercholesterolemia. Whether or not dietary nitrate supplementation has a beneficial effect on cholesterol profile is eagerly awaited [74, 83].

1.5 OxLDL and atherosclerosis

LDL oxidation is an important determinant involved in early and advancement stages of atherosclerotic plaques development [27, 30]. The oxidative modification hypothesis of atherosclerosis has emphasized the importance of oxidative modification step in atherogenesis [84]. This hypothesis proposes that small dense LDL is easily penetrate to sub-endothelial space and is more prone to oxidative modification by ROS prior to being engulfed by macrophages at sites of vascular cell activation [27]. The LDL oxidation is a complex process in which the protein and lipid components of LDL particles are susceptible to oxidative reactions during LDL oxidation [85, 86]. LDL is converted to minimally modified LDL (mmLDL) after infiltration in the artery [30]. This mmLDL induces the expression of intracellular adhesion molecules (ICAM) and vascular cell adhesion molecules (VCAM) to trigger the adhesion and recruitment of monocytes in the endothelial cells, and then undergoes oxidation to form fully or extensively oxLDL [58]. The exact mechanism of how and when oxLDL is formed and released to circulatory blood are still unclear [87]. However, there are different mechanisms contributing to the generation of oxLDL besides peroxynitrite including myeloperoxidase, lipoxygenase, phosophlipase A_2, metal ion-mediated oxidation (iron, copper), superoxide generators (xanthine oxidase, NADPH-oxidase), thiol-dependent oxidation, and other radical generation compounds [46, 85] followed by increased oxLDL recognition by the macrophages.

Some of the properties of oxidized LDL include the following: The new epitops of oxLDL inhibit LDL recognition by LDL receptors on macrophages [86]. OxLDL is a ligand for acetyl LDL receptor expressed in macrophages and termed as scavenger receptor and Lox-1[88]. These receptors are not down regulated as the native LDL receptors, and have high affinity to oxLDL leading to accumulation of intracellular cholesterol in the macrophages that turned to a foam cell formation. LDL oxidative modification plays a pivotal role in the development of cell apoptosis [27, 85].

Human studies clearly support that the concentration of oxidized LDL matters more than native LDL concentration since the former is the atherogenic form that enhance the impairment of the endothelial vasodilator response in endothelial cells more than does the native LDL levels both in large vessels and in the microcirculation [89]. Increased atherogenic lipids (oxLDL) promotes activation and dysfunction in endothelial cells in a dose dependent way that contribute mainly with the growth of atherosclerotic plaques and then CAD [27, 32, 37].

1.5.1 Plasma oxLDL

It has been long believed that oxLDL not merely accumulates in local inflammatory tissues, but can be transferred between vessel wall and the circulation [88]. Increased levels of circulating oxLDL have shown a positive correlation with severity of cardiovascular risk factors in some studies, specifically with age-related chronic metabolic diseases [46, 60]. A positive linear relationship has been established between plasma oxLDL and dyslipidemia in middle aged and elderly subjects [46]. Higher levels of oxLDL are positively correlated with LDL and apoB-100 concentrations [90]. Matsumoto et al. (2004) have suggested that circulating oxLDL could be used as an additional tool for coronary endothelial function. [91]. Matsumoto et al. observed a correlation between plasma oxLDL and endothelial function in coronary macro and micro vascular responses compared to conventional lipid parameters. Further studies are needed to clarify the mechanisms underlying the existence of oxLDL levels in the blood [92, 93]. The existence of oxLDL in the circulation is strongly confirmed by the presence of autoantibodies against oxLDL in atherosclerotic patients [91]. Evidence highlighted the importance of antibody selection to detect different epitopes of oxidized apoB or oxidized lipids [90]. To date, three monoclonal antibodies have been used in human circulating plasma to determine the relative changes in oxLDL levels using an enzyme –linked immunosorbent assay (ELISA) [92]. 1) oxLDL-DLH3 antibody and 2) oxLDL-E06 antibody recognize phosphatidylcholine moiety of oxidized phosphatidylcholine (ox-PC). 3) oxLDL-4E6 antibody is the most widely used antibody to detect the MDA lysine epitope of apoB 100 moiety of oxLDL [88, 90].

Evidence has shown that diet and lifestyle modification strategies play roles in atherogenesis as they can reduce LDL oxidation levels. For example, Barona et al. (2012) in a cross over clinical trial demonstrated that the consumption of polyphenol derived from grape juice reduces the oxLDL in a form of reducing I-CAM molecules after 4 weeks treatment compared to placebo [94]. As another example, Velmurugan et al. (2015) found a trend for a greater reduction of oxLDL levels after administering 250 ml of beetroot juice (~6 mmol of dietary nitrate) for 6 weeks in untreated hypercholesterolemia [74]. Of relevance to this dissertation, it is not well understood whether the specific dose or duration of dietary nitrate supplementation attenuates oxidative stress biomarkers in hypercholesterolemia subjects. Further studies are needed to draw a conclusion if dietary nitrate may regulate and/ or reduce plasma oxLDL concentrations itself [74].

1.6 Assessment of endothelial function

Loss of NO bioavailability is an index of ED that is associated with most, if not all, cv risk factors [78]. In clinical settings, there is neither a standard blood chemistry test nor prescribed therapy to diagnose and restore NO status effectively [18]. However, ED can be detected using

vascular testing that help with understanding vascular health prior to the development of AS lesion [95] .

There are different techniques have been developed to assess the pathophysiological function of endothelium in humans: 1) invasive technique via infusion of endothelial dependent vasodilator such as acetylcholine (ACH), serotonin, and bradykinin. 2) non-invasive technique via reactive hyperemia acts as a trigger to induce the release of endothelial dependent vasodilator NO[33]. The invasive techniques were not feasible for general population because they are expensive, time invasive, and only suited for patients requiring angiogram [13]. Therefore, non-invasive techniques have been introduced to assess macrovascular and microvascular endothelial function in peripheral arteries in response to reactive hyperemia [13].

1.6.1 Non-invasive assessment

The most common non-invasive method is flow mediated dilation (FMD), ultrasound based method [96], which reflects conduit artery vasodilation. Assessment of vascular function with FMD is the gold standard technique [96]. It measures the diameter changes in diameter of brachial artery (macro vascular beds) at rest and post reactive hyperemia [33]. The most common advantage of this method is that it measures the diameter changes of the brachial arteries response and is mainly dependent on endothelial NO release. FMD has been widely studied in clinical research, and has been correlated significantly with coronary artery endothelial dysfunction, and Framingham risk score [78]. Also, studies have demonstrated that impaired FMD has been associated with the presence of traditional cardiovascular risk factors [78]. However, this technique requires expensive equipment, is highly operator dependent, and needs an expert sonographer [97]. These methodological challenges may limit its application in research field and clinical practice [78].

Therefore, another simpler non- invasive technique has been developed and received greater attention recently [13]. The protocol of this technique is based on the same physiological mechanism as FMD called reactive hyperemia peripheral artery tonometry (RH-PAT) [97]. The assessment of endothelial function with PAT measurement began in early 2000 to measure vascular changes in subjects with obstructive sleep apnea, and then applied to vascular testing for cardiovascular research [95, 98]. RH-PAT is intended for detection of ED as measured by using the EndoPAT 2000 device (Itamar Medical Ltd, Caesarea, Israel) [99]. The main advantages of this system has encouraged researchers to use this technique in clinical trials are that it is operator independent, less expensive, automated analysis, and easily performed[99]. With this method, finger plethysmography records digital pulse wave amplitude (PWA) changes in the arterioles (microvascular beds) of the finger at rest and after reactive hyperemia to calculate the reactive

hyperemia index (RHI) [95, 97]. This RH-PAT method was chosen in this dissertation work and additional details describing endoPAT technique is provided in chapter 2.

1.6.2 Clinical significance of RH-PAT measurements

Similar to FMD, validation studies have demonstrated that impairment in peripheral finger endothelial function, which is translated into a reduction of RH-PAT ratio, was associated with cardiovascular risk factors including diabetes, smoking, overweight, low HDL, and high lipoprotein cholesterol [97, 100]. More importantly, PAT like FMD can predict independently the expression of cardiovascular disease events and detect the effect of disease on endothelial function [95] as shown in aged subjects with low risk of CVD [101] and healthy older adults [102].

Data describing the correlation between PAT and FMD is contradictory [97]. In spite of conflicting results that exist in the literature describing the correlation between these two measurements, the largest two cohort studies, Framingham heart study and Gutenberg heart study, have demonstrated a modest correlation between PAT and FMD [78, 103]. Both studies assessed the relation of brachial and digital measures of endothelial function with traditional cardiovascular risk factors. Each study recruited about 5000 subjects and demonstrated that PAT ratio as well as FMD was reduced when increasing the following factors: body mass index (BMI), fasting glucose, and lipid profile [95]. However, different responses of PAT and FMD have been observed. Furthermore, an attenuated PAT ratio was not associated with hypertension and inflammation whereas a reduced FMD was inversely associated with systolic blood pressure [103]. Moreover, while a reduced FMD was associated with increasing age as shown in the Framingham heart study [104, 105], the RH-PAT ratio was found to be positively associated with age [95]. This lack of correlation might be explained, in part, by the use of different measurement sites of the cuff position (Forearm rather versus upper arm). FMD performed with upper arm occlusion noted a better correlation than forearm compared with that of shown in previous studies. Another explanation could be that the different physiological mechanisms underlying the response of reactive hyperemia between these techniques [97]. The use of either FMD or RH-PAT comes with both strengths and weakness. Most of the clinical studies described that the FMD approach in conduit arteries is principally mediated by endothelium-derived nitric oxide in response to reactive hyperemia [106]. According to microvascular reactive hyperemia (PAT) and NO bioavailability, few studies investigated the important role of NO in regulation of digital RH in humans [98, 107-109]. However, number of studies reported that PAT responses is partly dependent on nitric oxide, and there is a possibility that other mechanisms could affect PAT results than strict NO dependent vasodilation [78, 97, 110, 111]. Thus, digital PAT is a promising approach in evaluating endothelial function but it may not be interchangeable to FMD [98, 101]. Therefore, additional studies are

required to evaluate the relationship of the PAT response to NO following therapeutic interventions that target NO pathway [98].

Reproducibility is one of the main concerns investigators should take into account when compared specific testing technique is employed [78]. Although data describing the test-retest reliability of PAT examinations are scarce [99, 112], published studies have reported a good reliability of PAT in healthy and metabolic syndrome samples. Two PAT measures across intervals greater than 1 week (mean interval of 19.5 ± 6.2 days), powered at 0.90 were highly repeatable of a relatively small sample size (15-30) [99]. Likewise, five repetitive PAT measures conducted 1 week a part for 20 patients with metabolic syndrome showed robust repeatability with intra-class correlation of 0.74 [101]. In addition, the between- day variability was similar (11%) between PAT and FMD measurements when performed within an interval of 2 days under same circumstances in a small study included 18 male patients with CAD [113].Thus, we can conclude that RHI has a good reliability for separate day measurements [113].

1.6.3 PAT response to dietary interventions

Current evidence in cardiovascular research and prevention of CVD is not sufficient to evaluate the utility of PAT measure to assess therapeutic interventions in clinical settings [78, 95]. Only few studies were published about the reversibility of PAT following therapeutic interventions [98] and yielded mixed results [107, 114-116]. In a RH-PAT study conducted by Kim and colleagues (2011), the effects of lycopene on markers of oxidative stress and endothelial function were examined [114]. In this study, 126 healthy men with impaired endothelial function, who frequently smoked cigarettes or consumed alcohol, were placed into three groups: 39 placebo, 41 on 6mg of lycopene supplement, and 41 on 15 mg of lycopene supplement. The intervention period was 8 weeks. Results revealed that RH-PAT index was increased from baseline by 23% (1.45 ± 0.09 vs 1.79 ± 0.12; $p < 0.05$) compared to the other groups after treatment, suggesting that RHI significantly responded to the dietary intervention [114]. Another study done by Kwak et al. (2012) looked at the effect of rice containing resistant starch on markers of endothelial function and oxidative stress using RH-PAT [116] . This study included 90 patients with prediabetes or newly diagnosed with type 2 diabetes. Patients were randomly assigned to two groups: either a group consuming rice containing 6.51 g resistant starch or a control rice group. The intervention period was 4 weeks. Results showed significant increases in serum NO levels and RH-PAT index from baseline in the interventional group compared to control group. The authors found positive correlation between serum total NO, RH-PAT, and superoxide dismutase (SOD) activity. Similarly, Nogueria et al, (2012) showed a significant improvement on RH-PAT post 4 weeks of 50 g high-polyphenol dark chocolate supplement in overweight and obese stage 1 hypertensive subjects with

no significant changes in lipid profile [115]. Changes in RH-PAT index over the 4-week of dietary treatment suggesting a good relationship between PAT response, and dietary intervention. Further research is needed to examine if PAT signals can be a surrogate endpoint for monitoring additional dietary supplement interventions targeting NO such as dietary nitrate or vitamin C, particulary if NO is the primary objective for investigation [98].

1.7 Beneficial dietary approaches for CVD

Over the past few decades, a number of studies have emerged describing the association between dietary patterns and metabolic cardiovascular risk factors. The main beneficiary dietary approaches for CVD is a diet rich in fruits and vegetables [117]. Mediterranean and vegetarian diets are associated with lower blood pressure and further reduced incidence of CVD in different populations [83, 118, 119]. Other studies have tested Dietary Approaches to Stop Hypertension (DASH) diet and supported the thesis that diet rich in fruits and vegetables lower blood pressure [120].

While the consumption of fruits and vegetables are well- known for the beneficial effect on cardiovascular health, the key dietary constituent for this effect is not established yet [83]. Possible contributors include antioxidant vitamins like vitamin C and E [121], potassium [122], fiber, bioactive phytochemicals such as polyphenol [117], and other bioactive components that are not classified as nutrients but have protective cardiovascular effects known as dietary nitrate [123]. Much research interest was focused on the effect of antioxidant vitamins, however large scale clinical trials failed to suggest that isolated supplements of antioxidant vitamins are the key mediators for cardiovascular benefits [83]. Additional large cohort studies conducted by Willet and colleagues have suggested that diet rich in green leafy vegetables have more potentially bioactive compounds than a single isolated dietary supplement constituent and thus provide a greatest protection against cardiovascular mortality and morbidity [83]. Therefore, researchers have analyzed different types of food to figure out the exact mechanism underlying the green leafy vegetables effect on cardiovascular health [124]. They have demonstrated that the highest component of green leafy vegetables is dietary nitrate with estimates indicating up to 20mmol of dietary nitrate per day [124]. Scientific research has grown exponentially in recent years due to compelling evidence of human studies showing that high levels of nitrate and nitrite of the vegetables provide favorable cardiovascular effects and could confer the lowering BP effect of green leafy vegetables on CV system [124-126]. These include enhancement of NO bioavailability in vasculature and vasodilation [124]. Theoretically, studies suggested that dietary nitrate is biologically active when it is bio-activated with agents such as vitamin C to release the vasodilator NO [127]. Despite of all these health benefits, research about nitrate supplementation in humans is relatively new [22]. In the following sections, the potential therapeutic roles of both dietary nitrate

and vitamin C in cardiovascular risk markers including NO signaling in endothelium, oxidative stress, and lipids will be discussed with more detail.

1.7.1 Dietary nitrate and nitrite

Dietary nitrate is mainly obtained from plant-based sources [123, 124]. Eighty-five percent of dietary nitrate is derived from green leafy vegetables such as arugula, spinach, lettuce, and kale as well as beetroot, and the remainder from drinking water (the amount permitted in the US is 44 mg nitrate/liter) [128]. There are environmental factors that may affect the concentration of nitrate content of vegetables from batch to batch such as temperature, fertilizer use, and sunlight exposure [126]. Beetroot juice is a rich natural source of dietary nitrate and the most common source used frequently in most human studies [126]. Dietary nitrite intake, on the other hand, comes from mainly animal-based products in which nitrite used as food preservatives in processed food, and cured meat along with processed grain and cereal products [127, 128].

In the past, it was believed that nitrate and nitrite content in vegetables was harmful to human beings because of an increased risk for cancer and methemoglobinemia. Animal and epidemiological studies in 1970s and 1980s have shown that high intake of dietary nitrate was associated to the formation of the carcinogenesis compound called nitrosamine in the stomach [126] that acts as a precursor for the development of cancer. Therefore, the dosage was strictly limited. The World Health Organization (WHO) Expert Committee on Food Additives and the European Food Safety Authority (EFSA) set the acceptable daily intake limit of dietary nitrate to 3.7 mg/kg; this is equivalent to 4.2 mmol for a 70-kg adult [129] [83]. However, it was pointed out that people who consume DASH or vegetarian diet that is rich in nitrate in which the dosage of nitrate consumption would result in 1222 mg [130]. This dosage exceeded the WHO acceptable daily intake by five times, and have been associated with lower risk of cancers [124, 131]. In regards to the studies showing a link between nitrate and nitrite intake in red meat and increased risk of gastric or colorectal cancer, most of these associations were relatively weak <2 [128]. A recent study in 2014 by Dellavalle et al, suggested that colon cancer development is associated with reduced antioxidant levels (vitamin C) and not related to high nitrate levels [126, 132]. The WHO Expert Committee concluded that epidemiological and human evidence have failed to show any link between increasing consumption of nitrate intake and cancer development [133]. Dietary nitrate and nitrite naturally interact with the essential nutrients in fruits and vegetables such as vitamin C and polyphenols to inhibit the nitrosamine effect [118]. In methemoglobenemia (also referred to as “baby blue syndrome”), nitrite reacts with hemoglobin and oxidizes ferrous iron in the heme to ferric state to form methemoglobin, and then no longer is able to bind with oxygen [126] . Methemoglobenemia was previously observed in infants due to their immature GI enzymatic

system and the low gastric acid production capacity. Later on, studies suggested that high nitrite was not the cause for methemoglobenemia but fecal bacteria in the well water mixed with infant formula was responsible for gut infection and produced NO,which further converted the hemoglobin to myoglobin in infants [118, 126]. Recent studies have clarified the safety of prolonged intravenous infusion of nitrite in healthy individuals [126]. Thus, evidence about the potential toxicity of dietary nitrate and nitrite was overstated [134]. In addition, it was approved that dietary nitrate beneficial effects outweigh any risks [133].

In the early 20th century, Chinese medicine used inorganic nitrate to treat hypertension, while the providers were unaware of the exact mechanism underlying the nitrate effect [83]. Recently, cardiovascular research studies are focusing on the mechanistic effects of this component among various populations. Interestingly, several primary studies supporting the view of plasma levels of bioactive nitrogen oxides (NOx) that were increased after NO_3 load, and were associated with potential cardioprotective effects [129]. Some examples of inorganic nitrate benefits apart from sparing NO signaling and vasodilator actions are improving endothelial function, inhibiting platelet aggregation, reducing oxidative stress and improving mitochondrial efficiency, and subsequently, improving cardiovascular health [135].

1.7.2 Pharmacokinetics of nitrate and nitrite

Nitrate and nitrite originate from two sources, endogenous (NO oxidation) and exogenous (dietary nitrate) [126]. Administration of dietary NO precursors (independent of NOS pathway) rely on sequential reduction of inorganic nitrate to nitrite and then NO to ensure that blood and tissue levels of NO and its metabolites are sufficient to mediate NO physiological functions [127, 136]. Total plasma NO availability reflects the sum of NO generated by endogenous and exogenous NO pathways. However, measuring NO levels is inconvenient because the half-life of this molecule is very short (i.e. milliseconds) [24]. Under normal and physiological PH and oxygen environment, NO is rapidly autoxidized in vivo to form stable compounds, nitrite and nitrate. The average plasma concentrations of nitrate and nitrite in healthy individuals is (0.01-0.6 µm), and (20-40 µm) respectively [128, 136]. Accumulating evidence supports that the half-life of plasma nitrate is 5-6 hour [126, 137] while the half-life of plasma nitrite is about 1-5 minutes [124]. Thereby, plasma nitrite plus nitrate (NOx) have been used as a surrogate biomarker to represent NO bioavailability for cardiovascular health and other diseases [18]. It is worth noting that plasma and tissue concentrations of nitrite and nitrate are inversely associated with cardiovascular risk load including age, hypercholesterolemia, hypertension, and other conditions known to reduce NO bioavailability [18].

Additionally, plasma levels of nitrate and nitrite are strongly influenced by changes in dietary NOx intake [138]. Studies show that the peak in plasma nitrate and nitrite is dose dependent [126, 139]. Bolus administration of dietary nitrate in various concentrations increases the circulatory markers of NO production dramatically within 25-30 minutes. As an example, 24, 12, and 4 mmol of potassium nitrate supplementation increased the endogenous levels of nitrate by 35, 27, and 7-fold, respectively. Also, plasma nitrite concentrations were raised by 4, 2, and 1.3 fold, respectively [140]. Furthermore, sources of dietary nitrate impact pharmacokinetics of plasma nitrate [141]. Potassium nitrate supplementation reaches NO_3 peak (~1 hr) faster than beetroot juice (~2-3hr) at a similar dosing load, and it sustained higher than baseline levels for about 24 hours after the last nitrate dose ingestion [142]. Nevertheless, the plasma nitrite attained its peak slower than nitrate, after approximately 3 hours post ingestion of dietary nitrate [141].

1.7.3 Exogenous pathway to produce NO (nitrate-nitrite-NO)

There are two sequential reductions required to convert nitrate to NO via exogenous pathway. First, reduction of NO_3 to NO_2: following an oral nitrate load, dietary nitrate is quickly absorbed from the upper gastrointestinal tract into the blood within 30 minutes of consumption, peaking after 1 hour [142]. Approximately 75% of the absorbed nitrate excretes in urine while approximately 25% of the plasma nitrates are taken up by the saliva and concentrated10-20-fold where it reduced to yield nitrite by the presence of oral commensal bacteria [128]. Certain oral anaerobic bacteria possess nitrate reductase enzyme to reduce 20% of nitrate to nitrite in saliva under hypoxic condition over a few hours [22]. Recent studies have provided evidence about the important role of nitrate reducing capacity of oral bacteria [143, 144]. Mouthwash or overuse of antibiotics that removes commensal bacteria could disrupt the exogenous NO production [20]. Moreover, Kapil et al. examined the effect of mouthwash treatment for 7 days on healthy individuals, and demonstrated a reduction in oral nitrite production by 90% with a decrease in plasma nitrite concentration by 25%, and thereby this reduction is associated with an increase in blood pressure [143]. These data suggest that oral bacteria is a primary factor for nitrate reduction to nitrite through this pathway, and thereby contributes to the systemic levels of nitrites. Second, reduction of NO_2 to NO: when swallowed, much of saliva nitrite (pKa 3.4) reaches the stomach and gets rapidly protonated to form nitrous acid [20, 145]. Subsequently, nitrous acid is further reduced to release large quantities of nitric oxide and other oxides of nitrogen [20]. NO production is pH dependent, therefore, low gastric pH is the second important factor for nitrite derived NO production. Gastric NO formation could be detected in expelled gastric air of healthy volunteers post NO_3 –rich lettuce ingestion and were significantly higher than those found in orally exhaled air [146]. In addition, pretreatment with antacid drugs called proton pump inhibitors (PPI) reduced gastric NO formation by 95% [146].

Some evidence have shown that long-term use of potent antacids medication such as proton pump inhibitor may abolish the cardiovascular benefits of dietary nitrate because it could prevent NO production [20]. Overall, the main two factors in this exogenous pathway to produce NO are oral bacterial and gastric acidic environment. Under hypoxic and acidic condition, unlike the NOS dependent pathway, investigators have noticed that this non-enzymatic pathway serves as a backup system for NO generation and works more efficient under these conditions in which the classical eNOS dependent pathway is dysfunctional/compromised due to NO scavenging by ROS with the presence of cardiovascular risk factors [24, 124].

However, it is now known that not all produced nitrite in the gut are converted to NO [23]. Some nitrite transport from the gut to the systemic circulation by unknown mechanism of how it across the gastric wall [147]. In tissues and circulation, there are numerous enzymatic and non-enzymatic pathways enhance the bioconversion of nitrite to NO and it is well –recognized that these agents participate in acidic and hypoxia conditions [135]. These pathways include xanthine oxidoreductase, carbonic anhydrase, aldehyde oxidase, polyphenol, and vitamin C. In the presence of vitamin C in gastric environment, additional reactions will occur between nitrite and this potent reducing agent to generate NO that can diffuse across the stomach wall and induce local muscle relaxation [148]. Published data about the relative contribution of these bioactive agents to generate NO is scarce [128].

NO_2+H+ HNO_2 (pKa 3.2-3.4)

$2HNO_2$ + vitamin C $\longrightarrow$ 2NO + $2H_2O$+ dehydroASC

Figure 1-1. Reactions of nitrite reactions

1.7.4 Therapeutic effect of dietary nitrate

a. Dietary nitrate has antioxidant effects

Some studies suggest that dietary nitrate supplementation target oxidative stress [149, 150]. Animal studies have promising findings about the dietary nitrate effect on different well established oxidative stress markers of rats with induced hypertension [151]and renal ischemia reperfusion rats [150]. Nitrate treatments increased the tissue levels of NOx and decreased the levels of oxidative stress markers including (malondialdehyde in plasma) and (F2-isoprostanes and 8-hydroxy-2-deoxyguanosinein in urine) [151] and (NADPH oxidase-mediated O2•− formation in bone marrow-derived macrophages) [150]. In agreement with these results, in vitro examination of NO_2 concentrations (12.5-200µmol/l) prevented myeloperoxide -mediated LDL modification in the absence of chloride [152]. Therefore, based on these findings, it could be speculated that dietary nitrate possesses antioxidant properties to reduce ROS generation in humans, and this property could contribute to preserved NO production from ROS scavengers [149].

However, scarce data are available demonstrating reduced vascular oxidative stress in human studies during dietary nitrate supplementation with controversial findings. For example, Vulmerurgan et al. (2015) recently found a trend for a greater reduction of oxLDL levels after administering 250 ml of beetroot juice (~6 mmol) at 6 weeks in untreated hypercholesterolemia [74]. Likewise, Ashor et al., (2016 &2019) examined the acute effect of dietary nitrate on endothelial function and oxidative stress markers in two studies for two different populations young and older adults with acute hyperglycemia [153], and healthy older adults [154], respectively. In the former study, results found that single dose of nitrate supplementation significantly increased plasma concentrations of NO metabolites (nitrate and nitrite) and reduced 3- nitrotyrosine as an index of oxidative stress (3NT) in older obese adults with acute hyperglycemia compared to younger adults. Contradictory to the previous findings, in the latter study, 3NT concentrations were not significantly reduced post dietary nitrate supplementation alone. In addition, Larsen et al. (2014) reported no changes on plasma oxidative stress biomarkers such as plasma isoprostanes F_2 following administration of inorganic nitrate supplementation for 3 days [155]. Collectively, most of these results have been observed with short duration studies of only 1- 3 days intervention and in healthy individuals without evidence of oxidative stress, which may make the observation of antioxidant properties of dietary nitrate less likely. Many other factors could contribute to this contradiction between these studies including dose and duration of nitrate therapy, population characteristics, and the use of different oxidative stress biomarkers. Further explorations are required to examine whether the use of dietary nitrate as a potential antioxidant source would reduce oxidative stress in hypercholesterolemia [74].

b. Dietary nitrate improves lipid profile

Preclinical studies have shown that inorganic nitrate and nitrite have potential to improve dyslipidemia by an unknown mechanism [156-158]. One group investigated the effect of sodium nitrite administration on C57Bl/6J mice after they fed them with high cholesterol diet for 3 weeks to induce ED and microvascular inflammation. The addition of sodium nitrate to drinking water did not change plasma total cholesterol (TC) levels but it reduced triglycerides (TG) levels compared to control mice [156]. Another group demonstrated that administration of dietary nitrate treatment (0.1 mmol/kg) for 7 weeks reduced visceral fat accumulation and TG levels in aged eNOS knock out mice with metabolic syndrome compared to untreated mice [157]. In addition, Khalifi et al. (2015) observed a significant improvement in glucose tolerance and the levels of TC, LDL, and TG by 22.9%, 54.2%, and 47.6% after nitrate treatment compared to control diabetic rats [149]. These findings support the nutrition-based strategies of oral nitrate ingestion against CVD in animal models [157]. In human studies, a small clinical study enrolled thirty subjects older than 40 years

with high plasma TG levels of >150 mg/dL and at least two cardiovascular risk factors. Findings showed a significant increase in plasma nitrate and nitrites, and reduction in elevated TG levels by 27% after 30 days of a unique formula of nitrate supplementation called Neo40 on empty stomach twice a day compared to pre-treatment levels [159]. However, evidence to support beneficial effects in humans with hyperlipidemia is lacking, and further human studies will be worthwhile [74] to assess whether dietary nitrate with or without other dietary supplements could improve the lipid biomarkers [159].

c. Nitrate as a NO sparing effect to restore endothelial function

In the last two decades, evidence has accumulated to support that ED is responsive to dietary interventions and can be partially restored following acute and chronic administration of diet-rich inorganic nitrate and/or nitrite [136, 160]. The mechanism underlying vasodilation response of nitrate and nitrate is yet to be fully elucidated [128]. The study duration for most published studies, that have examined the efficacy of inorganic $NO3^-$ and beetroot supplementation on endothelial function, was generally short ranged from 2 hours to 42 days. The majority of acute studies (1.5-4 hours) observed a significant improvement in endothelial function as measured by FMD [161-164]. In chronic setting, four published studies examined the nitrate intake for a duration of 2-6 weeks. Results showed an improvements in FMD in three different populations [74, 140, 165] with no effect was seen in individuals with type 2 diabetes[166].

Concerning nitrate benefits on vascular function among the aged population, nitrate supplementation has yielded mixed results in older adults who have an age-related decrease in NO and higher risk of cardiovascular disease. some studies have seen vascular responsiveness to chronic dietary nitrate intake in its various forms (4.5-6 mmol) and were not affected by age in the absence of obesity (body mass index ≤30 kg/m2) [140, 165]. Kapil et al. (2015) considered sixty-eight hypertensive patients ages 18-85 years, stratified to drug naïve and treated patients [140]. They were randomly assigned to nitrate or placebo group. Researchers found an increase in plasma nitrate and nitrite concentrations by 5.5 and 2.7 fold respectively, and an increase of plasma cGMP in both treated and untreated patients. These elevations were associated with reduction in BP, and 20% improvement in endothelial function as measured by FMD in treated hypertensive patients following ingestion of 250 ml beetroot juice (~6.4 mmol daily for 4 weeks compared with nitrate depleted beetroot juice (0.007 mmol nitrate). The elevations of plasma NOx were returned to baseline level after a 2-week of post-treatment. In line with this study, Rammos et al. (2014) looked at eleven normal and overweight elderly volunteers (average age = 63 years) with moderate cardiovascular risk [165]. They found that 4 weeks of sodium nitrate (~11.3 mmol of nitrate) consumption resulted in a modest increase in FMD of 0.5%, and thus an improvement in endothelial

function and reduction in systolic BP in the nitrate intervention group compared to placebo. Velmurugan and colleagues (2015) conducted a novel clinical randomized parallel 6-week study investigating the effects of inorganic NO_3^- (~6 mmol nitrate) in the form of beetroot juice on BP and endothelial function in untreated hypercholesterolemia patients between 18-80 years old with BMI range from 18.5 to 40 kg/m_2[74]. Compared to the control group, the beetroot juice group showed greater plasma concentrations of NO. Of note, the dietary nitrate group demonstrated absolute enhanced blood flow as measured by FMD, and significant reduction in the platelet aggregation. In this study, the authors stated that the improvements were related to the duration of treatment, not to the dosage. This evidence of this study contradicts the analysis of a systematic review in 2014 reporting that greater vascular effect was observed with higher nitrate doses [160]. In these studies, the population of interest exhibited at least one cardiovascular risk factors besides aging, which might suggest the ability to translate these findings to population at risk of CVD with NO insufficiency.

Conversely, some evidence suggests that vascular responsiveness in older adults (>52 years old) is less sensitive to dietary nitrate compared to younger individuals [167]. In 2013, Gilchrist and colleagues demonstrated that 14 days of supplementation with beetroot juice (250 ml, 7.5mmol NO_3^-) in older adults with obesity and type 2 diabetes had no significant effect on FMD or Doppler perfusion and therefore did not improve endothelial function [166]. Further, two trials by Ashor et al, assessed endothelial function using laser Doppler Flowmetry (LDF) in aged subjects after nitrate consumption. In 2015, Ashor et al. administered a 70 ml concentrated beetroot juice with nitrate concentrations of ~300-400 mg for three weeks among overweight and obese aged adults with an age of 55-67 years. No benefit of daily dietary nitrate was evident in aged-obese adults compared to placebo group [168]. Subsequently in 2016, Ashor et al., designed another study to examine the acute effect of potassium nitrate intervention (7mg/kg of KNO_3) versus its placebo in 10 young and 10 old obese adults with an age range of 18-70 years old and BMI range of 30-40kg/m_2 with acute hyperglycemia [153]. The washout period was seven days between the two interventions. Findings demonstrated no changes in microvascular blood flow in both groups during acute hyperglycemia. A better improvement response in plasma NOx was observed in young adults (31.4 ±1.8 years) compared to older subjects (64 ± 1.3 years) after the ingestion of inorganic nitrate, while a significant reduction in oxidative stress biomarkers was shown in older adults. The aforementioned studies suggest that obesity and vascular aging may independently modify the reduction of dietary nitrate effect. Some investigators have explained age-related factors that could possibly be the reasons behind no effect of nitrate supplementation on vascular health are the following: 1) high body mass index (>30 kg/m2); 2) low production of gastric acid, or low absorption of vitamin C

that may affect NO formation; 3) reduction of oral microflora that may influence the conversion of nitrate to nitrite; and/or 4) low sensitivity of vascular smooth muscle cells to the vasodilator NO [167, 169]. To conclude, it is currently unknown whether a larger dose and/or a longer duration of inorganic nitrate supplementation or both are necessary to have an effect in regressing ROS biomarkers and amplifying NO bioavailability to augment cardiovascular effects in older adults with underlying cardiovascular diseases such as hypercholesterolemia. Further research is needed to determine the effective dosage and duration of beetroot juice for preserving physiological function and optimal health for population with advanced aging and at risk of CVD [167]. Furthermore, identifying novel dietary nitrate strategies that might improve the oxidative stress and ED associated hypercholesterolemia in older adults are required [71].

1.7.5 Introduction to vitamin C (ascorbic acid)

Vitamin C known as L-ascorbic acid is an essential nutrient that consists of six-carbon compounds [29]. Ascorbic acid is a water-soluble vitamin that human being cannot synthesize it endogenously due to lack of enzyme L-glunolactone oxidase [170]. Therefore, vitamin C has to be obtained from diet or dietary supplement as tablet or powder. In the diet, it is naturally present in various plant-based food sources. Some examples of the vitamin C rich foods are papaya, green and red pepper, citrus fruit, cantaloupe, berries, and green leafy vegetables like broccoli, coriander [171]. Ascorbic acid plays a crucial role in many biological functions. One of the important roles is scavenging free radicals (antioxidant) [172]. Ascorbic acid can be reduced to ascorbate radical when it loses the first electron, which has a short half-life ranging with seconds to minutes, and then to dehydroacsorbic acid (DHA) after losing the second electron. Both forms are reversibly converted back to ascorbic acid [173]. Apart from the antioxidant activity, other benefits of vitamin C include serving as a mediator in collagen synthesis, and in protein metabolism, NO restoration, vitamin E regeneration, and other benefits that may mitigate the development of AS such as decreasing the LDL oxidation, and proliferation of vascular smooth muscle cells [174].

The recommended daily allowance (RDA) for adults are 75mg/day for women and 90 mg/day for men with an upper tolerable limit of 1-2 g/day for both [175]. The normal plasma concentration of vitamin C is typically range between 30-60 μM [176]. Plasma vitamin C concentrations between 11-28 μmol/L defined as inadequate or marginal vitamin C deficiency, while the levels < 11 μmol/L determined as sever deficiency. However, plasma vitamin C levels is not a dose dependent as dietary nitrate. At moderate intake of 30-180 mg/day up to 200mg/day, the intestine absorbs approximately 70-90 % of vitamin C [177]. An intake of 200mg/day of vitamin C is enough to raise plasma concentration of 70-80 μmol/L [175]. However, at very high dose of up to 3 g/day of vitamin C, the absorption declines significantly to less than 50% [177]. Results

from pharmacokinetics studies have shown that a dose of 1.25 g/day ascorbic acid is sufficient to raise the plasma level to 135 micromol/L [172].

Some observational studies suggest that western diet lacks of vitamin C [124, 178]. Data from 2003-2004 from National Health and Nutrition Examination Survey (NHANES) showed that numerous U.S. residents under-consumed vitamin C. About 10% of US adults consumed less than 30 mg per day of vitamin C, and suffer from vitamin C deficiency with a plasma level of <11 µmol/l [29], and 20% of American populations have marginal vitamin C deficiency [175]. Some studies have demonstrated that aging is associated with vitamin C deficiency[179]. The plasma concentration of vitamin C of older adults >65 years is 25% less than younger adults [180]. Reduced bioavailability with aging is attributed to low absorptive capacity for vitamin C, low vitamin C intake, or excessive oxidative stress [180]. Nevertheless, many studies have since looked at the favorable outcomes of high vitamin C intake above the RDA throughout the body [181]. The effect of vitamin C on oxidative stress, lipid profile, and endothelial function are discussed below.

1.7.6 Therapeutic effect of vitamin C

a. Vitamin C has antioxidant effects

Vitamin C has been documented to have powerful antioxidant activity [182]. In early animal studies, animal models of AS have demonstrated beneficial effect of antioxidant vitamins administration [183]. Results from animal studies have been correlated with delayed AS progression. These data support the antioxidant hypothesis that dietary supplementation with antioxidant vitamins have effect in reducing risk of CHD [183-185].

However, in human studies, abundant data from epidemiologic observational studies as well as randomized clinical trials have failed to provide clear-cut indications for antioxidant supplementation to combat CVD due to equivocal data [183, 186, 187]. There are extensive epidemiological studies that have shown that administration of antioxidant supplements such as vitamin C [188, 189], vitamin E, and/or beta-carotene have positive effect on cardiovascular event reduction as shown in the Cambridge heart study [190] and Nurses' Health study [191]. While the others failed to confirm this beneficial effect on cardiovascular outcomes as shown in Women's Health Study [192] and the Physicians' Health Study II [193]. By contrast, randomized clinical trials (RCTs) have not established the effect of vitamin C intake on CVD risk [25, 194].

Some researchers have explained the reason behind the discrepancy of the results of antioxidant vitamins like vitamin C between trials. They suggested possible limitations that might reduce the effectiveness of antioxidant vitamins over these studies include differences in subjects' characteristics, the selection dose of antioxidants, and the status of endogenous antioxidant defense system [183, 186, 195]. For example, healthy individuals are less likely to respond because of their

normal nutritional status. Another example for low-responders is smokers who they might require a higher dose of antioxidant vitamin C than others to get rid of the greater oxidative stress in their body [25]. other limitation is the failure to measure plasma vitamin C at baseline and after treatment in these studies [196]. In addition, the lack of true placebo in diet-related vitamin C studies [195]. Subjects taking placebo may continue to consume a high vitamin C diet through the study period [195]. Subgroup and meta-regression analyses have recently noted that certain population who experience a pro-oxidant state due to the high levels of oxidative stress may benefit more from vitamin C supplementation. For example, older adults, obese, patients with higher risk of CVD [197], and who have depletion of endogenous antioxidant (e.g. vitamin C deficiency) [25, 188]. Future clinical trials should take these limitations into consideration to achieve greater physiological response to vitamin C [29].

Moreover, it has been shown that vitamin C administration is effective in attenuating LDL oxidation. This fact is supported by in vitro studies. Shariat al., (2013) examined the inhibitory effect of vitamin C by incubating the LDL with 2 mg/ml MPO in the absence and the presence of vitamin C concentration between 50-150mM [198]. Resulted showed that vitamin C inhibited the LDL oxidation mediated by myeloperoxidase and increased the lag time of LDL oxidation up to 2.4 times. Another example of in vitro studies reported that 100 g of cranberries, which has the equivalent antioxidant capacity to 1012 mg of vitamin C, resists LDL oxidation [89]. Moreover, Das et al., (2006) used different concentrations of vitamin C, 40mMol/L and 80mMol/L, to modulate in vitro oxidation of LDL that are isolated from normal and hypercholesterolemic subjects[199]. Results showed that higher concentration of vitamin C was required to reduce LDL oxidation level from LDL derived from hypercholesterolemic subjects compared to isolated LDL from normocholesterolemic subjects. To our knoweledge, most of the aformentioned studies tested the susceptility of LDL oxidation in vitro however, few studies were published the vitamin C supplementation effect on circulating oxidized LDL in vivo [200, 201]. Therefore, the development of novel strategies to effectively reduce oxLDL itself in vivo is highly anticipated [87].

b. Vitamin C improves blood lipid profile

Vitamin C supplementation has been shown to be associated with more favorable lipid profiles in human observational studies [202]. However, conflicting results were observed in the RCTs of vitamin C supplementation on lipid profile [203, 204]. In regard to the mechanisms of action, vitamin C acts as a cofactor for the enzyme 7-a-hydroxylase to facilitate the conversion of cholesterol to bile acids. This conversion may enhance the expression of LDL receptors to increase the uptake of plasma LDL concentration, thereby, lower LDL in circulation [202]. Another possible

mechanism to reduce cholesterol concentration is related to the antioxidant protection of vitamin C [205]. Vitamin C supplementation causes inhibition of LDL oxidation, which eventually increases the LDL uptake by liver and lowers LDL concentration in blood [205]. Two meta-analysis studies have looked at the effects of vitamin C on lipids [202, 206]. The first analysis was performed of 13 trials investigated the effectiveness of vitamin C on blood lipids including total cholesterol, LDL, high density lipoprotein (HDL), and TG in adult subjects with hypercholesterolemia. The minimum dose of vitamin C supplementation was 500mg/day for duration of at least 3 weeks. Following vitamin C administration, results have shown a significant reduction in both LDL and TG levels with no significant increase on HDL levels [202].

On the contrary, another meta-analysis study of 40 trials failed to show significant effect of vitamin C supplementation on altering lipid profiles [206]. The duration of vitamin C intervention in the included studies were > 2 weeks. However, the reduction in cholesterol levels was observed only in specific subgroups including younger (< 52 years) adults, subjects with higher concentrations of lipids or lower vitamin C at baseline, or individuals at higher CVD risk (e.g. those with hypercholesterolemia or dyslipidemia). Because of this contradictory results, further studies are warranted to draw conclusions about the effectiveness of vitamin C on hypercholesterolemia condition.

c. *Vitamin C has NO sparing effect on endothelial function*

Vitamin C has been established as a great contributor for the maintenance of the systemic NO pool [207]. It is thought that ascorbate plays a beneficial role in endothelial vasodilator dysfunction through its ability to decrease oxidative stress biomarkers and spare NO availability [207]. Several proposed mechanisms linked with vitamin C modulate vascular tone. These mechanisms are 1) scavenging free radical and ROS which prevents them from reacting with NO to form peroxynitrite, leading to higher bioactive NO levels [207]; 2) upregulating NOS activity in endothelial cells to produce NO through increasing the levels of eNOS cofactor (BH4), inhibiting the enzyme arginase, and consequently increasing the L-arginine bioavailability, and/or inactivating S-nitrosylation, resulting in more blood flow; 3) acting as a cofactor for eNOS in hydroxylation reaction to release NO; 4) decreasing the toxic interaction of oxLDL with endothelial cells; 5) preserving the cGMP activity; and 6) enhancing the reduction of nitrite to NO through the dietary exogenous pathway of NO, and consequently may potentiate vasodilation [24, 25, 42]. Therefore, the antioxidant capabilities and NO probabilities of vitamin C, as shown previously, encourage the investigators to examine its potential vasoprotective effects along with nitrate effects on clinical and circulatory biomarkers of ED pre and post interventions.

However, clinical trials show inconsistent results with oral vitamin C supplementation on endothelial function [25]. Forty-four randomized clinical trials were compared in a systematic review [25]. Data analysis reported that two-thirds of these studies had significant improvement on endothelial function in response to acute vitamin C supplementation alone (<2 weeks). Interestingly, this evidence is limited to older adults and patients with cardio metabolic disorders [25]. Acute supplementation with vitamin C had an effect on the vascular function when given to individuals with Type II Diabetes (1000 mg) for 3 days measured by FMD [208] and to healthy subjects exposed to glucose load to induce ED (1000mg) for 10 days measured by forearm blood flow [209]. Taken together, the current studies published the effect of vitamin C on endothelial function in patients with metabolic syndrome or hypercholestrolemia are few [25]. A metanalysis review of 44 trials found a greater effect of vitamin C on endothelial function was demonestrated in experimental induced ED, and patients with diabetes or heart failure, or AS [188]. However, conflicting results were found in studies that measured the effect of vitamin C administration on endothelial function in hypertensive, smokers, or healthy subjects [210]. Additionally, investigators concluded that the improvement of endothelial function is affected by vitamin C dose and unaffected by the study design, duration, and route of administration [25]. According to vitamin C dose, data from meta-analysis of 9 cohort trials showed that 700 mg/day or higher, which exceeded the RDA, elicited substantial improvements in endothelial function and were associated with a 25% reduction in CHD risk [211].

Moreover, aging was linked strongly to the modifying effect of long-term vitamin C supplementation for more than 2 weeks on endothelial function [188]. Antioxidant vitamin C supplementation profoundly restored ED in older individual (56 years or older), but no benefit was shown in younger (age <56) or healthy individuals with no ED [18, 188]. The explanation for achieving greater benefits of vitamin C supplementation in older adults may be because they were more likely to have inadequate intake of vitamin C, their requirements were higher than young adults in order to remove oxidative stress that resulted from aging, or due to their lower baseline vitamin C status and higher oxidative stress [25, 195]. It has been proposed that systemic or localized ascorbate deficiency and oxidative stress generates ED in various diseases and conditions and is rapidly treatable with ascorbate repletion [207, 212]. Therefore, these data suggests that elevation of plasma vitamin C may restore endothelial function and enhance NO bioavailability [213]. Overall, although the antioxidants therapy of vitamin C and dietary nitrate are attributed to improvement in endothelial function separately, paucity data has been examined the combination effect of these compounds to augment endothelial function among CVD population [22]. Future studies exploring the combined effect of dietry nitrate and vitamin C on vascular function and used

biomarkers to assess patients risk may identify effects of these compounds on cardiovascular health.

1.8 Nitrate-nutrient interaction

As mentioned earlier, when NOS is impaired, NO can be produced via the non-enzymatic NO pathway. One of the factors that is likely increase nitrate effectiveness is the ingestion with additive or synergistic compunds such as polyphenols or antioxidants (e.g. vitamin C) [169]. To our knowledge, there is limited evidence towards the combined effects of deitary nitrate and these compounds on cardiovascular health, particularly, endothelial function. Polyphenol and dietary nitrate as a combination have been studied to look at their ability to augment NO status and endothelial function. In one study, the acute effectiveness of flavonoid as an additive to improve NO status, endothelial function, and blood pressure was tested in randomized controlled crossover study with healthy men and women [162]. Subjects were divided into four groups: receiving control, flavanoid-rich apple, nitrate-rich spinach, apple +spinach (nitrate and flavonoid in combination). The washout period was 1 week. They found that nitrate and flavonoid had lower systolic blood pressure when provided together compared to other groups. However, all treatment groups have higher NO status and FMD compared with control group. Another similar cross-over study examined the interaction between flavanols and dietary nitrate on endothelial function at low and high doses in healthy subjects [161]. Measurments were taken to determine FMD, intragastric NO formation, and plasma NO metabolites. Additive effects were observed when nitrate and flavanol was consumed together on endothelial function. An increase in NO formation in the stomach and a significant improvement in FMD response by 1% was observed 1 hour post-consumption of low intake of flavanols (2.7 mg/kg) in combination with inorganic nitrate (3 mg/kg) compared to higher intake. These data suggest additive effects between dietary nitrate and NO-releasing compounds which may exert beneficial cardiovascular effects.

With regard to the interaction between dietary nitrate and vitamin C, previous research focused on the individual cause- and- effect of dietary nitrate and vitamin C with vascular function improvement and biomarkers of cardiovascular risk [214, 215]. Studies support the notion that vitamin C enhances the efficiency of the enzymatic and non-enzymatic NO pathways as a potential way to preserve endothelial function in patients whom NO insufficiency and high concentration of ROS may be more pronounced [25]. These data support the hypothesis that introducing vitamin C with inorganic nitrate may enhance the formation of NO from dietary nitrate and thus increase the efficiency of NO bioavailability in endothelial cells. Thereby, possibly improve compensatory vasodilation in patients with impaired endothelial function [99, 160]. The possibility that simultaneous consumption of dietary nitrate and vitamin C could have an additive or even a

synergistic effect on cardiovascular health is derived from the observation that both nutritional constituents augment NO production via different mechanisms and from studies showing that vitamin C catalyzes the conversion/reduction of acidified nitrite to NO in the gastric acidic environment [22, 39, 216, 217]. Astudy of Dejam etal. (2007) Supported a role of vitamin C as an intravascular reductant for nitrite-induced vasodilation of the human forearm [218]. They found that vitamin C improved the forearm blood flow modestly by 10% when it co-infused intrabrachially with nitrite copmared to nitrite alone.

Recently in 2017, Ashor et al., demonstrated for the first time the potential of a beneficial synergistic interaction between inorganic NO_3^- (7mg/kg) and vitamin C (20mg/kg) on vascular outcomes. These dosage are corresponded to 150-250 g of nitrate and 1400 mg of vitamin C in 70 kg adult person. Results showed a significant decrease in arterial stifness measured by pulse wave velocity (PWV) (PWV:-2.0 m/s, P=0.02) and mean arterial BP (-2.6 mmHg, P=0.03) following a single dose of dietary nitrate combined with vitamin C at lower doses in healthy aged adult volunteers compared to vitamin C and nitrate alone [154]. However, the interaction of these compounds was not investigated in longer studies nor in vulnerable population with impaired vascular function. Based on this evidence, there is a reason to believe that supplemental vitamin C could have the potential to complement dietary nitrate effects for augmenting the NO pool, and amplifying vascular-protective benefits of dietary nitrate if introduced for longer duration [19]. Its important to take into consideration that the vascular responses to dietary nitrate coupled with vitamin C may not be the same among healthy compared to obese old individuals with higher risk of cardiovascular diseases (hyperlipidemia). Additionally, further studies are warranted to examine the optimal dosing regimen and study duration that maximize beneficial effects, if any, on endothelial function and oxidative stress biomarkers. Moreover, it is interesting to investigate whether the interaction between these could 1) promote addtive interaction resulting in greater increase in NO availability and further reduction in oxidative stress biomarkers, 2) achieve greater effects on markers of CVDs – including endothelial function– than do these dietary agents given individually [216].

1.9 Summary

Endothelial dysfunction and oxLDL are the main two hallmarks involved in the development and progression of AS and are associated with most cardiovascular risk factors including hypercholesterolemia. Hypercholesterolemia associated oxidative stress production and oxLDL have been found to reduce NO bioavailability and then promote ED. ED serves as an independent predictor of CVD and it can be treated by dietary therapeutic interventions. Therefore, studies targeting NO pathway to improve overall vascular health, as monitored by measures of

vascular response to post reactive hyperemia with non-invasive technique, and blood biomarkers should be of great interest. Evidence from clinical studies has suggested a novel nutrition intervention called dietary nitrate that serves to restore NO insufficiency through the nitrate-nitrite-NO pathway, thereby improve vascular health. While most beneficial effects of exogenous nitrate ingestion were mainly found in healthy and hypertensive populations, few have looked at the usage of dietary nitrate for hypercholesterolemia population. Although NO insufficiency is the hallmark feature of ED, it is unknown whether the dietary nitrate intervention for endothelial function with hypertension and healthy individuals will be suitable for other population like hypercholesterolemia. Four weeks of dietary nitrate supplementation were sufficient to produce favorable changes in endothelial function. However, there is no study published yet has examined the dietary nitrate effect on endothelial function using PAT assessment. Studies have shown that the antioxidant vitamin C enhances non-enzymatic conversion of inorganic nitrate to NO. Dietary nitrate and vitamin C are powerful nutrient constituents that have been studied independently in augmenting NO pool, reducing oxidative stress, and even lipid profile, but their combined effect requires further studies to elucidate potential effects on the cardiovascular system. To date, only one recent study of Ashor et al that investigated the acute potential of synergistic effect of dietary nitrate with vitamin C on vascular health in healthy individuals, yet no study has evaluated the efficacy of administering these anions together for longer duration nor in older adults with impaired vascular function. While the impact of dietary nitrate on vascular health is greater in young population, evidence of stronger response to vitamin C intervention > 2 weeks was observed in older adults ≥ 56 years of age and those with higher BMI compared to young adults. Overall, further studies to develop optimal effective dose and duration of dietary nitrate therapy combined with vitamin C could be of great benefit to explore the potential additive effects of these compounds on endothelial function and oxidative stress biomarkers in older adults with cardiovascular risk factors.

1.10 Scope of dissertation

1.10.1 Innovation

This dissertation is the first study examining the effect of dietary nitrate on endothelial function and cardiovascular biomarker with the use of 4 innovative approaches. (1) Combined supplement intervention examines the additive effect of antioxidants and NO properties of dietary nitrate with vitamin C supplementation in normal and obese older adults with impaired endothelial function. (2) Study technique: uses PAT method to examine the sensitivity of PAT hyperemic response to the therapeutic dietary nitrate intervention. The evidence supporting the vascular benefits of dietary nitrate using endoPAT method is understudied. (3) Plasma biomarkers: uses the ratio of oxLDL/NOx to assess ED. The effect of dietary nitrate on this ratio has not been

investigated [60]. (4) Combined clinical parameters with plasma biomarkers: This study assessed endothelial function from two perspective methods, noninvasive method (PAT) with the relevant biomarkers (oxLDL/NOx ratio).

1.10.2 Study purpose

The purpose of this study is to investigate whether adding vitamin C will complement the benefits of dietary nitrate supplementation and yield a robust effect on endothelial function compared to dietary nitrate supplementation alone. We anticipate that the addition of vitamin C will enhance the effects of dietary nitrate, resulting in the augmentation of the cumulative NO pools and inducing a greater improvement in vascular function and reduction in oxidative stress biomarkers as determined by RHI values and oxLDL to NOx ratio.

1.10.3 Study hypothesis

Our central hypothesis is that co-supplementation of nitrate and vitamin C for four weeks will improve blood biomarkers and clinical measures of endothelial vascular function over-time as determined by oxLDL/NOx ratio and RHI, respectively, compared to the provision of dietary nitrate supplementation alone.

1.10.4 Study aims

Specific Aim 1: Examine the combined effects of dietary nitrate and vitamin C supplementation on a clinical biomarker of vascular endothelial function (Peripheral Artery Tonometry) compared to dietary nitrate alone.

Specific Aim 2: Examine the combined effects of dietary nitrate and vitamin C supplementation on plasma biomarkers of oxidative stress and endothelial dysfunction (oxidized LDL/NOx ratio) compared to dietary nitrate alone.

Chapter 2: MATERIALS AND METHODS

2.1 Experiment design

The purpose of this study was to examine the efficacy of beetroot juice, with or without vitamin C supplementation on endothelial function and oxidative stress outcomes in older subjects between 50-70 years old with high cholesterol (> 130 mg/dL), and not on statin medication. This was a randomized; double blinded, placebo-controlled, crossover study. The study consisted of one 4-week intervention period and one 4-week placebo control period separated by a 2-week washout as shown in (Figure 2-1). 4 weeks intervention was hypothesized to be sufficient to detect changes in endothelial function based on previous work that considering either dietary nitrate intervention [140, 165] to produce favorable changes for vascular health in subjects at risk of CVD. In addition, this washout period was chosen based on previous work that documented this period was successfully returned NO metabolites and vitamin C to baseline level [219-221]. Subjects visited the laboratory a total of 4 times to complete study measures: during the screening visit/ baseline 1 (week 0), endpoint 1 (week 4), baseline 2 (week 6), and endpoint 2 (week 10). The timeline of the study visits along with the associated study measures at each visit is shown in (Table 2-1). Height and weight, endothelial function, BP, assessment of plasma vitamin C, nitric oxide species, oxidized LDL, lipid profile and dietary intake were measured pre and pose each intervention. All testing procedures were performed following a 12 hour overnight fast.

Before implementing the study, The University of Kentucky Institutional Review Board approved this study. Written informed consent was obtained from all subjects prior to participation at their first visit. Study data were saved in charts and in an electric data capture tool (REDCAP). REDCAP is a web application used to capture datasets for research studies.

2.1 Subjects characteristics (inclusion and exclusion criteria)

Males and post-menopausal females between 50-70 years old were recruited to participate. This age group was selected because older adults are more prone to have endothelial dysfunction [222], and the percent of hypercholesterolemia among adults aged 55 and over was higher than younger adults in both sexes [223]. The inclusion criteria included subjects with a body mass index (BMI) of 18.5–34.9 kg/m$_2$, who were non-smokers, with high cholesterol levels (low-density lipoprotein cholesterol >130 mg/dl), and without a history of lipid pharmacological treatment (statin). A BMI of 34.9 kg/m$_2$ was used as a cut off for this study because obesity might modify dietary nitrate response [153, 166]. We want to eliminate confounding factors with our sample size and elucidate whether dietary nitrate will have an effect on endothelial function with normal BMI to obese class I older adults.

Exclusion criteria included evidence of overt atherosclerotic disease (i.e., documented medical history of coronary artery disease, peripheral artery disease, cardiovascular disease, and stroke). Additional exclusion criteria included any surgery or medical history that could confound study results, such as a history of bowel resection, rheumatoid arthritis, inflammatory bowel disease, celiac disease, diabetes, uncontrolled hypertension (systolic blood pressure >160 mm Hg or diastolic blood pressure >100 mm Hg), controlled hypertension on clonidine medication, or tobacco use in the past month. Subjects with BMI less than 18.5 Kg/m^2 or greater than 34.9 Kg/m^2 were not eligible to take part in the study. Moreover, subjects were ineligible if they had been diagnosed with the following medical conditions such as active cancer, severe anemia (Hb < 10mg/dL), hepatic or renal disease, heart failure, or finger deformities. In addition, HIV-positive patients on combination antiretroviral therapy because of potential pharmacokinetic interactions with beetroot juice were excluded. Subjects were also excluded if they reported taking any form of hormone replacement therapy (estrogens, progesterone, and testosterone), multivitamin supplements that provides >100% of RDA, or any of the following medications that attenuate nitrate conversion to NO: proton pump inhibitors (PPI) and antibiotics. Finally, subjects who consumed greater than 6 drinks of alcohol (any form) per week, performed structured resistance or aerobic training for more than 1-hour four times per week, or participated in other clinical intervention within the previous 30 days were excluded from the study. Exclusion criteria were specified to eliminate different conditions or diseases because the effects of dietary nitrate may not uniformly affect endothelial function due to these various conditions, so the current work could focus on hypercholesterolemia- related endothelial dysfunction in older adults. In addition, they were asked to avoid using the mouthwash before and throughout the study duration. In addition, enrolled subjects were advised to consume low- vitamin C diet throughout the study. They were provided at the beginning of the study with a list of high vitamin C foods/fortified foods to avoid during the study.

2.2 Subjects screening procedures and description of study measures

Subjects were identified and recruited through the medical centers of the University of Kentucky campus using an IRB approved recruitment flyer (Appendix 1.a) that were distributed to the physicians' offices of outpatient internal medicine clinic at the University of Kentucky, and internet and social media (using UK and departmental/lab pages), Family medicine at Turfland clinic. Other sources for recruitment were internet and social media (using UK Facebook page), and UK HC television screens found in high-traffic hallways. The study consisted of two screening visits and three laboratory visits to complete study measures:

Screening visit stage 1: The first screening stage consisted of a screening interview followed by a vascular screening visit. This stage of the screening process was coordinated by a trained graduate student and included one of the following: 1) face-to face interview with potential study subjects visiting the outpatient clinics to gauge volunteer interest followed by assessing height/weight for BMI, and checking inclusion and exclusion criteria with brief questions about medical history to check for potential eligibility. 2) Phone screening for potential subjects recruited from ResearchMatch and social media were included same screening questions as the face- to face interview. Potentially eligible subjects who passed the first stage of screening were asked to sign the informed consent form before inclusion in the trial. Following the informed consent process, subjects were scheduled for the second stage of screening.

A medical history questionnaire (Appendix. B) about current alcohol and smoking status, physical activity, specific chronic illnesses or medical; conditions that could confound the study results, past bowel surgeries, or any evidence of overt atherosclerosis disease. In addition, this questionnaire documented if subjects were currently seeing their primary healthcare provider for any reason, and if they were on any of the following: prescribing drugs, and over the counter medications including dietary supplements, hormonal replacement therapies, antibiotics, and antacid medications. Potential subjects who are on proton pump inhibitor (PPI) medications were asked to discuss switching to an H2 blocker medication with their primary care provider and then reported back to the study team.

Screening visit stage 2: this second stage determined the final eligibility with a confirmation of poor endothelial function RHI$\leq$ 2 by non-invasive vascular testing (PAT). This vascular screening visit occurred within a month after the screening visit stage 1 (Figure 2-1). According to the study measures, it entailed three processes: 1) anthropometrics and vascular testing 2) blood draw, 3) 24-hour dietary recalls were completed by the research coordinator and graduate research assistant in the nutrition program. All study visits were conducted at Center for Clinical and Translational Science (CCTS) clinic. Each visit to the UK campus lasted for 60-75 minutes. The following are the timeline for each study visit.

Baseline visit 1: Once screening stage 2 was completed, and the study volunteer was eligible to take part in the study based on initial screening results, vascular testing during the screening visit 2 served as part of the baseline measures on the first day of intervention 1. During the same visit, the study coordinator asked the CCTS nurse to draw a blood sample for measuring baseline plasma levels of vitamin C and NO metabolites concentrations (NO3, NO2, and NO), lipid profile, and oxidized LDL. Subjects who did not pass the vascular testing in the screening stage were thanked for their interest and participation in our screening visits.

Baseline visit 2: Following the washout period on the first day of intervention 2, subjects returned to the clinic for repeating study measures. All baseline 1 testing procedures were repeated, except the blood measures for plasma NO metabolites and vitamin C levels. After that, the alternate treatment were distributed and the subjects were asked to start taking them from this day.

Endpoint study visits (Endpoint 1 and Endpoint 2): To determine the efficacy of the intervention, all baseline 1 testing procedures outlined above were repeated at the end of each study intervention (one day after the last intake of dietary supplements or placebo).

2.3 Blinding and randomization

On baseline testing days after baseline testing complete, subjects were assigned randomly to start with either inorganic nitrate + vitamin C, or inorganic nitrate + vitamin C placebo. Subsequently, a washout period of two weeks preceded the alternate treatment as shown in **Figure 2-1**. The randomization was conducted using the Investigational Drug Service (IDS) at our institution, which assisted with storage, supply management, quality control, and transfer of the study supplement. Random numbers that corresponded to each of the study subject were generated by statistical software and individually placed in sealed envelopes for treatment assignment. Vitamin C and its placebo packaging were identified by the letter "A" or "B", and only IDS staff was unmasked to supplement assignment.

2.4 Dietary supplement interventions

2.4.1 Supplemental nitrate (NO_3)

The nitrate supplement was provided in the form of commercial beetroot juice (Sport Beet IT shot, Heartbeet Ltd) for all subjects. Sport Beet IT was a concentrated beetroot juice (70ml) that delivered on average 300-400mg of inorganic nitrate. This product was made from using conventional non-organic concentrated beetroot juice. The product had no added flavors. This supplement was served either cold or at room temperature. Subjects were asked to consume one shot of Sport Beet IT per day. Subjects were asked to not mix beetroot juice with anything and particularly citrus, because the low pH of citrus juice can convert nitrite (pKa 3.4) to NO prior to consumption and thereby reduce the efficacy of the product. All subjects were encouraged to consume the supplements regularly during the morning hours between 7 and 11 am each day if possible and drink it as fast as possible within 2-3 minutes so it is all in the stomach at the same time. Additionally, subjects were asked to avoid chewing gum after drinking beetroot juice for a minimum of 3 hours.

2.4.2 Vitamin C and matching placebo

Vitamin C and the placebo were provided as supplement capsules. The preparation and labeling of the vitamin C and its placebo were performed by the IDS in such a way that both pills

were identical in shape, color, and size to ensure adequate blinding of both subjects and investigator. All investigational supplements were stored in a locked cabinet with limited access at room temperature and were protected from light until distribution. Vitamin C supplements were either active capsules that contained 500 mg of Nature Made product in each or corn starch placebo capsules. Subjects were asked to consume a total of 1000 mg of vitamin C every day. This 1000 mg dose was divided into two capsules; each capsule delivered 500 mg of vitamin C. The dose of vitamin C was based on previous work by Ashor et. al who reported in meta-analysis study that 700 mg of vitamin C or higher elicited an improvement in endothelial function [25].

Based on subjects' supplement assignment, supplements were distributed to subjects to self-administer at home with breakfast. However, the first dose was administered in the clinic right after the study measures have been completed. Subjects were asked to consume supplements in the morning every day during the four-week study period, except for the test days and washout weeks. Aligned with recommendation from Rodriguez-Mateos et al., subjects were asked to consume the concentrated beetroot juice with breakfast meals, and then 2 vitamin C capsules or placebo at the same time one-hour post beetroot juice supplementation. One-hour provides enough time to permit dietary nitrate to be absorbed completely and then re-entered the entero-salivary circulation and thereby maximize salivary nitrite levels in the saliva at time of vitamin C ingestion (Rodriguez-Mateos et al., 2015). If the subject reported skipping breakfast or forgetting to take the supplements with breakfast, they were instructed to ingest the supplements at lunchtime. At each baseline visit, subjects received a 14-day supply of supplements that contain of 30 pills and 15 bottles of beet juice (2 pills/day + 2 extra pills, and 1 BRJ/day + 1 extra bottle). Study subjects were asked to retain all the used and unused study packaging to turn in to the study coordinator at their next scheduled supply distribution. The redistribution of supplements occurred every two weeks to ensure that subjects remained in contact with the study team every two weeks. Subjects were asked to record the daily date and time of supplement consumption with the amount consumed.

2.5 Study procedures

2.5.1 Clinical assessment

Measurements for height and weight were collected for each subject at each visit using digital wall-mount stadiometer (DETECTO 3P, Webb City, MO) and Tanita 800S scale (Tokyo, Japan) at the CCTS. Subjects were instructed to remove shoes and not hold anything before stepping on the scale to determine their height and weight. The study coordinator calibrated the scale to zero and then asked the subject to step on the scale to record their body mass in kilograms (kg). Further, subjects were instructed to stand up against the wall of the stadiometer as the coordinator lowered the headpiece to touch firmly on top of the subject's head and recorded to

measure height in centimeters (cm). BMI was calculated using the formula of numerical values of weight and height as weight (kg)/ height (meters^2).

Blood pressure measurements were obtained with automatic BP cuff 5-10 minutes before each vascular testing visit [224]. Blood pressure measurements were taken from the non-dominant arm in a relaxed position with arm rested at the sides of the subject to determine the supra-systolic blood pressure for endoPAT. Subjects were asked to not cross their feet during the blood pressure measures and the study coordinator recorded the systolic and diastolic values (mmHg).

2.5.2 Vascular testing

Endothelial health was assessed using endoPAT-2000 device (Itamar Medical, Caesarea, Israel) to measure digital pulse wave amplitude (PWA) pre and post flow stimulus. We focused on assessing vascular function as a reliable outcome measure described in previous cardiovascular intervention studies [78, 95]. Since the decline of endothelium-derived NO bioavailability is reflected by impaired PAT vascular responses [78].

Prior to each vascular testing, including the screening visit stage 2, subjects were asked to do the following: 1) consume a low-nitrate diet for 48 hours to control diet variation between study subjects [219]. Study personnel provided potential subjects with reference table that clarifies all high nitrate sources in the screening visit stage #1; 2) refrain for 24 hours from taking anti-hypertensive agents and long- acting vasoactive medications including steroids, beta-blockers, antacid, anticoagulants, calcium channel blockers, angiotensin converting enzyme inhibitors (ACE-inhibitors), and aspirin; 3) abstain from consuming alcoholic beverages, coffee, a high fat diet, chewing gum; 4) and abstain from any strenuous exercise for 24 hours before study visit [74]; 5) fast overnight for 12 hours before morning study visits [99]. The presence of the aforementioned medications and dietary compounds has been shown to influence endothelium dependent NO vasodilation and may affect the study outcomes.

EndoPAT tests occurred at CCTS for each visit at approximately 10 AM. Measures were performed in a temperature-controlled room (70–75°F [21–24°C]) and the subject was asked to lie in a supine position before the procedure [99]. This test lasted for 20 minutes. The endoPAT device measured pulsatile changes of the occlusion and control arms via finger probe plethysmography. A blood pressure measurement with a standard electronic BP cuff was taken, and then a manual blood pressure cuff was placed on the upper arm of the non-dominant hand (occlusion arm) to start performing PAT measure. Then, two probes plethysmography that lined with sensitive balloons were placed on the both index fingers of each hand and inflated. The metal tips inside these probes record/assess changes of pulse volume amplitude (PWA) at the fingertip in response to reactive hyperemia [225]. Reactive hyperemia acts as a stimulus to release the vasodilator, NO. The PAT

endothelial test consisted of three phases: baseline (pre occlusion), occlusion, and post occlusion (hyperemia). Subjects were asked to stay still as possible during the entire testing period. Following a 10 min quiet rest period, the blood pressure cuff was inflated to suprasystolic pressures by adding at least 60 mmHG above systolic blood pressure for five minutes during occlusion then the cuff deflated quickly and endoPAT device continued recording reactive hyperemia response for five minutes [95, 224]. The RHI ratio of the study arm was instantly calculated automatically by dividing post-occlusion PWA to the pre-occlusion value of PWA of the same arm, normalized to the control arm, and then multiplied by baseline correction factor to determine RHI (**Figure 2-2**). RHI greater than 2 is indicative of normal endothelial function and RHI< 2 is considered poor endothelial function. Validation studies reported that lower RHI is associated with clinical population of cardiovascular risk factors.

2.5.3 Blood sampling and analysis

Fasting blood samples were drawn by trained staff at University of Kentucky's CCTS immediately following the vascular testing at all visits. Venous blood samples were collected in three specific tubes with three different colored tops from all subjects during the morning of the testing days to measure plasma vitamin C (red top), nitrate/ nitrite content and oxidized LDL (purple top), and lipid profile (green top). Blood specimen were taken mostly from the antecubital vein but if arm vein attempt was not successful, we used the dorsal surface of the wrist. A 21 gauge x ¾" butterfly needle was used for our blood draws. The volume of each tube was as follows: lipid panel: 3 mL, vitamin C: 4 mL, and NO metabolites and oxLDL tube: 3 mL. The latter tube containing ethylenediaminetetraacetic acid (EDTA) and was centrifuged immediately, and then transferred to three 0.5mL cryo tubes to analyze the relevant samples. Sample storage and analysis were completed using standard procedures by CCTS staff. The plasma was separated from the whole blood by centrifugation at 20°C for 10 minutes and then stabilized with metaphosphoric acid. Plasma samples were stored at −80°C for analysis at end study. The university Biospecimens Core of the CCTS and UK clinical lab processed all blood samples and completed all analyses. Chemiluminesence/colorimetric assay, high performance liquid chromatography (HPLC), roche analyzers a cobas 311 or cobas 702, and sandwich enzyme-linked immunosorbant assays (ELISA) with the antibody 4E6 were used to asses circulating plasma levels of NO metabolites, ascorbate concentrations, lipid profile, and OxLDL respectively.

2.5.4 Dietary assessment

Dietary intake was monitored throughout the study by collecting three- day food diaries in each study period to examine the extent to which differences in dietary intake influenced study outcomes. Upon enrollment in the study, subjects were instructed to maintain their lifestyle

including physical activity, and diet throughout the study duration as these factors have been shown to influence endothelial function [226-228]. Subjects were asked to keep track of what they consumed including all food, beverages and snacks for three non-consecutive days, two weekdays and one weekend. Trained nutrition graduate students in dietary assessment conducted an interview by phone to complete the three 24-hr dietary recalls per subject during the midpoint of each interventional period (week 2&3, and week 8&9). The interviewer asked subjects to report their food and beverage intake consumed throughout the interview. The interviewer recorded all details about food and beverage items including portion size, methods of preparations, ingredients, and brand name if known. In addition, subjects were asked about their use of any dietary supplements and to provide a description of their composition and dose. Data were recorded using Nutrition Data System for Research software (NDSR version 2017, Minneapolis, MN). This method is a standardized recall strategy designed to improve recall accuracy [229-231].

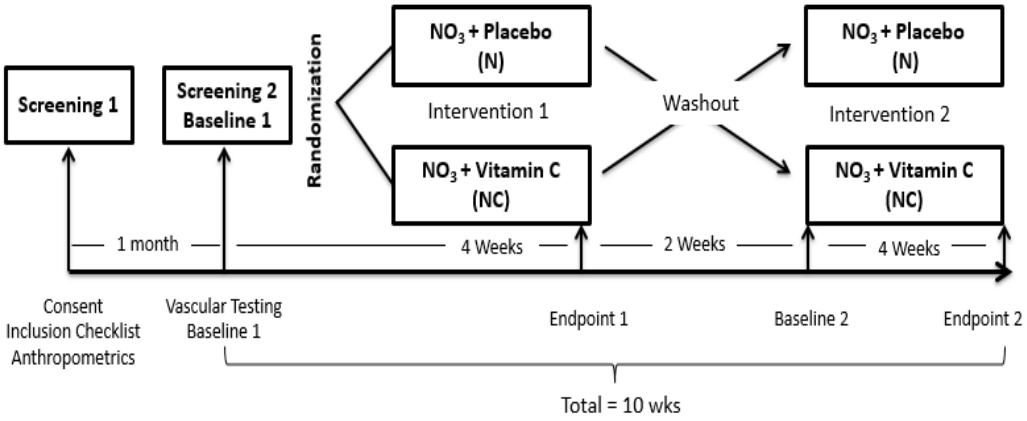


Figure 2-1. Schematic representation of the study design

NO_3: inorganic nitrate provided in concentrated beetroot juice dietary supplement; (N): inorganic nitrate and vitamin C placebo; (NC): inorganic nitrate and active vitamin C

Table 2-1. Timeline of visits and associated measures

Study Measures	Screening Visits		Weeks 1-4	Visit 2 (EP1)	Visit 3 (BL2)	Weeks 7-10	Visit 4 (EP2)
	screening1	Screening2 /BL1					
Anthropometric measures		X		X	X		X
Inclusion/ exclusion screening	X						
Medical history questionnaire	X						
Blood pressure and Vascular testing		X		X	X		X
Blood draw		X		X	X		X
24-hr dietary recalls			X			X	
Supplement distribution		X	X		X	X	

BL: baseline; EP: endpoint.

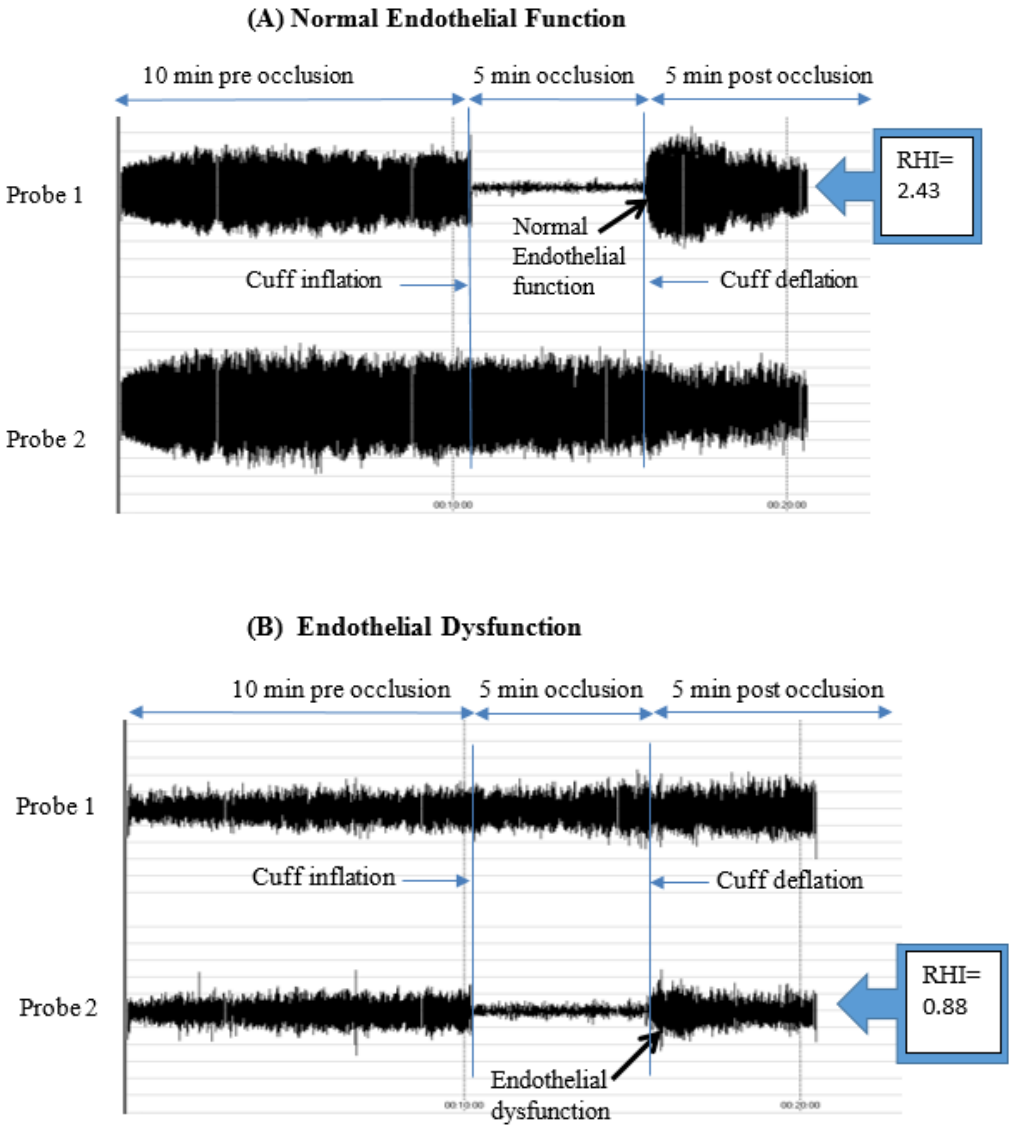


Figure 2-2.PAT testing showing normal (A) and abnormal (B) hyperemia response.

A healthy blood vessel dilates notably in response to ischemic stimulus as shown in figure (A) and the PWA increased post cuff deflation compared to baseline. An artery with endothelial dysfunction lacks the ability to release sufficient NO resulting in low reactive hyperemia response post-deflation as shown in figure (B).

Chapter 3: **AIM 1**

3.1 Abstract

Introduction: Endothelial dysfunction (ED) serves as a marker of atherosclerotic risk. The key mechanism of ED is limited nitric oxide (NO) bioavailability. Dietary nitrate represents an alternative source of NO and improves endothelial function through its sequential reduction to nitrite and then NO. It is believed that vitamin C contributes to nitrite reduction in the gastric cavity to boost NO production. The combined effect of providing both dietary supplements on ED is relatively unexplored.

Purpose: The aim of this study was to compare the effect of inorganic nitrate in the form of concentrated beetroot juice (CBJ) with and without vitamin C on ED as measured by the reactive hyperemia peripheral artery tonometry (RH-PAT) in subjects at risk for cardiovascular disease.

Methods: Untreated hypercholesterolemic Subjects, aged 50-70 years with RHI ≤2 were enrolled in this randomized crossover double-blind study. Two 4-week interventions were separated by a 2-week washout. Subjects were assigned to daily CBJ (70mls) with 1000 mg of vitamin C (NC) (sequence1; N-NC) or CBJ with matched placebo (N) (sequence2; NC-N), then switched to alternate treatment. The change in endothelial function was assessed using the reactive hyperemia index (RHI) at 4 weeks compared to the study baseline for each intervention, and then the score change between the two interventions was compared (RHI change of N treatment vs RHI change of NC treatment). Secondary outcome measures included plasma vitamin C concentration and plasma NO metabolites as indices of NO production.

Results: Eighteen subjects aged 53-70 years completed the study. No differences in RHI change score (N: 0.21±0.46; NC: 0.20±0.67; p> 0.05) were observed following 4 weeks of each intervention. Eight out of 18 subjects responded with greater improvement in RHI following NC (High-responders: 0.73±0.52; Low-responders: -0.32±0.23; p<0.001) compared to N. It was found that subjects who showed a better response in RHI after NC entered the study with mean baseline RHI of <1.67 than those who less respond with greater mean RHI scores >1.67 (1.23; n=8 v. 1.75; n=8; p=0.02). Significant differences were observed between overall treatment in the mean change of plasma nitrate (N: 78.64±15 μmol/L; NC: 128.66±29 μmol/L; p<0.001) and nitrite N: 0.08±0.02 μmol/L; NC: 0.04±0.03 μmol/L; p=0.03). Likewise, the mean change of plasma vitamin C was greater following 4 weeks of NC treatment compared to N treatment (N: -0.14±0.53 mg/dL; NC: 0.45±0.66 mg/dL; p<0.001).

Conclusions: Vitamin C, when combined with inorganic nitrate for 4 weeks promotes an additive effect on the NO pool and may potentiate greater improvements in endothelial function in subjects with significant endothelial dysfunction compared to inorganic nitrate supplementation alone.

3.2 Introduction

Vascular endothelium produces several vasoactive substances to maintain vascular homeostasis [232]. One of the most important mediators that promotes vaso-relaxation is nitric oxide (NO)[233]. Endothelial dysfunction (ED) represents a well-established response to cardiovascular risk factors such as aging and hypercholesterolemia, and acts as a primary driver of atherosclerosis (AS) development [39, 42]. ED is characterized by a reduction in the bioavailability of the vasodilator NO and an enhancement of oxidative stress production resulting in an impairment of endothelium-dependent vasodilatation [45]. Endothelial function can be assessed by measuring the vascular response to physiological stimuli, reactive hyperemia, in the coronary and peripheral arteries [42]. Flow mediated dilation (FMD) is the most prevalent method utilized in most clinical trials to assess for ED in conduit arteries because it is non-invasive technique and principally mediated by endothelium-derived NO [106]. An alternative technique with high reproducibility called reactive hyperemia peripheral artery tonometry (RH-PAT), based on the same principle as FMD, has emerged for assessing peripheral endothelial function using fingertip probes and has resulted in associating ED with cardiovascular risk factors in three studies [33, 99, 104]. Unlike FMD, RH-PAT testing is more affordable, easily trained, and operator independent as automated calculation of reactive hyperemia index (RHI) [96].

In order to mitigate the deleterious effect of ED and further reduce the risk of major cardiovascular disease (CVD) events among vulnerable populations, we must better understand the role of modifiable lifestyle factors on vascular health. Modifiable lifestyle factors including healthful dietary choices [94, 162, 209] and exercise [227, 228] have been shown to positively restore NO bioavailability and further ameliorate ED. In epidemiological studies, high fruit and vegetable intake has been associated with lower risk of CVD [122, 234]. Bioactive constituents present in fruits and vegetables may be responsible for these cardioprotective effects. For example, vitamin C and E [121], potassium [122], and other bioactive components may produce protective cardiovascular effects such as inorganic nitrate [123]. Supplementation with inorganic nitrates or with vitamin C (ascorbic acid) has been shown to augment NO production via different mechanisms, which in turn, is thought to increase vasodilation and improve endothelial integrity [25, 160, 235]. Inorganic nitrate is a dietary NO precursor derived from natural sources such as green leafy vegetables and beets and relies on sequential reduction to nitrite and then NO through entero-salivary circulation [20, 123, 236]. This non-enzymatic pathway serves as a backup system for NO generation and works more efficiently under acidic and hypoxic conditions than the endothelial nitric oxide synthase (eNOS) dependent pathway. The classical eNOS pathway is often dysfunctional in the presence of cardiovascular risk factors [24, 124]. Under hypoxic and acidic

conditions in the blood and gastric lumen, the presence of vitamin C may augment the reduction of nitrite to NO [236]. The interaction between nitrate and vitamin C has been recently described in the literature and has indicated that that vitamin C acts as an enhancer to catalyze the conversion of acidified nitrite to NO, thus, increasing the NO pool [147, 218, 236-238].

$NO_2^- + H^+ \rightarrow HNO_2$

$2\ HNO_2$ + vitamin C $\rightarrow 2 \bullet NO$ + dehydroascorbic acid + $2\ H_2O$

Therefore, the combination of dietary nitrate and vitamin C is a potentially promising dietary supplement strategy to combat ED associated CVRFs. However, evidence describing their combined effect on cardiovascular health, particularly, endothelial function is limited. Recently, synergistic acute effects were observed when a single dose of inorganic NO_3^- was administered in combination with vitamin C on vascular outcomes [154]. Results showed improvements in arterial stiffness as measured by pulse wave velocity (PWV), heart rate variability (HRV) indices, the cofactor BH4 compared to nitrate or vitamin C given separately. However, this combination did not improve endothelial function in all groups as measured by post-occlusion reactive hyperemia (PORH). It is not yet known if longer duration studies designed to combine these dietary supplements would serve to improve endothelial function outcomes over time in vulnerable populations.

We aimed to examine whether the co-administration of vitamin C and nitrate for a longer duration would yield an additive effect on endothelial function. The purpose of this study was to investigate the combined effect of a 4 week vitamin C and dietary nitrate supplementation regimen on post-occlusion reactive hyperemia, a clinical biomarker of vascular endothelial function as measured by PAT ratio, compared to nitrate supplementation alone. On the basis of their complementary mode of action to augment NO and improve endothelial function, we hypothesize that that 4 weeks co-administration of inorganic NO_3^- and vitamin C would elicit a greater improvement in endothelial function as determined by reactive hyperemia index (RHI) as a result of changes in plasma levels of NO metabolites compared to nitrate alone.

3.3 Methods and materials

3.3.1 Subjects

Male and female non-smokers with a body mass index (BMI) range of (18.5-34.9 kg/m^2) between the ages of 50 and 70 years of age with high LDL concentrations (>130 mg/dL) were enrolled in the study. Subjects were not eligible if receiving lipid lowering therapy, multivitamin supplements exceeding >100% of recommended daily allowance (RDA), or on medications at the time of screening that would interfere with the study outcomes (e.g. hormone replacement therapy, proton pump inhibitors (PPI) and antibiotics). Additional exclusion criteria were history of

cardiovascular disease, diabetes mellitus, chronic inflammatory disease, celiac disease, uncontrolled hypertension (systolic blood pressure >160 mm Hg or diastolic blood pressure >100 mm Hg), controlled hypertension on clonidine medication, hepatic or renal disease, and active cancer. Moreover, subjects who consumed greater than 6 servings of alcohol per week, performed structured resistance or aerobic training for more than 1-hour four times per week, or participated in other clinical interventions within the previous 30 days were excluded from the study. Final enrollment decisions were contingent upon vascular testing results (RHI is ≤ 2).

The study was approved by the university Institutional Review Board, and the informed written consent was obtained from all subjects prior to engaging in baseline measures. All institutional and governmental regulations concerning the use of human volunteers were followed during this research. All study visits were conducted in the Center for Clinical and Translational Sciences (CCTS), University of Kentucky, from February 2019 to September 2019.

3.3.2 Design and randomization

This was a 10 week randomized, cross-over, double-blinded, placebo-controlled study. This study was designed to compare the magnitude changes in RHI (primary outcome), plasma nitrate and nitrite levels as biomarkers for NO (secondary outcome), and plasma vitamin C following the 4-week treatment of inorganic nitrate with vitamin C placebo (N) and inorganic nitrate combined with vitamin C (NC). Eligible subjects were randomized to receive either treatment (N) or treatment (NC) for 4 weeks (period1), and subjects were then crossed over to the alternate treatment for another 4 weeks (period 2). There was a 2-week washout at crossover. Throughout the intervention periods, enrolled subjects were advised to consume low- vitamin C diet throughout the study. They were provided at the beginning of the study with a list of high vitamin C foods/fortified foods to avoid during the study.

3.3.3 Dietary supplementation intervention

The nitrate supplement was provided to all subjects in the form of commercial beetroot juice beverage (Sport Beet IT shot, Heartbeet Ltd). Sport Beet IT was a concentrated beetroot juice (CBJ) (70ml) that delivered an average of 300-400mg of inorganic nitrate. Vitamin C and the placebo were provided as supplement capsules. The study coordinator was blinded to the treatment assignment and the randomization was conducted by investigational Drug service (IDS) at University of Kentucky, which prepared the list of randomization treatment sequence and then provided to the study coordinator with the treatment allocation. The preparation and labeling of the vitamin C and its placebo were performed in such a way that both pills were identical in shape, color, and size to ensure adequate blinding of both subjects and investigator. Vitamin C supplements were either active capsules that contained 500 mg of ascorbic acid (Nature Made ®)

or corn starch placebo capsules. The dose of vitamin C provided in this study was based on previous work by Ashor et. al who reported in meta-analysis study that 700 mg of vitamin C or higher elicited an improvement in endothelial function [25]. The study coordinator instructed subjects to self-administer CBJ for 7 days a week at the same time by mouth during the morning hours and then consume 2 pills (1000 mg of vitamin C or placebo) one hour later.

Supplement adherence was assessed by counting the unused pills and beer juice bottles. Subjects were asked to return any unused CBJ bottles and vitamin C pills at the following endpoint visits of each intervention for pill counts.

3.3.4 Study protocol

Screening visits: The study consisted of two screening visits: a screening interview followed by a vascular screening visit. At screening visit stage 1, phone screening or a face-to face interview was performed with potential study subjects to gauge subject's interest followed by assessing height/weight for BMI and checking inclusion and exclusion criteria with brief questions about medical history to assess eligibility. At screening visit stage 2, a non-invasive vascular testing using PAT test was employed to determine final eligibility for subject selection: only subjects with RHI score ≤2 were included in the study. This visit served as part of the baseline measures for enrolled subjects and other study measures were assessed once vascular testing was completed.

Study visits: Subjects completed 4 visits at the beginning and the end of each interventional period for anthropometric measures, blood pressure, vascular testing, and for blood collection as indicated in the subject timeline (**Figure 3-1**). Vascular function was defined as measures of RHI ratio (primary outcome). Biochemical markers of ED included in this study were plasma nitrate/nitrite as indices of NO production and plasma vitamin C (secondary outcome). Prior to each visits, including screening visit stage 2, subjects were asked to perform the following to prepare for vascular testing: 1) consume a low-nitrate diet for 48 hours to control diet variation between study subjects [219]; 2) refrain from taking anti-hypertensive agents and long- acting vasoactive medications including steroids, beta-blockers, antacid, anticoagulants, calcium channel blockers, angiotensin converting enzyme inhibitors (ACE-inhibitors), and aspirin for 24 hours; 3) abstain from consuming alcoholic beverages, coffee, high fat foods, chewing gum; 4) abstain from any strenuous exercise for 24 hours [74]; and 5) fast overnight for 12 hours before morning study visit [99].

Subjects were instructed to maintain their usual dietary patterns throughout the duration of the study and dietary intake was assessed via 24-hr dietary recall. Three 24-hours dietary recalls were collected and analyzed using Nutrition Data System for Research (NDSR, Version

2019, University of Minnesota, Minneapolis, MN, USA) at midpoint of each interventional period, covering 1 weekend and 2 weekdays for a total of 6 recalls.

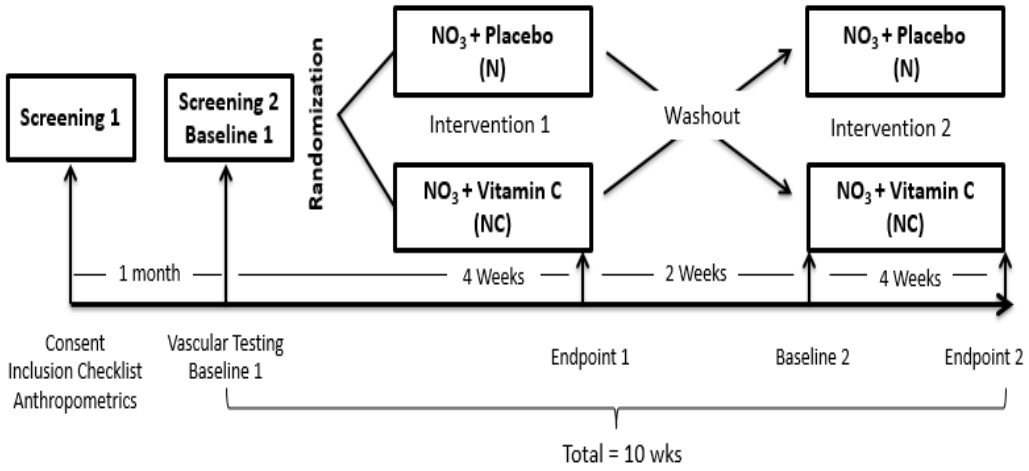


Figure 3-1. Schematic representation of the study design. NO3= inorganic nitrate provided in concentrated beetroot juice dietary supplement

3.3.5 Vascular testing using peripheral artery tonometry

Endothelial health in peripheral arteries was assessed using EndoPAT-2000 device with finger plytheysmographic probes (Itamar Medical, Caesarea, Israel) to measure finger pulse wave amplitude (PWA) changes pre and post flow stimulus. EndoPAT tests occurred at approximately 10 AM for each visit. Measures were performed in a temperature-controlled room (70–75°F [21–24°C]) and the subject was asked to lie in a supine position for the procedure [99]. To start performing PAT measure, a blood pressure measurement (BP) with a standard electronic BP cuff was taken, and then a manual BP cuff was placed on the upper arm of the non-dominant hand (occlusion arm). The PAT endothelial test consisted of three phases: baseline (pre occlusion), occlusion, and post occlusion (hyperemia). Subjects were asked to stay still as possible during the entire testing period. Following a 10 min quiet rest period, the blood pressure cuff was inflated to suprasystolic pressures by adding at least 60 mmHG above systolic BP for five minutes then the cuff was deflated quickly and EndoPAT device continued recording pulse wave response for five minutes [95, 224]. The RHI ratio of the study arm was calculated automatically by dividing post-occlusion PWA to the pre-occlusion value of PWA of the same arm, normalized to the control arm, and then multiplied by baseline correction factor to determine RHI score.

3.3.6 Blood sampling and analysis

Fasting blood samples were drawn by trained staff at University of Kentucky's CCTS immediately following the vascular testing at all visits. Venous blood samples were collected in three specific tubes with three different colored tops from all subjects during the morning of the testing days to measure plasma vitamin C (red top), nitrate/ nitrite content (purple top). Plasma samples were stored at −80°C for analysis at end study. Plasma nitrate and nitrites were analyzed using the commercial assay kits according to the manufacturer's instructions. Nitrate/Nitrite were measured by Parameter assay kit KGE001. Nitrate/nitrite are measured by the Griess Reaction [239], a reaction that produces a diazonium ion which subsequently couples to amine 1-naphthylamine to produce a red-violet color (absorbance @ 540-570 nM). Plasma vitamin C concentrations were analyzed using HPLC.

3.3.7 Statistical analysis

Using the nQuery Advisor program, sample size calculations were based on the work of Kwak et al. (2012) who assessed endothelial function by PAT measure in subjects with newly diagnosed type 2 diabetes [116]. Based on these data, nineteen subjects were needed for 80% power with a two-sided p-value of ≤ 0.05.

Statistical analysis was performed using Statistical Package for Social Sciences (SPSS) software (version 22, IBM, Armonk, NY). This study was designed to detect significant differences in RHI between the two treatments (NC vs N). Distribution of continuous variables were examined by Shapiro-Wilk test. Within treatment group, comparisons at baseline and 4-weeks for each treatment were conducted by Paired Student t-tests (for normally distributed data) and Wilcoxon signed rank-test test (for non-normally distributed data) for two period data separately. Changes in measures over the 4-week period were assessed by calculating the difference between the pre and post-intervention measures. Between treatment group, the treatment effect was evaluated by the two-sided one-sample t-test which is based on the mean of the differences between the two changes for each pair (Δ) [240]. An independent sample t-test was used to assess the changes in RHI between high-responders and low-responders. Additionally, based on work by Wellek et al. [241] we examined whether there were any carryover effects from one treatment period to another. Linear mixed models were used for secondary analysis to assess the potential interaction effect of variables such as BMI and BP medication, sex, and age with the dependent variables in the two treatments. Pearson and Spearman correlations were used to assess the relationship between the dependent variables. Spearman correlations were used for variables that were not normally distributed. Data are presented as mean ± standard deviation in tables and mean ± standard error of the mean in figures.

3.4 Results

3.4.1 Subjects

A total of 162 subjects were assessed for eligibility and 23 eligible subjects were randomized. Of these, 18 completed both interventions and all study visits (Figure 3-2). Subjects had a mean age of 59±4 years and a BMI of 28.3±3 kg/m2. Thirteen subjects were normal to overweight with a BMI range from 22.5 to 29.9 kg/m2. Five subjects were obese (class I) with a BMI range (30.8 to 34.4 kg/m2). There were no significant differences in baseline characteristics between the two treatment groups (sequence 1: N-NC and sequence 2: NC-N) (**Table 3-1**). No significant changes in body mass index were observed between the two treatment periods.

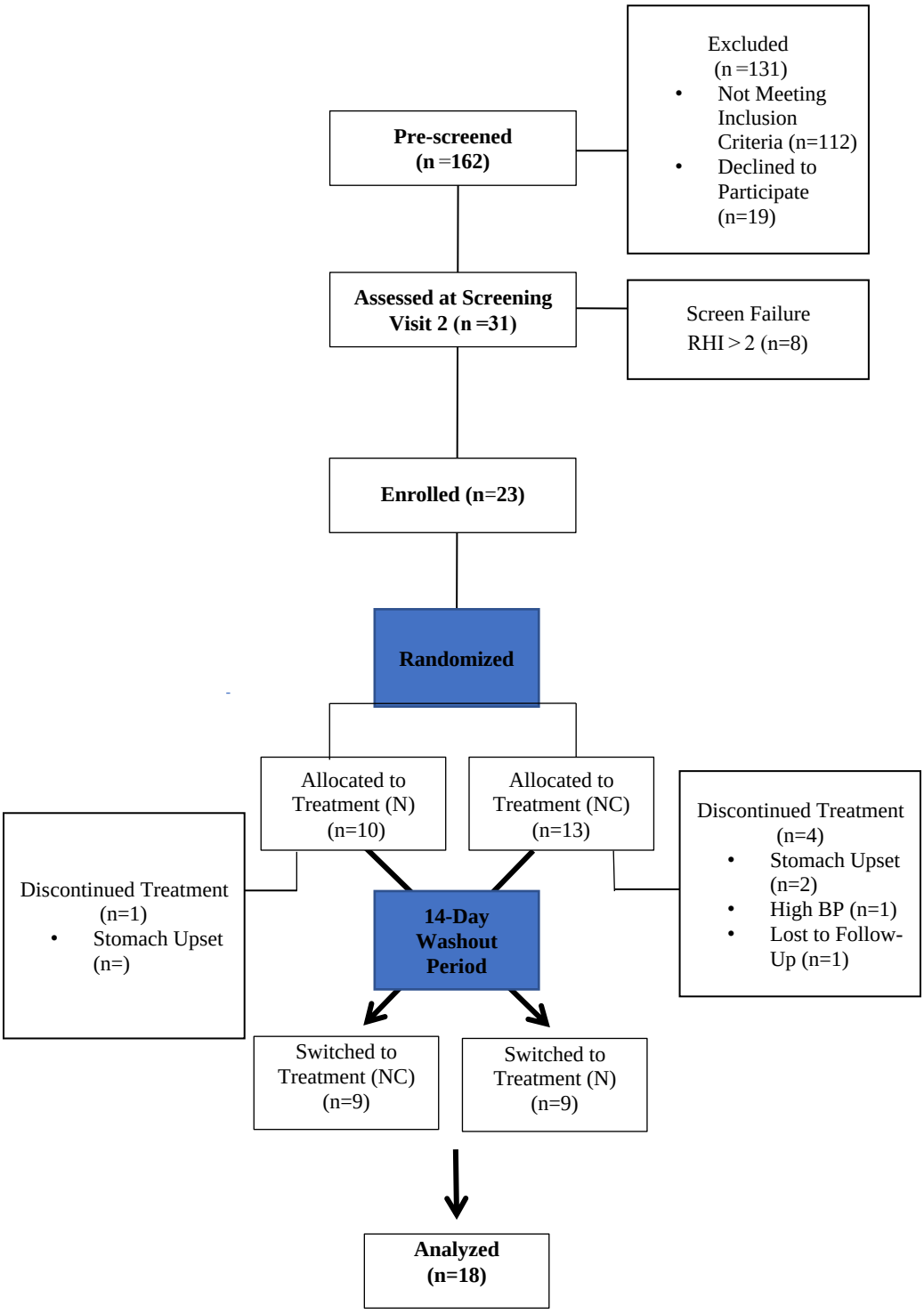


Figure 3-2. Consort Diagram

Table 3-1. Baseline Characteristics

Variables	Treatment N (sequence1; N-NC)	Treatment NC (sequence2; NC-N)	Total
	(n=9)	(n=9)	(n=18)
Male sex, n (%)	3(33%)	4(44%)	7(39%)
Age (years)	58 ±3	59 ±5	59±4
BMI (kg/m$_2$)	29.6±3.1	27.1±3.0	28.3±3.2
Systolic BP (mmHg)	130±15	125±11	127.5±13.5
Diastolic BP (mmHg)	84±10	81±6	85.5±8.2
Subjects on BP medications, n (%)	3 (33.3%)	3 (33.3%)	6 (33.3%)
Plasma vitamin C (mg/dL)	0.73±0.4	0.88±0.5	0.81±0.41
Plasma NO_3 (µmol/L)	13.4±5.3	18.5±8.5	16.0±7.3
Plasma NO_2 (µmol/L)	0.44±0.2	0.46 ±0.3	0.45±0.25
RHI	1.55±0.3	1.66±0.3	1.60±0.3

BMI: body mass index; BP: blood pressure; NO_3: nitrate; NO_2: nitrite; NO: nitric oxide; RHI: reactive hyperemia index. (sequence1; N-NC): subjects were assigned to randomly start with inorganic supplementation alone in period 1 and then crossed over to inorganic nitrate with vitamin C in period 2. (sequence2; NC-N): start with inorganic supplementation with vitamin C in period 1 and then crossed over to inorganic nitrate supplementation alone in period 1. Quantitative variables were described as the mean±SD. ($P \leq 0.05$, student t-test, Wilcoxon Rank-Sum test, and fisher exact test). Values were not significantly different between groups at baseline.

3.4.2 Endothelial function

Two subjects who started NC treatment were excluded from RHI statistical analysis due to poor RHI signals at endpoint. For our primary outcome of comparing differences between our two treatment groups (N vs NC), there were no significant differences in RHI change (N treatment: 0.21±0.46; NC treatment: 0.20±0.67; $p > 0.05$). No evidence of carryover effect was observed from

one treatment course to the other (p=0.64). Within treatment group comparisons for both treatment N (p=0.09) and treatment NC (p=0.24) revealed that the RHI score over time did not significantly change (**Figure 3-3A**). In addition, no notable interactions were observed between any of the dependent and independent variables.

We observed that the RHI of eight subjects with mean RHI baseline value of (1.23± 0.43) responded to the NC treatment greater than the N treatment (mean 1.75± 0.32). Following these observations, subjects were grouped into "high-responders" and "low-responders" based on their RHI response to NC treatment. High-responders demonstrated significant increases in RHI score pre to post NC treatment intervention (1.23± 0.43 to 1.96 ±0.53; p<0.01), while the low-responders had a significant decrease in RHI after the NC treatment intervention (1.75± 0.32 to 1.43 ±0.29; p<0.01). The RHI change in responders was 0.73±0.52, while the RHI change in less-responders was -0.32±0.23; p<0.001. The change of RHI over time following NC treatment between high-responders and low-responders is found in **Figure 3-4**.

3.4.3 Plasma biomarkers

a. Nitrate and nitrite levels

Significant differences were observed in the mean change of plasma nitrate (N: 78.64±15 μmol/L; NC: 128.66±29 μmol/L; p< 0.01) and nitrite (N: 0.08±0.02 μmol/L; NC: 0.04±0.03 μmol/L; p=0.03) between both treatment groups. Within treatment group comparison showed that the endpoint plasma nitrate levels_were significantly increased compared to baseline (p<0.001) (**Figure3- 3B&C**). Likewise, the endpoint plasma nitrite levels were significantly increased after N (p=0.006) but did not increase after 4 weeks of NC (p=0.23). Secondary correlational analysis indicated that post-supplementation changes in plasma vitamin C correlated significantly with changes in plasma nitrate levels following NC treatment (r=0.49, p=0.044). In addition, RHI change between N and NC was inversely related to changes in plasma nitrite (r=-0.68, p<0.001), but not with plasma nitrate (r=0.07, p=0.81).

b. Vitamin C concentrations

One subject who started with N treatment was excluded from statistical analysis due to missing data at endpoint. There was a significant difference in plasma vitamin C change between the treatments (N: -0.14±0.53 mg/dL; NC: 0.45±0.66 mg/dL; p<0.001). Within treatment group comparison showed that plasma vitamin C concentrations increased significantly over time following 4-week administration of NC (**Figure3-3D**).

3.4.4 Supplement adherence

Overall, subject reported adherence for taking dietary supplements in both interventional periods was 99.7% in period1 and 99% in period2 for CBJ, and 98% for period1 and 97.5% in period2 for vitamin C.

3.4.5 Dietary patterns

Analyses of three 24-hour dietary recalls indicated that no significant differences in nutrient intake and dietary pattern (**Table 4-2**). Three subjects were removed from the analysis because their dietary recalls were missed in period 2.

Table 3-2. Nutrient Intake and Dietary Patterns.

	N Treatment (n=15)	NC Treatment (n=15)	P-value
Energy (Kcal)	1757.2±505.2	1571.8±537.7	0.17
Protein (g)	65.6 ± 16.2	62.8±15.1	0.49
Carbohydrate (g)	202.9 ± 81.0	177.6±88.7	0.26
Fat (g)	80.9 ± 31.9	69.9±23.9	0.12
MUFA (g)	27.5 ± 11.6	26.1±9.7	0.58
PUFA (g)	17.7 ± 10.2	13.8±5.2	0.13
SFA (g)	29.2±14.0	24.0±10.7	0.057
Vitamin C (mg)	46.5±23.9	59.6±49.4	0.23
Vitamin E (mg)	10.3±5.4	9.8±3.5	0.69
Alcohol (g)	3.4±6.1	2.6±4.4	0.24
Caffeine (mg)	244.1±132.2	243.6±120.9	0.98
Dietary patterns			
Dark-Green vegetables Servings/day	0.56±0.7	0.59±0.7	0.92
Cured meat Servings/day	0.05±0.1	0.07±0.1	0.22

MUFA: mono-unsaturated fatty acids; PUFA: Poly-unsaturated fatty acids; SFA: Saturated fatty acids. P value were calculated from paired t-tests between N vs. NC. N Treatment: inorganic nitrate-rich beet juice and vitamin C placebo or NC Treatment: inorganic nitrate-rich beet juice and active vitamin C (1000mg). Values were not significantly different between treatment groups. Values are presented as mean ± SD.

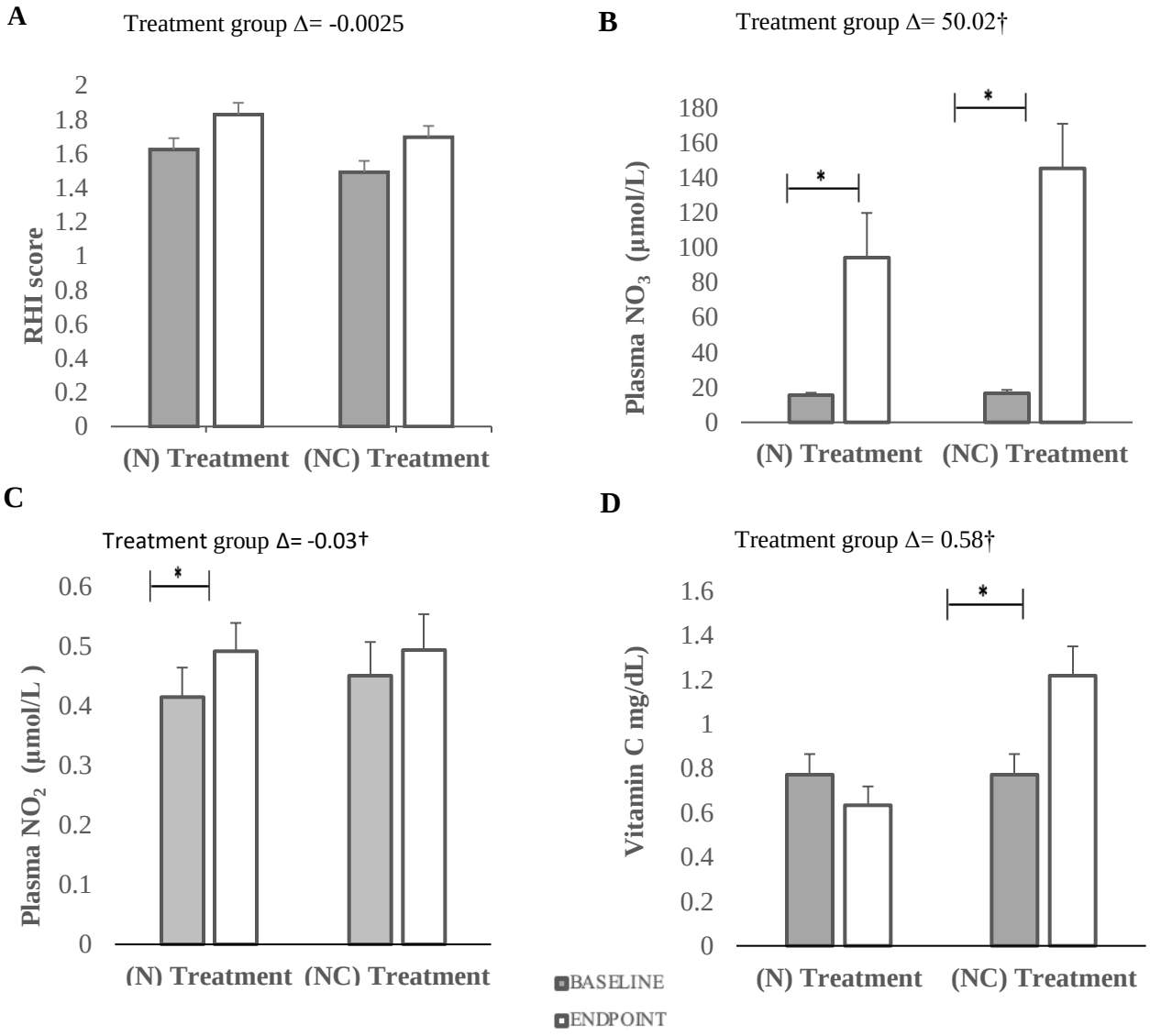


Figure 3-3.Endothelial function measurements in response to 4 weeks interventions of dietary nitrate supplements with and without vitamin C.

RHI score (n=16) (A), NO_3: plasma nitrate (n=18) (B), NO_2: Plasma nitrite (n=18) (C), and plasma vitamin C (n=17) (D) before (dark gray panel) and after 4-weeks of supplementation (white panel) with (N) Treatment: inorganic nitrate-rich beet juice and vitamin C placebo or (NC) Treatment: inorganic nitrate-rich beet juice and active vitamin C (1000mg). Values represent means ± SEM. Data were analyzed using paired t-test for RHI and vitamin C, and Wilcoxon-test for plasma nitrate and nitrite levels, *p ≤0.05. Δ= mean difference between the two changes for each pair. Data were analyzed using one sample t-test, †P<0.05.

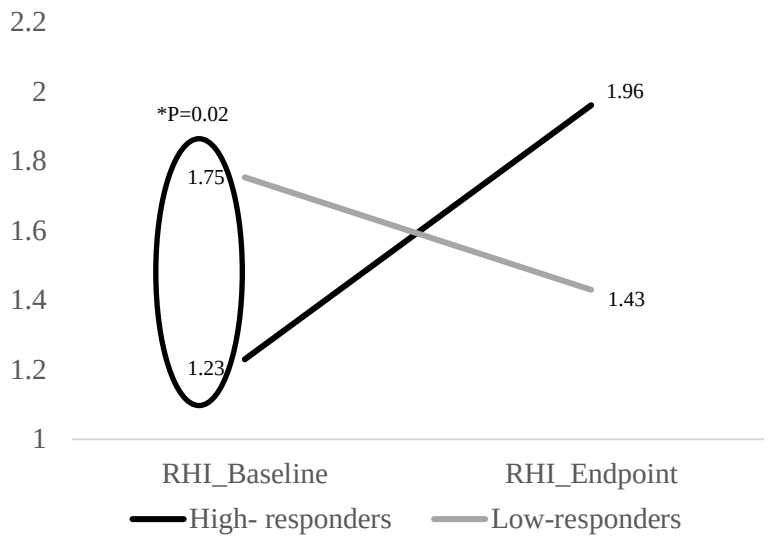


Figure 3-4. Change of RHI over time following NC treatment between high-responders and low-responders

RHI score response was compared between high-responders (n=8) and low-responders (n=8) before and after 4 weeks of NC treatment. NC: inorganic nitrate -rich beet juice and active vitamin C (1000mg). RHI: Reactive hyperemia index. Subjects with greater change score in NC were defined as high responders. Data were analyzed using independent sample t-test to assess RHI baseline score between high-responders and low-responders, *p ≤0.05.

3.5 Discussion

3.5.1 Vascular testing using RHI

The primary finding from this study was that there were no significant differences in endothelial function, as measured by RHI, between NC and N treatment groups. These findings do not support the first half of our hypothesis that 4 weeks co-administration of inorganic NO_3^- and vitamin C would elicit a greater improvement in endothelial function as determined by reactive hyperemia index (RHI) compared to nitrate alone. Previous work has shown a positive improvement in endothelial function following 4-6 weeks administration of inorganic nitrate in subjects with CVRFs [74, 140, 165]. All of these RCTs reported improvements in vascular reactivity following dietary interventions of nitrate have assessed endothelial function via FMD, a reliable method for the measurement of NO-mediated endothelial function [96, 111]. Improvements in FMD reported in these studies were achieved through an increase in NO bioavailability [242].

The absence of a significant change in RHI in the present study may have many explanations. Like FMD, RH-PAT measures are inversely related with traditional CVRFs;

however, previous studies have shown that correlations between FMD and RH-PAT measurements are controversial [97, 98, 103, 113, 224, 243]. It has been proposed that a key difference between these two measurement techniques is partially due to the fact that the RH-PAT method is in part dependent on NO [108] and different vasodilation regulators such as prostaglandins, adenosine, and other non-endothelium-dependent mediators underlying the post-reactive hyperemia response [107, 244]. As PAT measures do not solely assess NO mediated endothelial function, it has been suggested that PAT may not be sensitive enough to detect the influence of lifestyle modifications on microvascular beds [97, 107, 224]. This is in line with former work conducted by Wray et al. [213] that demonstrated significant improvement in endothelial dependent -vasodilation via FMD in conduit arteries following an acute-administration of an antioxidant cocktail but failed to show detectable changes when measured by reactive hyperemia in different vessels (resistance vessels) of elderly subjects. Moreover, it was suggested by Wray et al. that their vitamin C intervention could possibly improve endothelial function without influencing the microvascular function [213]. It has been postulated that endothelial improvement following vitamin C supplementation occurs through a reduction of oxidative stress that subsequently improves bioavailability of NO. However, the current study did not look at changes in oxidative stress associated with improvements in RHI and therefore cannot support this evidence.

An additional explanation for no alterations in RHI could be that EndoPAT manufacture guidelines established a threshold value for ED of <1.67 to define endothelial dysfunction [245]. An RHI value below 1.67 was found to have 80% sensitivity and 85% specificity to detect subjects with coronary endothelial dysfunction [246]. Therefore, another possible explanation for why no improvements were seen in our primary outcome could be that the baseline RHI values of some subjects were >1.67. Endothelial function scores greater than this cut point value may be less responsive to improvements in endothelial function. In our secondary subgroup analyses, it was shown that the eight "high responder" subjects who showed an individual increase in RHI following NC treatment (~60% improvements from baseline) had a baseline mean of RHI score below the threshold value of 1.67. Fifty percent of the high responders achieved an increase in RHI to normal or above normal values, while the other eight subjects (defined as low-responders), had a baseline mean of RHI >1.67. Our findings were supported by two different studies that noticed an improvement in RHI was substantial following dietary interventions in subjects with impaired endothelial dysfunction of <1.67 RHI value at baseline [114, 247]. For example, Maki et al. (2013) [247] demonstrated a significant improvement in RHI values following 5 weeks of consuming low-fat dairy products compared with low-fat non-dairy products among subgroup analyses of hypertensive subjects with RHI less than 1.67. Similarly, Kim and colleagues divided subjects into

two groups based on their initial levels of RHI using 1.67 as a cut value. They demonstrated that lycopene intervention for 8 weeks significantly increased RHI in subjects with impaired endothelial function at baseline [114]. Furthermore, although it is important to note that adherence data for vitamin C supplementation was excellent in all subjects, we were not able to determine if all subjects took vitamin C exactly one hour following CBJ consumption. Proper timing of vitamin C ingestion 1 hour after the nitrate dosing is important to permit adequate nitrate to enter the enterosalivary circulation, thereby maximizing the reduction of nitrite to NO in the gastric cavity as vitamin C consumed [161]. Improper timing of vitamin C intake with respect to CBJ intake may partially explain our results of no significant differences in the treatments combination versus nitrate alone. Our findings may suggest that responders adhered with the timing of vitamin C ingestion more than less-responders.

3.5.2 NO metabolites and vitamin C

In the current study nitrite and nitrate were used as surrogate measures of vascular NO bioavailability [18]. As expected, our NC intervention produce significant differences in plasma NO_3 and NO_2 levels compared to nitrate supplementation alone. Additionally, plasma vitamin C was significantly increased after 4 weeks of NC treatment. Our findings were inconsistent with recent work by Ashor et al., [154] who found no modifying effect of vitamin C on plasma nitrate and nitrite following supplementation with an acute single dose of potassium nitrate (5.1-8.7 mmol) combined with vitamin C (20mg/kg) in healthy individuals. This disparity in findings could be due to the study duration and the differences in the populations studied. Our results suggest that the addition of vitamin C to the consumption of inorganic nitrate supplementation for a longer duration of 4-weeks yielded a substantially greater improvement in circulatory markers of endothelial function in older adults with hypercholesterolemia. Continued supplementation of dietary nitrate and vitamin C in the present study elevated plasma nitrate to 8.8 fold in systemic circulation, while dietary nitrate alone caused a 6.0 fold increase in plasma nitrate after 4-weeks of intervention. When comparing our findings with a recent study that examined a similar nitrate supplement dosage (~6mmol) on hypercholesterolemia for 6 weeks [74], our work demonstrated that combined NC administration resulted in an 8.8 fold increase compared to a 7.5 fold increase following dietary nitrate alone after a longer duration of 6 weeks. These findings support the second-half of our hypothesis that exogenous vitamin C supplementation modifies the effect of dietary nitrate resulting in greater changes in plasma levels of NO metabolites compared to nitrate alone. Furthermore, the significant increase of plasma nitrate levels from baseline within each treatment group with or without vitamin C confirmed an excellent adherence with CBJ and supported what have been demonstrated in literature that plasma nitrate and nitrite are strongly

influenced by changes in dietary nitrate intake and the exogenous pathway NO_3-NO_2- NO is a good precursor for augmenting systemic NO production in subjects with CVRFs [74, 248].

When assessing plasma nitrite levels within each treatment group, we showed that 4 weeks of inorganic nitrate supplementation alone increased the nitrite circulatory levels to 1.2 fold which reflected that nitrite can be derived from dietary nitrate intake, while vitamin C combined with nitrate supplementation produced no significant changes in plasma nitrite over time. These findings following NC treatment could be due to the increased reduction of nitrite to NO and other nitrogen oxygen species through the exogenous pathway in the acidic environment of stomach with the presence of vitamin C resulted in substantial augmentation of NO and less availability of nitrite to be absorbed into circulatory system [162]. The second explanation could be due to the auto-oxidation of nitrite to nitrate as a stable end product of NO in a circulatory system [249, 250]. Another potential possibility for the observed plasma nitrite not increased significantly as the N treatment could be that vitamin C scavenges superoxide radicals, thus, increasing the bioavailability of the NO produced from nitrate [161].

3.6 Strengths and limitations

To our knowledge, this was the first study to examine the relationship between RHI and inorganic nitrate supplementation individually and in combination with vitamin C supplementation. This was also the first study investigating the therapeutic co-effect of inorganic nitrate and vitamin C supplementation among subjects at risk of CVD. This work has many strengths including the crossover study design, strict inclusion and exclusion criteria, and high supplement adherence. An additional strength is that dietary intake appeared to be consistent between the two interventions. There are also limitations in this work that should be taken into considerations for the future trials. There is wide inter-individual variation in RHI scores in response to study supplementation that is largely due to our small sample size. Small sample size may have limited our ability to observe significant differences between treatments or potential interactions between the dependent and independent variables when analyzing our outcomes. An additional explanation for the wide inter-individual variation of our PAT results may be the sensitivity of probes to movements which may have resulted in excessive signal noise exposure. Another limitation is that not all the subjects studied had a high prevalence of endothelial dysfunction. Subjects were selected based on their LDL concentrations of >130 mg/dL and their baseline RHI score of <2. The results of a subgroup subjects with ED (RHI≤1.67) suggest a significant association between NC and vascular measure. These observations deserve further attention in future studies given the potential additive impact of combined supplementation on restoring vascular health in subjects with impaired endothelial function. In addition, it is thought that FMD response is dependent on endothelium- derived NO

[242]. Therefore, examine the combined effect on endothelial function in large clinical trials using appropriate gold standard method like FMD to evaluate the treatment efficacy would draw a firm conclusion whether vitamin C could modify the effect of dietary nitrate supplementation on clinical measures of endothelial function.

3.7 Conclusion

The current study did not find a significant change in RHI between treatment groups among older adults with hypercholesterolemia, though a subgroup of subjects with low RHI at baseline have significant improvements in RHI. The addition of vitamin C to dietary nitrate may have resulted in the greater augmentation of systematic nitric oxide synthesis from the exogenous NO pathway. These improvements may positively influence the downstream pathways affected by NO production and improve endothelial function.

Chapter 4: **AIM 2**

4.1 Abstract

Introduction: Oxidative stress is a key mediator contributing in the promotion of endothelial dysfunction (ED) and atherosclerosis (AS), which may be counteracted by preserving endothelial nitric oxide (NO). Evidence has shown inorganic nitrate supplementation can improve NO bioavailability. It is conceivable that vitamin C may enhance the exogenous NO production through stepwise reduction of nitrate to nitrite to NO. **Purpose:** Our aim was to compare the change in plasma oxidized low density lipoprotein (oxLDL) to the sum of NO metabolites (NOx) ratio following the combined treatment of vitamin C and inorganic nitrate (NC) compared to inorganic nitrate alone (N).

Methods: Male and female subjects, aged 50-70 years with low-density lipoprotein (LDL) >130 mg/dL, and not treated with statin drugs were included in this randomized double-blind crossover study. Subjects were randomly assigned to start with either N (concentrated beetroot juice (CBJ) and placebo) (sequence1; N-NC) for 4 weeks or NC (CBJ with vitamin C (1000 mg)) (sequence2; NC-N) for 4 weeks in period1 and then crossed over to the alternate treatment for another 4 weeks in period2. At baseline and 4 weeks of each treatment, vascular NO bioavailability was assessed by plasma NO nitrate and nitrite (NOx) while oxidative stress was measured by plasma oxLDL to compare the magnitude change of oxLDL/NOx ratio between the two treatment groups. Plasma lipid profiles served as secondary outcome measures.

Results: NC treatment reduced oxLDL/NOx ratio compared to N (N: -3.97±2.83U/L; NC: -4.29±3.07U/L; p= 0.02). When analyzing the elements of the ratio separately, mean plasma oxLDL change was not different between treatments (N: -1.08±9.8 U/L; NC: -6.07±9.1 U/L; p= 0.19) although the reduction in oxLDL was evident over time for NC only (p=0.01). The mean plasma NOx change was significantly different between treatments (N: 94.24±82.9 μmol/L; NC: 128.70±123.5 μmol/L; p=0.01) and within group improvements in NOx were evident in both treatments ($p<0.05$). Four-weeks of NC treatment elicited significant reductions in the mean change of circulating concentrations of total cholesterol (N: 0.17±22; NC: -16.06±26; p=0.05), LDL cholesterol (N: 2.2±15; NC:-10.67±23; p=0.049), and triglycerides (N: 14.6±43; NC: -43.7±45; p=0.03). The mean change of HDL did not differ between treatments (N: 0.05±4; NC: 0.33±6; p=0.81).

Conclusions: NC reduced the oxLDL/NOx ratio, a new biomarker of ED, and showed promise in improving atherogenic lipids (oxLDL), and non-HDL lipid parameters in human subjects at risk for cardiovascular disease. These findings may have implications to reduce the incidence of cardiovascular events in diseases with ED.

4.2 Introduction

Hypercholesterolemia represents a major risk factor for the development of atherosclerosis (AS) [4, 60, 251]. Elevated low-density lipoprotein (LDL) concentrations are associated with both impairment of endothelial dependent vasodilation and stimulation of atherogenesis [37, 69, 70, 72, 74]. Vascular oxidative stress is also strongly associated with elevated LDL cholesterol in aging [46, 60]. Increased LDL particles are susceptible to oxidative modification when exposed to extra release of reactive oxygen species (ROS), particularly, superoxide radicals [27] that contribute to scavenging of nitric oxide (NO) and subsequent decrease in biological activity. In general, this imbalance between reduced NO bioavailability and overproduction of oxidative stress shifts the balance toward increasing ROS to further promote endothelial dysfunction (ED) [151]. During this process, oxidized low-density lipoproteins (oxLDL) are made in the vessel wall through oxidative modification. Mature oxLDL in the early stages of AS are known to precede the formation of foam cells, induce greater impairment in the endothelium than does non-oxidized LDL and consequently stimulates the progression of AS [32] when taken up by macrophages [87]. As oxLDL is a key determinant involved in all stages of AS [27], reducing the oxidative modification of LDL could slow the progression of future cardiovascular events [252].

Oxidized LDL and NO are the two characteristic features of ED, and share significant antagonistic roles in overall vascular health [60]. While oxLDL is a pro-atherogenic biomarker for cardiovascular risk factors, NO is a master signaling anti-atherogenic molecule that maintains vascular homeostasis and modulates *in vitro* lipid peroxidation reactions [52]. NO, presents in circulation from two potential sources but direct in vivo measurement of NO cannot be measured due to its short half-life (milliseconds) [22, 129, 253]. NO gets rapidly oxidized to nitrate and nitrite when it synthesized from the endogenous sources (arginine) by endothelium nitric oxide synthase (eNOS) enzyme. Dietary sources (dietary nitrate) is an alternative source of NO generation when eNOS is compromised [249]. Thus, nitrite and nitrate acts as stable end products that reflected total systemic NO levels from both pathways [129]. Combined measurements of NO metabolic pathway products (nitrate+nitrite) termed as NOx [46]. However, these plasma NO metabolites may not be good direct biomarkers of long-term nitrate exposure when measured on their own [23]. Likewise, plasma oxLDL as an individual parameter is not a strong independent predictor of coronary heart disease (CHD) after controlling for other lipid markers [60]. Borsa et al. (2012) [46] reported that systemic NO levels were negatively correlated with circulating oxLDL levels in hyperlipidemic elderly patients. They also found that the ratio of oxLDL/NOx is strongly correlated with important lipid biomarkers: (oxLDL/HDL), and (TC/HDL) [46], and has been considered as relevant biomarker of ED and aging [60].

There is a growing interest in examining the utility of dietary antioxidants and dietary nitrate on the management and reversal of ED and attenuating oxidative stress biomarkers [24, 125, 182]. Dietary supplementation with inorganic nitrate has been studied as a potential non-pharmacological therapeutic intervention to restore the biological activity of vascular NO in hypercholesterolemia, and increased NO status has been positively associated with improvements in endothelial function [74]. Preclinical studies have shown that inorganic nitrate supplementation has the potential to reduce well-established markers of vascular oxidative stress [150, 151], but the mechanism is unknown. However, scarce data are available demonstrate reduced oxidative stress markers in human studies following dietary nitrate supplementation [74, 153-155]. To date, Velmurugan et al. [74] found a trend for a greater reduction of oxLDL levels after administering 250 ml of beetroot juice (~6 mmol) at 6 weeks in patients with untreated hypercholesterolemia [74]. A better understanding of the use of dietary nitrate in relation to circulatory oxLDL in hypercholesterolemia is needed to further elucidate the dietary nitrate benefit on oxidative stress associated ED.

Vitamin C (ascorbic acid) is another dietary therapeutic intervention that may augment the NO pool and further improve endothelial health through different mechanisms [25]. By scavenging oxygen-derived free radicals and thereby preventing interaction with NO, and the subsequent formation of toxic oxLDL mediator peroxynitrite, vitamin C supplementation may protect LDL against oxidative changes [182, 207]. Another plausible mechanism is that vitamin C plays a role in enhancing the exogenous pathway of NO production through dietary nitrate reduction [154]. Under hypoxic and acidic condition in blood and gastric lumen, the presence of vitamin C may augment the reduction of nitrite to NO. The interaction between nitrate and vitamin C has been recently described in the literature and has indicated that vitamin C acts as an enhancer to catalyze the non-enzymatic nitrite reduction to NO, thus, increasing the NO pool [147, 218, 236-238].

$NO_2^- + H^+ \rightarrow HNO_2$

$2\ HNO_2 + \text{vitamin C} \rightarrow 2 \bullet NO + \text{dehydroascorbic acid} + 2\ H_2O$

Although dietary nitrate and vitamin C have been independently linked to augmentation of NO and reducing oxidative stress, there is a paucity of data that have examined the combination effect on cardiovascular health among patients with risk factors for cardiovascular disease. Recently, Ashor et al. (2019) demonstrated a significant synergistic effect following single dose co-administration of vitamin C with dietary nitrate supplementation on the reduction of 3-nitrotyrosine (3NT) levels compared to nitrate and vitamin C given alone in healthy individuals. These data shed light on the importance of further investigating the additive effect of these two dietary components on biomarkers of ED and oxidative stress among subjects with overload

oxidative stress. We aimed to target NO-substrate for oxidation with inorganic nitrate and vitamin C as a NO dietary supplement strategy in individuals with risk factors of CVD to better improve endothelial function by modulating the circulatory detectable markers of ED and oxidative stress [46, 88].

Thus, the purpose of this study was to investigate the combined effects of dietary nitrate supplementation with vitamin C for 4-weeks on a biomarker of endothelial function and oxidative stress as measured by oxLDL/NOx ratio compared to inorganic nitrate given alone in older adults with hypercholesterolemia. We hypothesized that the addition of vitamin C to nitrate supplementation would reduce the ratio of oxLDL to NOx status better than nitrate supplementation alone. Our secondary outcome was to identify changes in lipid profile between the interventions. We expected that the provision of nitrate and vitamin C together for 4 weeks would be associated favorable changes to the lipid profile [159, 254].

4.3 Materials and methods

4.3.1 Subjects

Male and female non-smokers with a body mass index (BMI) range of (18.5-34.9 kg/m_2) between the ages of 50 and 70 years of age with high LDL concentrations (>130 mg/dL) were enrolled in the study. Subjects were not eligible if receiving lipid lowering therapy, multivitamin supplements exceeding >100% of recommended dietary allowance (RDA), or on medications at the time of screening that would interfere with the study outcomes (e.g. hormone replacement therapy, clonidine, proton pump inhibitors (PPI) and antibiotics). Additional exclusion criteria included history of cardiovascular disease, diabetes mellitus, chronic inflammatory disease, celiac disease, uncontrolled hypertension (systolic blood pressure >160 mm Hg or diastolic blood pressure >100 mm Hg), controlled hypertension on clonidine medication, hepatic or renal disease, and active cancer. Moreover, subjects who consumed greater than 6 servings of alcohol per week, performed structured resistance or aerobic training for more than 1-hour four times per week, or participated in other clinical interventions within the previous 30 days were excluded from the study.

The study was approved by the university Institutional Review Board, and the informed written consent was obtained from all subjects prior to engaging in baseline measures. All institutional and governmental regulations concerning the use of human volunteers were followed during this research. All study visits were conducted in the Center for Clinical and Translational Sciences (CCTS), University of Kentucky, from February 2019 to September 2019.

4.3.2 Study design and randomization

This was a 10-week cross-over, double-blinded, placebo-controlled study. Subjects selected at random to compare the magnitude of change in the oxLDL/NOx ratio (primary outcome)

and a lipid profile (secondary outcome) following the 4-week treatment of the dietary nitrate supplementation alone (N) and combined with vitamin C (NC) interventions.

Eligible subjects were randomized to receive either treatment (N) or treatment (NC) for 4 weeks (period1), and subjects were then crossed over to the alternate treatment for another 4weeks (period 2). There was a 2-week washout at crossover. Throughout the intervention periods, enrolled subjects were advised to consume low- vitamin C diet throughout the study. They were provided with a list of high vitamin C foods/fortified foods at the first visit to avoid during the study **(Figure 4-1)**.

4.3.3 General clinical procedures

After showing interest in the study, potential volunteers were scheduled for a phone screening or face-to face interview to assess height/weight for BMI and check inclusion and exclusion criteria with brief questions about medical history to check for potential eligibility. Anthropometrics measurements and blood draws were performed 4 times throughout the study at baseline and endpoint visits of each intervention period. Venous blood samples were collected after an overnight fast during the study visit by trained staff at University of Kentucky's CCTS to measure plasma nitrate and nitrite, plasma vitamin C, plasma oxLDL, and a lipid profile. Plasma samples were stored at −80°C for analysis at end study. Plasma levels of NO metabolites and oxidized LDL were measured pre and post intervention of each treatment to calculate the ratio of atherogenic marker (oxLDL) to the biomarker of ED (NO related species ratio (NOx)). We assessed this ratio oxLDL/NOx as it could be a more reliable marker than individual parameters. Other biomarkers for secondary outcomes were obtained on the same testing days as anthropometric measures and included a lipid profile (serum total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL), and high-density lipoprotein (HDL). All blood draws were measured at baseline and endpoint visits of each intervention periods except for the plasma vitamin C concentrations at the second baseline visit (baseline2) following the washout period. A washout period of two weeks was chosen based on previous work that documented this period was successfully returned NO metabolites and vitamin C to baseline level [219, 221].

Subjects were instructed to maintain their usual dietary patterns throughout the study and dietary intake was assessed via 24-hr dietary recall. Three 24-h dietary recalls were collected and analyzed using Nutrition Data System for Research (NDS-R, Version 2019, University of Minnesota, Minneapolis, MN, USA) at midpoint of each interventional period, covering 1 weekend and 2 weekdays for a total of 6 recalls.

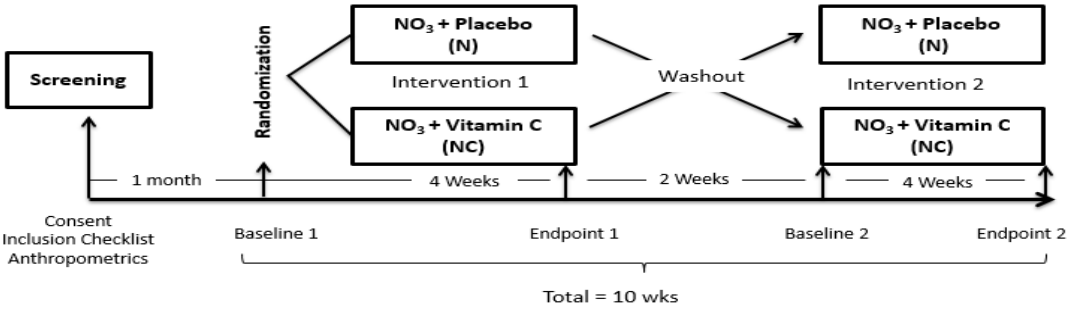


Figure 4-1. Schematic representation of the study design.

NO_3: inorganic nitrate provided in CBJ dietary supplement. (N): inorganic nitrate and vitamin C placebo; (NC): inorganic nitrate and active vitamin C.

4.3.4 Plasma biomarkers

a. Oxidized LDL and NO metabolites

Plasma oxidized LDL and nitrate/nitrite were measured by the UK CCTS bioanalytic core lab using commercially available assay kits according to the manufacturer's instructions. A commercially available sandwich chemiluminescent immunoassay was used to measure plasma oxidized LDL (Mercodia kit, Uppsala, Sweden) based on the monoclonal antibody 4E6 [255]. Nitrate/nitrite are measured by the Griess Reaction [239], a reaction that produces a diazonium ion which subsequently couples to amine 1-naphthylamine to produce a red-violet color (absorbance @ 540-570 nM). The assays were done according to manufacture instructions.

b. Vitamin c and lipid profile

Plasma vitamin C and plasma lipid profiles were measured by the UK Hospital Clinical Lab. Plasma concentrations of ascorbic acid were analyzed by HPLC. For this measurement, 4.5 mL of 5% metaphosphoric acid was added to 0.5 mL of plasma within 1 hour after venipuncture and stored at -80°C [200].

Fasting plasma TG and TC concentrations were determined enzymatically using the Specific Clinical Chemistry Analyzer. Heparin– manganese chloride precipitation of plasma was used to measure high-density lipoprotein (HDL) cholesterol. LDL concentration was calculated by the Friedewald formula [256].

4.3.5 Dietary supplementation intervention

The nitrate supplement was provided to all subjects in the form of commercial beetroot juice beverage (Sport Beet IT shot, Heartbeet Ltd). Sport Beet IT was a concentrated beetroot juice (CBJ) (70ml) that delivered an average of 300-400mg of inorganic nitrate. Vitamin C and the placebo were provided as supplement capsules. The study coordinator was blinded to the treatment assignment and the randomization was conducted by investigational Drug service (IDS) at University of Kentucky, which prepared the list of randomization treatment sequence and then

provided to the study coordinator with the treatment allocation. The preparation and labeling of the vitamin C and its placebo were performed in such a way that both pills were identical in shape, color, and size to ensure adequate blinding of both subjects and investigators. Vitamin C supplements were either active capsules that contained 500 mg of ascorbic acid (Nature Made ®) or corn starch in the placebo capsules. The dose of vitamin C provided in this study was based on previous work by Ashor et. al who reported in meta-analysis study that 700 mg of vitamin C or higher elicited an improvement in endothelial function [25]. The study coordinator instructed subjects to self-administer CBJ for 7 days a week at the same time by mouth daily during the morning hours and then consume 2 pills (1000 mg of vitamin C or placebo) one hour later.

Supplement adherence was assessed by counting the unused pills and beet juice bottles. Subjects were asked to return any unused CBJ bottles and vitamin C pills at the following endpoint visits of each intervention for pill counts.

4.3.6 Statistical analysis

Using the nQuery Advisor program, sample size calculations were based on the work of Kwak et al. (2012) who assessed endothelial function in newly diagnosed type II diabetes subjects [116]. Based on these data, nineteen subjects were needed for 80% power with a two-sided p-value of $\leq$ 0.05 set for statistical significance. Statistical analysis was performed using Statistical Package for Social Sciences (SPSS) software (version 22, IBM, Armonk, NY). Distribution of continuous variables were examined by Shapiro-Wilk test. Within treatment group, comparisons at baseline and 4-weeks for each treatment were conducted by Paired Student t-tests (for normally distributed data) and Wilcoxon signed rank-test test (for non-normally distributed data) for two period data separately. Changes in measures over the 4-week period were assessed by calculating the difference between the pre and post-intervention measures. Between treatment group, the treatment effect was evaluated by the two-sided one-sample t-test which is based on the mean of the differences between the two changes for each pair (Δ) [240]. Additionally, based on work by Wellek et al. [241] ,we examined whether there were any carryover effects from one treatment period to another. Linear mixed model was used for secondary analysis to assess the potential interaction effect of variables such as BMI and BP medication, sex, and age with the dependent variables in the two treatments. Pearson and Spearman correlations were used to assess the relationship between the dependent variables. Spearman correlations were used for variables that were not normally distributed. All tables reflect data in means ± standard deviation. All graphs reflect data in means ± standard error of the mean (SEM).

4.4 Results

4.4.1 Subjects

A total of 162 subjects were screened for eligibility and 23 eligible subjects were randomized. Of these, 18 completed both interventions and all study visits (**Figure 4-2**). Subjects had a mean age of 59±4 years and a BMI of 28.3±3 kg/m2. Thirteen subjects had a BMI ranging from normal to overweight ranging from 22.5 to 29.9 kg/m2. Five subjects were obese (class I) with a BMI range (30.8 to 34.4 kg/m2). There were no significant differences in baseline characteristics between the two treatment sequence groups (sequence 1: N-NC and sequence 2: NC-N) (**Table 4-1**). No significant changes in body mass were observed between the two treatment periods.

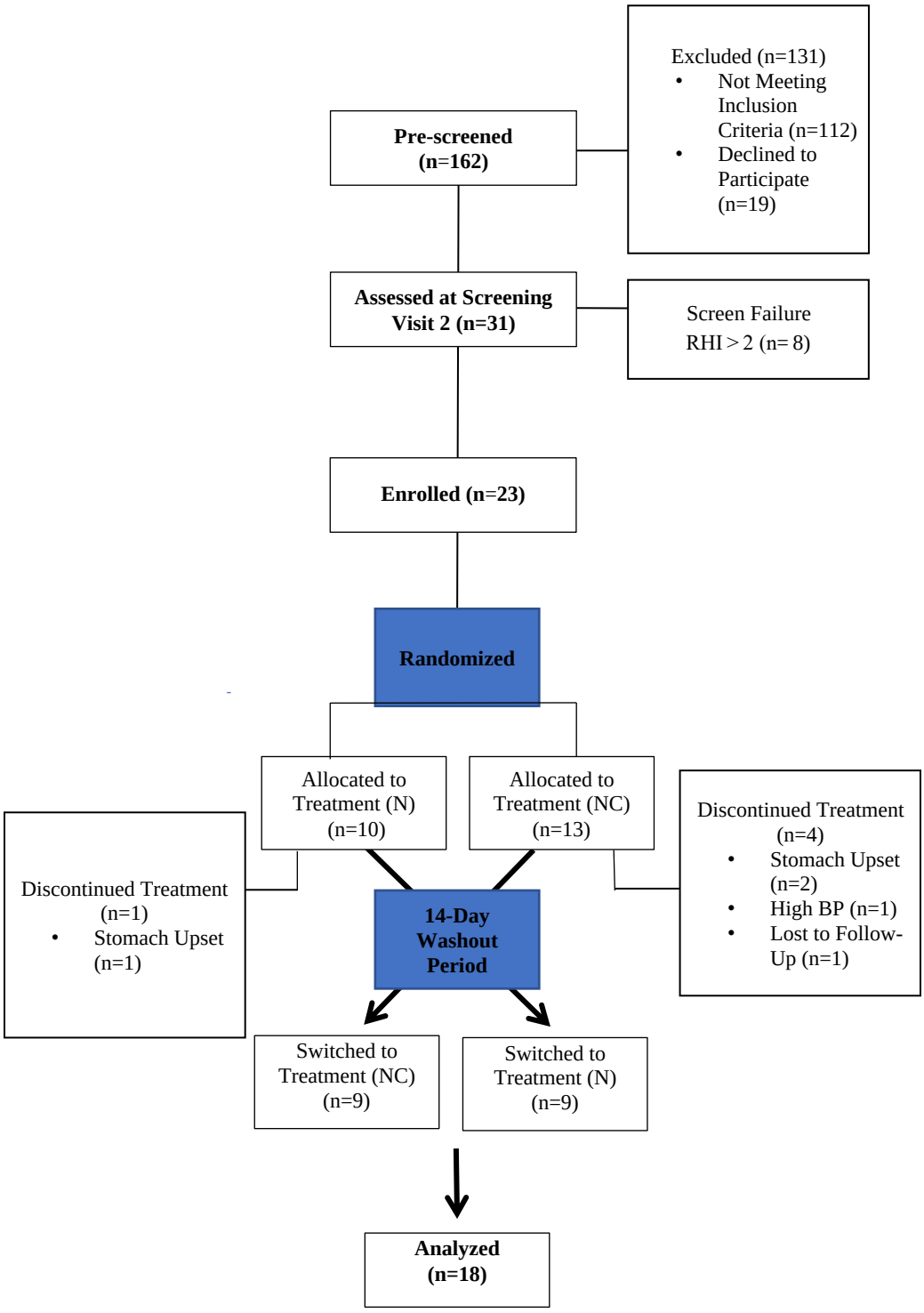


Figure 4-2. Consort Diagram

Table 4-1. Baseline Characteristics

Variables	Treatment N (sequence1; N-NC)	Treatment NC (sequence2; NC-N)	Total
	(n=9)	(n=9)	(n=18)
Male sex, n (%)	3(33%)	4(44%)	7(39%)
Age (years)	58±3	59±5	59±4
BMI (kg/m2)	29.6±3.1	27.1±3.0	28.3±3.2
Systolic BP (mmHg)	130±15.3	125±11.9	127.5±13.5
Diastolic BP (mmHg)	84±10.0	81±6.1	85.5±8.2
Subjects on BP medications, n (%)	3 (33.3%)	3 (33.3%)	6(33.3%)
Plasma vitamin C (mg/dL)	0.66±0.5	0.88±0.5	0.8 ±0.4
Plasma NOx (µmol/L)	14.4±5.3	18.9±8.4	16.7 ±7.2
Plasma oxLDL (U/L)	71.7±8.7	80±14.5	75.9±12.4
Plasma TC (mg/dL)	235.4±19.9	249.1±48.4	242.3 ±36.6
Plasma LDL (mg/dL)	135.3±18.4	167.9±41.0	160.6 ±31.7
Plasma HDL (mg/dL)	55.1±6.5	54 ±11.9	54.56±9.3
Plasma TG (mg/dL)	119.2±49.1	136±53.4	127.6±50.5

BMI: body mass index; BP: blood pressure; NOx: nitric oxide metabolic pathway products (nitrate (NO_3) + nitrite (NO_2)); TC: total cholesterol; LDL: low-density lipoprotein; HDL: high-density lipoprotein; TG: Triglycerides. (sequence 1; N-NC): subjects were assigned to randomly start with inorganic supplementation alone in period 1 and then crossed over to inorganic nitrate with vitamin C in period 2. (sequence2; NC-N): start with inorganic supplementation with vitamin C in period 1 and then crossed over to inorganic nitrate supplementation alone in period 2. Quantitative variables were described as the mean±SD. Variables were not significantly different between treatment groups at baseline.

4.4.2 Ratio of oxidized LDL to nitric oxide metabolites

For our primary outcome of examining differences in the ratio of oxLDL/NOx, there was a significant reduction following NC compared to N (N treatment: -3.97±2.83 U/L; NC treatment: -4.29±3.07U/L; p=0.02). No evidence of a carryover effect was observed from one treatment course

to the other (p=0.74). When ratio elements were analyzed separately, no significant difference was observed in the change of plasma oxLDL by treatment (N: -1.08.±9.8 U/L; NC: -6.07±9.1 U/L; p= 0.19) but a significant difference was observed in the change of plasma NOx status between the two treatments (N: 94.24±82.9 μmol/L; NC: 128.7±123.5 μmol/L; p=0.01). Secondary analysis did not reveal significant interactions between any of the dependent variables and subject characteristics such as age, sex, BMI, and BP medications.

Within treatment group comparisons revealed that the endpoint oxLDL/NOx ratio was significantly lower compared to baseline (p<0.001) **(Figure 4- 3A)**. When oxLDL and NOx variables were analyzed separately, NC endpoint oxLDL was significantly lower than baseline (p=0.01) **(Figure 4- 3B)**. However, N endpoint oxLDL showed no change over time compared to baseline (p=0.64). N and NC endpoint measures of plasma NOx levels were significantly higher by time (p<0.001) **(Figure 4- 3C)**. Our secondary analysis indicated that post-supplementation changes in plasma NOx level correlated significantly with changes in plasma vitamin C following NC treatment (r=0.49, p=0.04) with no correlation seen following N treatment (r=0.29, p=0.26).

4.4.3 Vitamin C concentrations

One subject who started with N treatment was excluded from statistical analysis due to missing data at endpoint. There was a significant difference in plasma vitamin C change between treatments (N: -0.14±0.53 mg/dL; NC: 0.45±0.66; p<0.001). Within treatment group comparison showed that plasma vitamin C concentrations at endpoint increased significantly over time following 4-week administration of NC (p=0.01) **(Figure 4- 3D).**

4.4.4 Lipid profile

Fasting lipid profile analysis indicated that the mean change in lipids was significantly different following NC and N treatments for LDL (N: 2.2±15; NC: -10.67±23; p=0.049) and TC (N: 0.17±22; NC: -16.06±26; p=0.05). Serum HDL was unaltered between treatment groups (N: 0.05±4; NC: 0.33±6; p=0.81). No carry over was evident in LDL (p=0.78), TC (p=0.73), and HDL (p=0.93). However, there was a significant carryover effect in TG (p=0.03). Therefore, we compared only data of period 1 for TG variable between the two treatments. We demonstrated a significant difference in the mean change of TG between treatments (N: 14.6±43; NC: -43.7±45; p=0.01).

Within treatment group comparison showed no change in TC (p=0.97), LDL (p=0.52), TG (0.33), and HDL (p=0.95) from pre- to post following N treatment. In contrast, there were significant decreases in TC (0.02) and TG (0.02) with a trend towards a reduction in LDL concentrations from pre- to post following NC (p=0.06) (**Figure 4-4A-C)**. HDL did not change by time after NC treatment (p=0.83) **(Figure 4-4D)**. Secondary, exploratory correlational analysis

demonstrated changes in plasma oxLDL levels correlated significantly with changes in plasma LDL ($r=0.84$, $p<0.001$) and TC ($r=0.85$, $p<0.001$) following NC and N, ($r=0.59$, $p<0.001$) and TC ($r=0.64$, $p<0.01$) respectively.

4.4.5 Supplement adherence

Overall, subject reported adherence for taking dietary supplements in both interventional periods was 99.7% in period1 and 99% in period2 for CBJ, and 98% for period1 and 97.5% in period2 for vitamin C.

4.4.6 Dietary patterns

Analyses of three 24-hour dietary indicated that no significant differences in nutrient intake and dietary pattern (**Table 4-2**). Three subjects were removed from the analysis because dietary recalls were missed in period 2.

Table 4-2. Nutrient Intake and Dietary Patterns

	N Treatment (n=15)	NC Treatment (n=15)	P-value
Energy (Kcal)	1757.2±505.2	1571.8±537.7	0.17
Protein (g)	65.6 ± 16.2	62.8±15.1	0.49
Carbohydrate (g)	202.9 ± 81.0	177.6±88.7	0.26
Fat (g)	80.9 ± 31.9	69.9±23.9	0.12
MUFA (g)	27.5 ± 11.6	26.1±9.7	0.58
PUFA (g)	17.7 ± 10.2	13.8±5.2	0.13
SFA (g)	29.2±14.0	24.0±10.7	0.057
Vitamin C (mg)	46.5±23.9	59.6±49.4	0.23
Vitamin E (mg)	10.3±5.4	9.8±3.5	0.69
Alcohol (g)	3.4±6.1	2.6±4.4	0.24
Caffeine (mg)	244.1±132.2	243.6±120.9	0.98
Dietary patterns			
Dark-Green vegetables Servings/day	0.56±0.7	0.59±0.7	0.92
Cured meat Servings/day	0.05±0.1	0.07±0.1	0.22

MUFA: mono-unsaturated fatty acids; PUFA: Poly-unsaturated fatty acids; SFA: Saturated fatty acids. P value were calculated from paired t-test between N vs. NC. N Treatment: inorganic nitrate-rich beet juice and vitamin C placebo or NC Treatment: inorganic nitrate-rich beet juice and active vitamin C (1000mg). Values were not significantly different between treatment groups. Values are presented as mean ±SD.

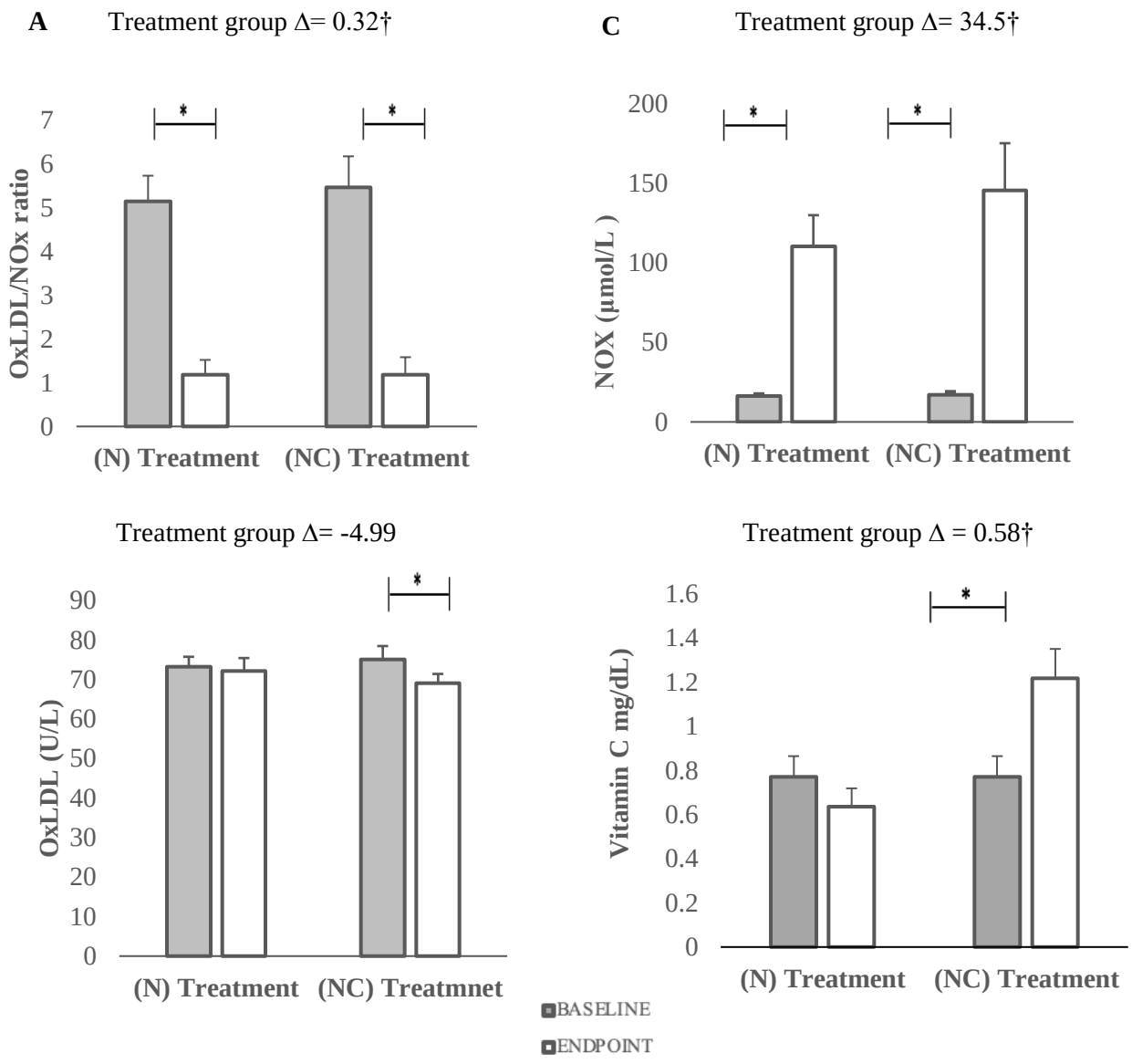


Figure 4-3. Circulatory biomarkers in response to 4 weeks interventions.

OxLDL /NOx: Oxidized low-density lipoprotein to nitric oxide metabolites ratio (n=18) (3A), NOx: plasma NO metabolites (n=18) (3B), oxLDL: plasma oxidized low-density lipoprotein (n=18)(3C), and plasma vitamin C (n=17) (3D) before (dark gray panel) and after 4-weeks of supplementation (white panel) with (N): inorganic nitrate and vitamin C placebo or (NC): inorganic nitrate and active vitamin C. Values represent means ± SEM. Data were analyzed using paired t-test for oxLDL and vitamin C, and Wilcoxon-test for the ratio and plasma NOx, *p ≤0.05. Δ= mean difference between the two changes for each pair. Data were analyzed using one sample t-test, †P<0.05.

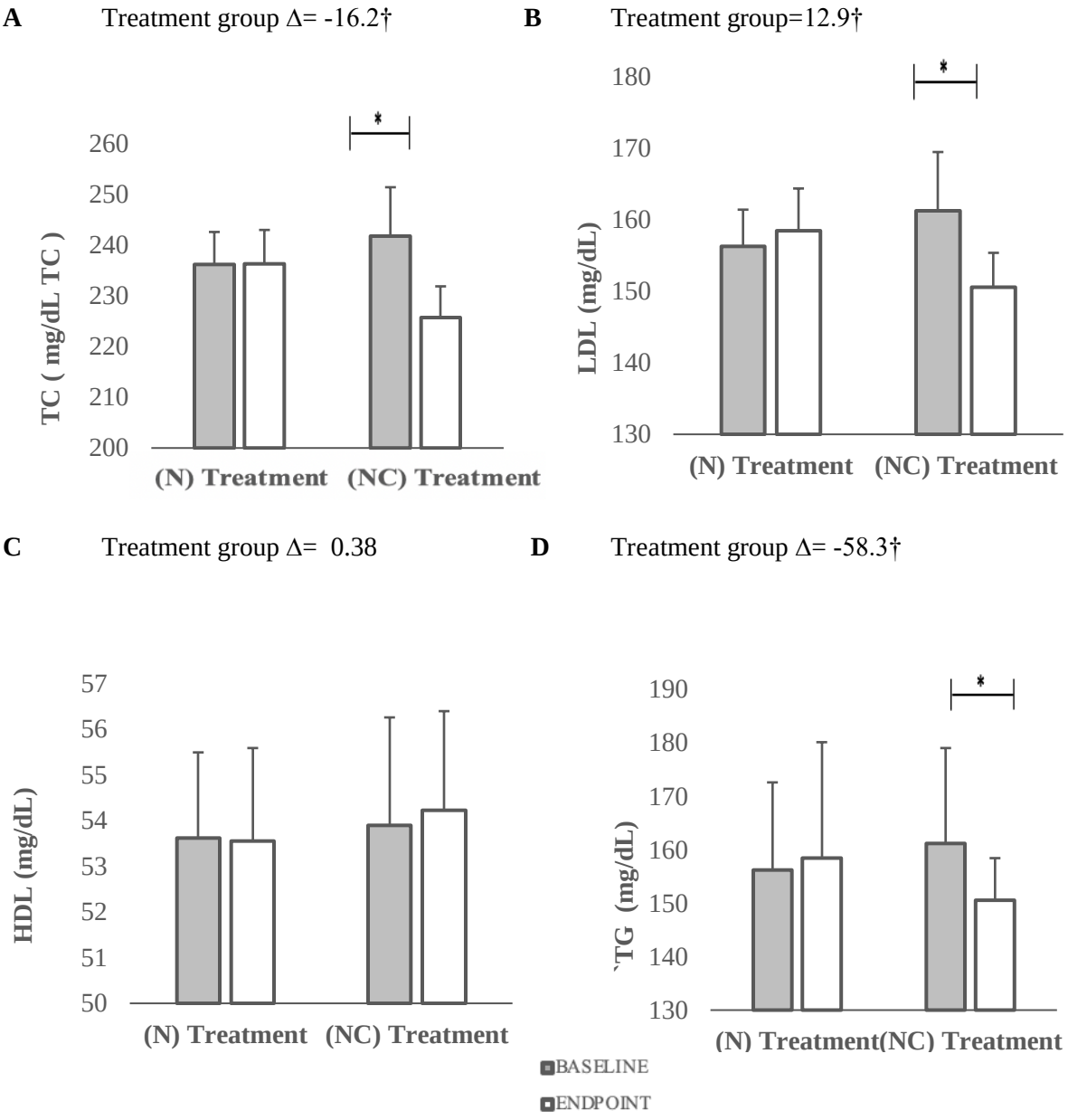


Figure 4-4. Serum Lipid profile in response to 4 weeks interventions.

TC: Total cholesterol (n=18) (4A), LDL: low-density lipoprotein (n=18) (4B), HDL: high-density lipoprotein (n=18) (4C), and TG: triglycerides (n=9) (4D) before (dark gray panel) and after 4-weeks of supplementation (white panel) with (N): inorganic nitrate and vitamin C placebo or (NC): inorganic nitrate and active vitamin C. Values represent means ± SEM. Data were analyzed using paired t-test for all lipid profile parameters, *p ≤0.05. Δ= mean difference between the two changes for each pair. Data were analyzed using one sample t-test, †P<0.05.

4.5 Discussion

4.5.1 Endothelial dysfunction and oxidative stress biomarkers

This study was the first examination of a potentially therapeutic dietary supplement approach investigating the impact of NC versus N supplementation on the magnitude of change in the oxLDL to NOx ratio and associated cardiovascular biomarkers. The ratio of oxLDL/NOx is a new marker of endothelial dysfunction associated with aging [46, 60]. The most significant finding from this work was the reduction in the ratio of oxLDL to NOx status following NC treatment. This finding supports our hypothesis that the addition of vitamin C to nitrate supplementation yielded a greater reduction in the ratio of oxLDL/NOx status compared to nitrate supplementation alone.

The significant treatment group effect on our oxLDL/NOx ratio outcome appears to be largely driven by NOx changes. We observed that the greatest reduction in oxLDL/NOx ratio over 4 weeks was associated with NOx levels between treatment groups. NOx levels were ~2 fold higher in NC than in N treatment. Our finding suggests that the addition of vitamin C supplementation to inorganic nitrate supplementation produced greater improvements in plasma NOx bioavailability resulting in greater reduction in oxLDL/NOx ratio. The elevation of total plasma NOx and vitamin C concentrations following NC confirm the capacity of vitamin C as a potent reducing agent to nitrite reduction [24-28], which in turn appears to increase the circulatory NO pool greater than nitrate supplementation alone.

Based on promising findings from both animal [150, 151, 257, 258] and human studies [153, 154] that reported a reduction in well-established blood markers of oxidative stress, we expected to see treatment group differences in oxLDL change following N versus NC treatment. However, no treatment group differences were observed. When evaluating the effects of individual treatments over time, N showed no changes in oxLDL levels which are in line with a previous report by Velmerurgan et al. (2015) [74], showing that the administration of a similar dosage of dietary nitrate supplementation (~6mmol) for 6 weeks had no effect in untreated hypercholesterolemic adults. Similarly, Larsen et al. [155] reported no changes in total antioxidant capacity and plasma isoprostanes F_2 following 3 days administration of inorganic nitrate supplementation. One factor that could contribute to the disparity between studies examining dietary nitrate and oxidative stress could be that studies evaluate oxidative stress with different blood markers [153]. The second factor could be differences in the magnitude of oxidative stress between subjects in various studies that may require higher antioxidant dose to reduce LDL oxidation [153]. The strong association we observed between circulating oxLDL and plasma lipid profile (TC and LDL) in both treatments underscore the value of oxLDL as a biomarker for atherosclerosis in subjects with hypercholesterolemia. Based on our findings and work from others

[74] who studied a similar oxidative stress marker, 6mmol of dietary nitrate for a 4-week study duration may not be sufficient to modulate oxLDL. In contrast, inorganic nitrate favorably modified oxLDL levels when examining within-group changes over time following NC treatment. After only four weeks, the oxLDL reductions suggests that the addition of vitamin C to inorganic nitrate produced a clinically significant change in oxLDL by -6.07 U/L in hypercholesterolemic adults. These findings are in accordance with a recent study that found a synergistic effect of acute single dose of combined nitrate and vitamin C supplementation on the oxidative stress marker of 3-NT [154]. We speculate that the combined antioxidant capacity of inorganic nitrate and vitamin C complement each other to augment NO production as well as a decrease of oxidative stress in subjects at risk of CVD. Furthermore, to our knowledge, many previous studies have demonstrated benefits of vitamin C supplementation in the absence of inorganic nitrate have shown that vitamin C reduces the susceptibility of LDL to oxidation in vitro [198, 199]. However, the effect of vitamin C supplementation on circulating oxLDL levels have been rarely examined. Two previous studies have examined same dose of 500 mg for 4-weeks [200] and 12weeks [259] in subjects with CVRFs. The former found no association with changes in circulating oxLDL, while the latter found a chronic supplementation of vitamin C associated with lowering oxLDL by -18.32 U/L. Although the current study did not look at the changes following vitamin C alone, we found a short-term intervention of NC treatments for 4 weeks reduced circulating oxLDL by 6.07U/L. Therefore, it is worthwhile to design a study that compared the NC effect versus vitamin C alone on oxidative stress marker and lipids.

The potential therapeutic benefits of NC for the reduction of oxLDL to NOx ratio can be attributed to two proposed mechanisms: the abundance of NO availability surrounding oxidants may have a direct effect on attenuating the lipid peroxidation reaction into the vascular wall [46]. Some studies have suggested that NO derived from exogenous nitrate supplementation possesses antioxidant properties that may reduce vascular oxidative stress *in vivo* in humans [153]. However, this is less clear and still requires further investigation to examine the antioxidant therapeutic potential effect of dietary nitrate alone in humans *in vivo* oxidative stress. The second mechanism could be explained by the indirect effect of a strong antioxidant vitamin C on augmenting NO bioavailability through inhibiting the ROS. This inhibition might shift the balance back toward NO while in turn protecting its bioavailability from ROS interaction and oxLDL formation [182, 207]. Overall, the sustained efforts must be undertaken to translate these results to be clinically significant before applying this ratio in clinical practice. Although the statistical significance between the two treatments highlighted that this ratio was influenced with short-term dietary supplementation, it

would be worthwhile to investigate whether a long- term dietary supplementation intervention could make a clinically significant difference.

4.5.2 Lipid profile

This was the first study that examined the combined effect of NC on lipid profiles in older adults with CVRFs. NC produced a significant reduction in many lipids compared to N. We found that NC treatment provided substantial reductions in TC (-16.1 mg/dL), LDL (-10.7 mg/dL), and TG (-43 mg/dL) but failed to produce a significant increase on HDL cholesterol. This translated to approximately a 6.6 % reduction in TC and LDL and a 32% reduction in TG in 4 weeks. These results support the second half of our hypothesis suggesting that the addition of vitamin C to inorganic nitrate supplementation produced favorable changes in lipid profiles. Our findings are of particular interest because the magnitude of change on different blood lipids are greater when compared with the magnitude of change seen in a recent meta-analysis [202]. This meta-analysis investigated the effect of vitamin C supplementation (500 mg) for 4 to 24 weeks on blood lipids concentrations only in hypercholesterolemic adults with a pooled mean age of 58.9 years. Thirteen trials which included subjects with hypercholesterolemia found that TC, LDL, and TG decreased by -10.76 mg/dL, -7.9mg/dL, and -20.1 mg/dL. This translated to approximately a 4.5% reduction in TC, a 5% reduction in LDL and an 8.8% reduction in TG.

Some preclinical studies have shown that inorganic nitrate and nitrite administrations have the potential to reduce TG concentrations [156-158]. To our knowledge there are limited clinical trials that have investigated these outcomes, including a few studies with equivocal findings [39, 74, 159, 163]. Three trials did not show an effect of dietary nitrate on lipid biomarkers [39, 74, 163] and these studies support our findings following 4 weeks of N treatment. However, two trials reported that inorganic nitrate supplementation had favorable effects on dyslipidemia [159, 254]. One study found that 250 ml of raw beetroot juice for 14 days in hypertensive subjects reduced plasma TC by ~15 mg/dL and LDL by ~14mg/dL compared to baseline [254]. Another study examined the 30-day effect of tablet containing nitrate derived from beetroot along with the antioxidant hawthorn berry [159]. The authors found a significant increase in plasma nitrate and nitrites, and a 27% reduction in TG concentrations compared to pre-treatment concentrations in hypertriglyceridemia subjects. When comparing our results with the Zand et al. [159], it is important to note that the percentage of the TG lowering effect (-32%) seen in our work after only 4 weeks of NC was similar to the percentage of TG reduction shown with neo40 [159]. We have also noted from our findings that NC improved not only TG but all non-HDL parameters (TC, LDL, and TG). Collectively, the improvements in blood lipids from Zand et al. [159] and our work suggests that dietary nitrate supplementation in the form of beet juice may be a valuable dietary

supplement to combat dyslipidemia. However, the addition of the antioxidant vitamin C may serve as a significant enhancer for augmenting the dietary nitrate effect to promote improvement in plasma nitrate levels and induce reduction in non-HDL parameters which could positively impact overall cardiovascular health. In regards to mechanism of action, studies have revealed that nitrate and nitrite may affect fat metabolism and energy utilization [156, 260]. Roberts et al. demonstrated that nitrate induced mitochondrial "browning" and increased fat oxidation in white adipocytes [260]. However, the exact mechanism explaining the nitrate effect on blood lipids remains to be determined. Combined with vitamin C, it is unknown if systemic NO accumulation has a direct effect on blood lipids or if the direct beneficial effect is explained by vitamin C supplementation. In addition, it has been suggested that vitamin C supplementation may enhance the reduction of serum lipids through two mechanisms either by enhancing the conversion of cholesterol to bile acids or by counteracting the oxidation of LDL. As a result of both mechanisms, vitamin C increases the uptake of LDL by the liver cells, hence, promoting LDL removal from the plasma concentrations [202, 205, 206, 261]. Future studies with four arms will determine if the reduction of oxidative stress and lipid parameters was a result of the co-administration of inorganic nitrate with vitamin C or was a response to vitamin C intervention alone. Further animal studies are required to elucidate the mechanism behind the observed effects.

4.6 Strengths and limitations

This was the first study targeting the ratio of oxLDL/NOx with therapeutic interventions to evaluate the status of endothelial function in an older adult population with CVRFs. The strengths of this study include the study design that controlled variability between subjects, and high supplement adherence. There were also some limitations to consider when interpreting these results. The major limitation in this study was the small sample size. The study was powered for the primary outcome measure of the absolute change in the RHI response at 4 weeks from baseline. Although we were able to demonstrate differences between placebo and active vitamin C interventions in most variables, comparison interpretations between N and NC should be made cautiously. It is important to note while the carryover effect may not have reached statistical significance in most of the variables, the small sample size may have driven that. Although we tested that carry over effect between the two treatment periods and no effect was observed in most variables, a number of exploratory analyses in addition to the carryover effect of some variables were conducted for which the study was not powered including all of the outcomes of study aim 2. Another limitation was the inability to compare NC outcomes with the vitamin C supplementation only group. The current study did not look at the changes following vitamin C alone, therefore we cannot compare our findings with very limited published studies that

examined the link between vitamin C and circulating oxidized LDL. In addition, the small sample size with a short study duration of 4 weeks may have not been sufficient to detect the responsiveness of oxLDL to dietary nitrate supplementation. Furthermore, the lack of measuring additional biomarkers of oxidative stress such as plasma concentration of isoprostane, a stable *in vivo* marker of lipid peroxidation prevents us from further elucidating the benefit use of dietary nitrate on oxidative stress.

4.7 Conclusion

In conclusion, the provision of vitamin C combined with dietary nitrate supplementation is a natural, cost effective strategy that reduced oxLDL/NOx ratio and blood lipids in our sample of older adults with hypercholesterolemia. Our results suggest that targeting endothelial function and restoring the reduction of NO with a novel combination of dietary supplements promoted favorable changes on markers associated with cardiovascular disease. Dietary nitrate and vitamin C have been identified as key components in the dietary pathway to augment NO production. Eating a diet rich in high nitrate vegetables combined with vitamin C rich foods, may also provide a greater NO reservoir in diseases associated with ED and decreased eNOS activity and require further investigation.

Chapter 5: **GENERAL DISCUSSION**

This dissertation work focused on a novel dietary supplement intervention for the purpose of improving endothelial dysfunction (ED) in older adults at risk of cardiovascular disease. We compared the effectiveness of two, 4-week supplementation treatments consisting of inorganic nitrate in the form of concentrated beetroot juice with and without vitamin C on the magnitude changes of endothelial function (EF), oxidative stress, and lipid profile outcomes. We also compared the treatment effect on the aforementioned outcomes within each treatment group (before and after each treatment).

The primary outcomes of this dissertation were changes in clinical measures (Chapter 3) and blood biomarkers of endothelial function (Chapter 4) as determined by reactive hyperemia peripheral arterial tonometry index (RHI) and the ratio of oxidized LDL (oxLDL) to nitric oxide metabolites (NOx), respectively. Comparing the two treatments groups, we found no significant differences in the change of RHI in the overall sample of older adults with hypercholesterolemia and a baseline RHI less than 2. We also found that NC promoted greater augmentation of nitric oxide (NO) bioavailability that may have potentiated greater improvements in endothelial function in older adult subjects with hypercholesterolemia. In addition, we evaluated whether RHI was influenced by subjects' characteristics (age, blood pressure medications, baseline BMI, baseline RHI, and sex), and found that RHI significantly improved in subjects with greater endothelial dysfunction at baseline (RHI <1.67) than those who entered the study with mean RHI greater than 1.67. No other interactions between subject characteristics and our dependent variables were found.

Although no treatment differences were observed with our clinical measure of endothelial function, we found in the second aim of the study (Chapter 4 significant improvements in the change of oxLDL/NOx ratio, plasma vitamin C, and circulatory biomarkers of NO metabolites (nitrate and nitrite) were observed following nitrate and vitamin C co-supplementation (NC) compared to nitrate supplementation alone (N). In addition, vitamin C co-administration with inorganic nitrate showed a better response in reducing serum lipid profiles, particularly, total cholesterol (TC), low-density lipoprotein cholesterol (LDL), and triglycerides (TG) concentrations compared to nitrate alone. No differences were observed between treatment groups in HDL and oxLDL concentrations in hypercholesterolemic older adults.

Regarding to the dose-response -relationship and study duration, we used similar doses of NC to the previous work by Ashor et al. [154] and found that the addition of vitamin C for a longer duration (4 weeks) modified the effect of nitrate supplementation on the plasma nitrate response and produced additive improvements in the NOx accumulation. These results suggest that the

treatment effect of nitrate with vitamin C on NO metabolites may have been attributed to both the dose of the supplementation and the longer duration of treatment. However, when examining within treatment comparison of dietary nitrate alone on oxidative stress and lipid profile, we demonstrate that 6 mmol of nitrate in the form of CBJ for a duration of 4 weeks may not be sufficient to contribute to a meaningful alteration in the oxidative stress marker of oxLDL, and lipid profile. This findings was consistent with previous work by Velmerurgan et al. [74], that showed 6 mmol of dietary nitrate in the form of beet juice for a longer duration of 6 weeks was not effective to reduce oxLDL when examining within treatment comparison. The results presented in Chapter 4 demonstrated that introducing a daily 1g dosage of vitamin C with 6 mmol of dietary nitrate was associated with significant increases in both plasma ascorbate and plasma nitrate concentrations. We noticed that this combination over 4 weeks showed signs of ameliorating oxidative stress. We speculated that antioxidant protection provided by vitamin C may be complementing the dietary nitrate effect to reduce the oxidative damage and blood lipids in hypercholesterolemic adults greater than 50-year-old. Our results suggested that the provision of vitamin C with inorganic nitrate can protect against *in vivo* lipid peroxidation measured as oxLDL. This signifies the potentiation of the effects of both supplemental agents when introduced together.

In regards to the impact of aging on dietary nitrate interventions, we found the administration of inorganic NO_3 with and without vitamin C to our selected population of older adults was associated with a significant increase in the levels of plasma NOx with no effect on endothelial function. Aging is a risk factors associated with a number of functional changes in multiple organ systems, including the vasculature, that affect the functioning of all body systems [262, 263]. Previous evidence has demonstrated that low gastric acid, low vitamin C absorption, and changes in oral and intestinal microflora associated with aging [264] may modify the reducing steps in the conversion of dietary nitrate to NO, and the efficiency of dietary nitrate to generate a beneficial microvascular response [167]. Our findings suggest that a 6mmol dosage of inorganic nitrate derived from CBJ produced substantial changes in NO metabolites that led to increasing the NO pool in adults greater than 50-year-old in 4 weeks. This augmentation may be clinically significant by helping to overcome endothelial nitric oxide synthase (eNOS) dysfunction associated with cardiovascular risk factors. It seems that aging has no impact on the ability to increase levels of NO metabolites as long as adequate exogenous substrates are provided [124]. Our results support recent studies that have demonstrated beneficial effect of inorganic nitrate supplementation on NO metabolites in older adults at risk of cardiovascular disease [140, 165].

Data synthesis of the two aims in my dissertation revealed greater improvements in the NO bioavailability after supplementation with large doses of NC for short-term intervention compared

to N. These results align with previous studies that both dietary nitrate and vitamin C have potential roles to improve NO availability through different mechanisms [24, 207], and both have antioxidant properties to reduce oxidative stress and protect NO from being oxidized by reactive oxygen species [155, 182]. NO improvement following the daily administration of NC was the reason behind the successful improvements of endothelial function, oxidative stress marker, and blood lipids which may have positive impact on cardiovascular health. Moreover, we found out that the changes of oxLDL/NOx ratio and non-HDL lipid markers were not associated with the change of RHI score suggesting that some of the beneficial effects of inorganic nitrate and vitamin C co-supplementation were not dependent on changes in clinical measures of endothelial function. Findings from this dissertation provided evidence of concept that NO can be increased as a result of CBJ supplementation, particularly when combined with vitamin C, in subjects with cardiovascular risk factors.

5.1 Strengths:

There are several key strengths in this dissertation.

- The major strengths of this study are the double-blinded placebo controlled cross-over design, the low dropout rate, and the high adherence to treatment in both periods and the restricted inclusion and exclusion criteria.
- Another strength was the examination of numerous markers associated with cardiovascular disease.
- This study demonstrated the potential of beneficial effect of nitrate combined with vitamin C on markers associated with cardiovascular outcomes.
- Subjects adherence to maintaining their usual diet during the two study periods. Our dietary analysis showed no difference in dietary intake or dietary pattern between treatment sequences. This suggests that diet did not influence our between treatment study outcomes.

5.2 Limitations:

Many of the study limitations were related to data collection methodology that need consideration when designing future studies.

- A major limitation in both aims was that the data was underpowered for some variables due to the small sample size.
- Our study protocol did not emphasize the importance of recording the exact timing of vitamin C ingestion. This precluded the ability to conduct a secondary analysis aimed at analyzing the interaction of timing of vitamin C ingestion with endothelial function outcomes.
- Another limitation was related to the uses of the peripheral arterial tonometry (PAT) for assessment of endothelial function. RHI scores were highly variable and limited statistical power given the small sample size. The sensitivity of endoPAT test to NO production and its contributions of other

molecules besides NO to assess endothelial function may limited our ability to detect the differences between treatments.

- Additional limitations were due to study budget constraints. We were unable to collect specific biomarkers such as expelled air of stomach NO, or circulatory endothelial function markers including endothelin1, soluble endothelial marker with adhesion molecules, and asymmetric dimethylarginine. The intra-gastric NO marker may provide more insight to the interaction site and the amounts of NO formed from the entero-salivary circulation, while the other markers may provide a clearer picture on how the NC intervention influence key molecules that contribute improvement in endothelial function.
- Another study limitation was the lack of dietary assessment during the washout phase. The dietary assessment during this phase might clarify if the subjects changed their quality of diet that could affect their results in baseline visit post washout period.
- Finally, although all subjects agreed to maintain their physical activity throughout the study duration and to restrict their exercise to no more than 1 hour a day for 4 times a week, the role of physical activity as a potential confounding variable was not assessed throughout the study. Due to physical activity being a factor influencing positively the integrity of endothelial function [227, 265, 266], changes in activity patterns throughout the study duration could have affected the study outcomes, particularly, RHI.

5.3 Clinical implications

The NC combination may have implications in improving endothelial function through restoring NO availability in populations with impaired endothelial function. This combined supplementation strategy may have utility of other patient populations with disease etiologies that include endothelial dysfunction and oxidative stress such as aging, young adults with untreated hypercholesterolemia, and hypertension. It is unknown whether our intervention will have similar effect on different populations with higher risk of cardiovascular complications such as diabetes. There is a paucity of studies examined inorganic nitrate effect on vascular health among diabetic patients. Targeting those populations may have public health implications contribute to improve their vascular health and further their quality of life.

Based on the Atherosclerotic Risk in Communities Study [267], a reduction of LDL concentrations by -7.9 mg/dL equated to a decrease in the coronary heart disease (CHD) risk of 6.6%. A reduction in TG by -20mg/dL could be translated to a 2.4% reduction in CHD risk [202]. It is worthwhile to note the percentage of reduction in our findings exceeded these values and have significant clinical benefits in reducing the incidence of coronary heart disease, especially when combined with a healthy lifestyle. My dissertation research suggests dietary changes that include

increasing consumption of dietary nitrate sources such as green leafy vegetables and beet juice with vitamin C foods that would be helpful –"food-first" approach which may have implications to lower lipid profile and oxidative stress and further slow the progression or the incidence of cardiovascular events in diseases with ED.

5.4 Future directions

While this project provides preliminary evidence that combination of nitrate and vitamin C elicited improvements on markers of endothelial function, oxidative stress, and lipid profile, several questions remain to be unanswered. These questions can be answered questions in the future studies.

- The primary need is for this study to be replicated in a larger sample in a RCT. In addition, identifying the mechanism related to how these supplements interact when consumed together to result in favorable changes in blood lipids and reduced LDL oxidation needs to be determined in future animal studies.
- It would be interesting to stratify the co-administration of vitamin C with dietary nitrate into two groups: together at the same time versus introducing vitamin C an hour after beetroot juice supplementation to identify whether the timing of vitamin C ingestion would matter or not on the same study outcomes.
- To gain a better understanding on the impact of nitrate and vitamin C on subjects with poor endothelial function, identifying older adults subjects with reduced RHI scores (<1.67) at baseline should be considered to detect a treatment effect to examine the effect of dietary nitrate on vascular health. Another suggestion to further explore the NC treatment effect would be testing the endothelial function in a larger study population since FMD is a gold standard method to determine a potential role of this intervention in endothelial function.
- Most of the published studies examined dietary nitrate on vascular function were used small sample size and have excluded all vasoactive medications from the study. Focusing on diet modifications as complements to conventional pharmacological therapy may improve cardiovascular health. Future research efforts should be given to the design of trials testing co-supplementation of dietary nitrate and vitamin C in hypercholesterolemic subjects with and without cholesterol lowering medication (statin) to evaluate whether NC intervention provides additive effects beyond pharmacological therapies on the same outcomes we used in our study.
- In addition, it would be interesting to investigate the following in future studies: 1) whether the dietary nitrate and vitamin C supplements when consumed together would sustain their efficacy and sustainability of the physiological effects in longer chronic interventional trials > 4 weeks; 2) whether smaller supplemental doses, that correspond to vitamin C and nitrate intake expected in a

plant-based diet, would produce similar effect on endothelial function, lipid profile and oxidative stress markers as the doses we used in our study; 3) whether oxLDL reduction may require either a longer time course or higher dosage of nitrate supplementation alone since the data describing dietary nitrate use on circulating oxLDL is still scarce.

- It is worthwhile to examine the effect of introducing vitamin C-rich foods an hour after dietary nitrate consumption. Vitamin C from food may complement the beneficial effects of dietary nitrate on cardiovascular health and may reduce the supplement burden for consumers.
- Moreover, some recommendations I suggest when designing future studies are the following 1) design effective ways to encourage subjects to report the exact time of daily dietary supplement ingestion. 2) continue to explore and refine other dietary supplementation strategies with dietary nitrate that target the NO exogenous pathway to work more efficient and overcome the deficiency of endothelial NO derived from arginine before the presence of clinical manifestations of atherosclerosis [24, 124]. There are several options for dietary nitrate supplementation instead of the CBJ such as tablets or beet bars. These options might be more palatable and feasible than the beet juice. However, very little studies have tested their effects.

5.5 Conclusion

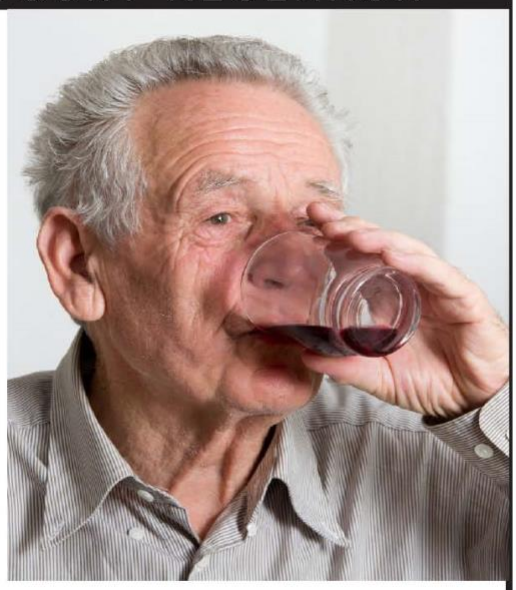
My dissertation research sheds light on the beneficial use of vitamin C with dietary nitrate supplementation as a therapy for improving endothelial function. The results from this valuable experience have encouraged me to continue developing research projects in the future to tackle cardiovascular disease with innovative dietary supplement approaches.

APPENDICES

Appendix. A

UNIVERSITY OF KENTUCKY RESEARCH

Dietary Supplementation Strategies That May Improve Cardiovascular Health



Researchers at the University of Kentucky are inviting you to participate in a study investigating how beetroot juice with vitamin C supplementation affects vascular health. All research will be conducted on the UK campus.

Participants will be reimbursed $60 upon completion of the study.

You may be eligible for this study if you:

- Have high cholesterol levels and are not on cholesterol medication
- Are between 50-70 years of age
- Do not smoke
- Are not diabetic

For more information, please contact:
Reem Basaqr
reem.basaqr@uky.edu
859-323-2042

UK Research

An Equal Opportunity University

www.UKclinicalresearch.com

Beetroot CLINSCI-005_flyer #

Appendix. B

	The Effect of Dietary Nitrate and Vitamin C on Endothelial Function and Oxidative Stress Biomarkers	Subject # Gender	
	PI Name: Travis Thomas	Subject Initials	
	IRB # and date: 17-0412-F3R	Subject DOB	
		Visit Date	

INCLUSION/EXCLUSION CRITERIA

Inclusion: *Subjects will **not** be considered eligible for the study if any of the following inclusion criteria is answered "no".*		
1.	Age between 50 and 70 years old. Age:	☐ Yes ☐ No
2.	Body mass index 18.5-34.9 kg/m^2 BMI: kg/m2	☐ Yes ☐ No
3.	Having LDL concentration of >130 mg/dl)	☐ Yes ☐ No
4.	Having poor endothelial function RHI≤2	☐ Yes ☐ No
5.	Willing to stop using mouthwash before and during the study duration.	☐ Yes ☐ No
6.	Fluent in English and able to understand and provide informed consent.	☐ Yes ☐ No
7.	Agree to avoid high rich sources of vitamin C throughout the study.	☐ Yes ☐ No
8.	Willing to participate in the study and comply with study requirements as evidenced by signed IRB-approved informed consent.	☐ Yes ☐ No

EXCLUSION: *Subjects will **not** be considered eligible if any of the following exclusion criteria is answered "yes".*		
1.	Any chronic illness which, in the opinion of the investigator, precludes study participation such as: History of previous CAD, peripheral vascular disease, stroke, or history of CVD.	☐ Yes ☐ No
2.	Surgery or medical condition that might confound study results such as: bowel resection, inflammatory bowel disease, and celiac disease.	☐ Yes ☐ No
3.	Uncontrolled hypertension (systolic blood pressure >160 mm Hg or diastolic blood pressure >100 mm Hg).	☐ Yes ☐ No
4.	Liver or kidney diseases.	☐ Yes ☐ No
5.	Active cancer.	☐ Yes ☐ No
6.	Severe anemia. Hemoglobin less than 10.0 g/dL at the time of enrollment.	☐ Yes ☐ No
7.	Using at least one of the following medications: Antibiotics or antacids (PPI) within 48 hours of the screening visit #2.	☐ Yes ☐ No
8.	Receiving sex of hormone replacement therapy (testosterone, estrogens, progesterone).	☐ Yes ☐ No
9.	Currently receiving multivitamin that provide > 100% of RDA for vitamin C.	☐ Yes ☐ No
10.	Currently Participating in any other study that involved investigational drugs or regimens.	☐ Yes ☐ No

11.	Performing Structured resistance or aerobic training for 1 hour 4 times/week.	☐ Yes ☐ No
12.	Inability or willingness of participant to comply with study protocol or procedures.	☐ Yes ☐ No
13.	Having positive HIV.	☐ Yes ☐ No
14.	Having finger deformities.	☐ Yes ☐ No
15.	Using any type of tobacco product within the past 1 month.	☐ Yes ☐ No
16.	Having heart failure.	☐ Yes ☐ No
17.	Consuming alcohol of more than 6 drinks (any form) per week.	☐ Yes ☐ No

VITALS		
Height	_____._____	☐ Inches ☐ Centimeters
Weight	_____._____	☐ lbs. ☐ Kilograms
Blood Pressure	_____/_____ mmHg	☐ Controlled ☐ Uncontrolled

SCREENING STAGE2/BASELINE 1

Appointment date: _____ **Time:** _____

Date completed: _____ **RHI:** _____

VITALS		
Height	_____._____	☐ Inches ☐ Centimeters
Weight	_____._____	☐ lbs. ☐ Kilograms
Blood Pressure	_____/_____ mmHg	☐ Controlled ☐ Uncontrolled

☐ **Eligible** ☐ **Ineligible** _____

Form completed by

_____ _____

Principal Investigator/Sub-Investigator signature ***Date***

MEDICAL HISTORY QUESTIONNSAIR

YES NO 1. Is your doctor currently prescribing drugs (for example, water pills) for your cholesterol level, blood pressure, heart, or any other condition?

Medicines/dosage: Taken For:

_____

_____

YES NO 2. Are you currently seeing your doctor or health care provider for any reason?
If yes, please explain any findings (heart murmur, abnormal pulmonary findings etc):

_____

_____

_____

YES NO 3. Do you currently smoke (or have a history of smoking) or use tobacco products?
When did you last quit?

_____

_____

YES NO 4. Have you had any surgeries of any kind? If yes, please list type, reason and year:

_____

_____

YES NO 5. Are you taking any over the counter medications, dietary supplements (vitamins or minerals) (multivitamins)?
Please list both type/dosage and reason for taking:

_____

_____

Appendix. C

Supplement Sheet Log

Patient Name:_____
Patient ID#:_____
Date Range:___/___/_____ to ___/___/___
Study period: 1 or 2
Week #: ____

WEEKLY SUPPLEMENT LOG

Instructions for the participant: This is a weekly log on which you are to record each supplement dose you take. For each dose write down the day, time, and amount consumed. Bring the log and all supplement packaging with you to your next scheduled supply pick-up.

Days of the week (Example: Monday)	Date	Supplement	Amount Consumed (Example: 1 small container of beetroot juice: 100%, all, 50%, half) 1pill, 2 pills, half pill)	Time taken? (Example:12:00 pm)				With or without food? Yes or No	Notes
		Small amount of beetroot juice			AM	Noon	PM		
		Blinded Pills			AM	Noon	PM		
		Small amount of beetroot juice			AM	Noon	PM		
		Blinded Pills			AM	Noon	PM		
		Small amount of beetroot juice			AM	Noon	PM		
		Blinded Pills			AM	Noon	PM		
		Small amount of beetroot juice			AM	Noon	PM		
		Blinded Pills			AM	Noon	PM		
		Small amount of beetroot juice			AM	Noon	PM		
		Blinded Pills			AM	Noon	PM		

Section to be completed by research personnel:

Distribution Date:___/___/_____
Total beetroot supplements distributed: _____
Total blinded pills distributed: _____

Return Date:___/___/_____
Total beetroot supplements returned:_____
Total blinded pills returned: _____
Number of Empty beetroot juice container:_____
Number of empty strips of pills: _____

Comments:_____
_____
_____

_____ _____
Signature (Research Personnel) Date

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VITA

Reem Othman Basaqr

I. GENERAL INFORMATION

Languages: Arabic and English

CERTIFIED DIABETIC EDUCATOR — Year Certified: 2011
King Abdulaziz Medical City, Jeddah, Saudi Arabia — National Certification

II. EDUCATION

UNIVERSITY OF KENTUCKY — 2015-2020
Lexington, Kentucky
Doctor of Philosophy, Nutritional Sciences-Clinical Nutrition Track
College of Medicine
Dissertation: The Effect of Dietary Nitrate and Vitamin C on Endothelial Function and Oxidative Stress Biomarkers
Mentor: Travis Thomas

UNIVERSITY OF KENTUCKY — 2013-2014
Lexington, Kentucky
Master of Science, Nutritional Sciences-Clinical Nutrition Track
College of Medicine

UNIVERSITY OF KENTUCKY — 2012-2013
Lexington, Kentucky
Study Abroad, English as a second language

KING ABDULAZIZ UNIVERSITY — 2010-2011
Jeddah, Saudi Arabia
Dietetic Internship (Clinical Dietitian)
Rotations at three different hospitals: King Abdulaziz University Hospital, Maternity and Child Hospital, and National Guard Hospital

KING ABDULAZIZ UNIVERSITY — 2006-2009
Jeddah, Saudi Arabia
Bachelor of Science, Clinical Nutrition
College of Applied Medical Sciences

III. PROFESSIONAL POSITION

KING SAUD BIN ABDULAZIZ UNIVERSITY FOR HEALTH SCIENCES — 2010-current
Jeddah, Saudi Arabia
Teaching Assistant

IV. Research Position

UNIVERSITY OF KENTUCKY 2015-2020
Graduate Research Assistant,
Laboratory of Dr. D. Travis Thomas
Experienced in clinical trials (emphasis: dietary nitrate), Endothelial Function Technique, Clinical Research and Institutional Review Board, Study Coordinator for two clinical trials.

V. Abstracts and Posters

1. **Basaqr Reem,** Thomas DT, Biddle M, Lennie T. Diet monotony is associated with dietary micronutrient deficiencies in patients with heart failure. American Heart Association November, 2019
2. **Basaqr Reem,** Schnell DM, Skleres M, Jayswal J, Thomas DT. The Effects of Dietary Nitrate and Vitamin C on Endothelial Function and LDL Cholesterol in Older Adults with Cardiovascular Risk Factors. Barnstable Brown Diabetes and Obesity Research Day abstract/poster May, 2019
3. **Basaqr Reem,** Kudrimoti M, Shelton B, Dressler E, Jayswal R, Yan D, Thomas DT. A Pilot Study of Concentrated Beet Root Juice in Participants Being Treated for Locally Advanced Squamous Cell Cancer of the Head and Neck. University of Kentucky Markey Cancer Center Research Day May, 2018
4. **Basaqr Reem,** Kudrimoti M, Shelton B, Dressler E, Jayswal R, Yan D, Thomas DT. A Pilot Study of Concentrated Beet Root Juice in Participants Being Treated for Locally Advanced Squamous Cell Cancer of the Head and Neck. University of Kentucky CCTS April, 2018
5. **Basaqr Reem,** Kudrimoti M, Shelton B, Dressler E, Jayswal R, Yan D, Thomas DT. A Pilot Study of Concentrated Beet Root Juice in Participants Being Treated for Locally Advanced Squamous Cell Cancer of the Head and Neck. American Society of Nutrition January, 2018

VI. SCHOLARSHIPS AND AWARDS

Full Scholarship to study abroad in the United States
Sponsorship: King Saud Bin Abdulaziz University for Health Sciences 2012-current
Thornton Scholarship-University of Kentucky 2017-2019

VII. HONORS

College of Nursing Travel Awards November 2019
to present an abstract at American Heart Association
National Lipid Association travel awards March 2016
San Diego, California
6th Applied Medical Science Annual Meeting April 2009
Best Oral Presentation Award
Jeddah, Saudi Arabia
King Abdulaziz University Dean's List 2006-2009

VIII. WORKSHOPS and WEBINARS

Beyond the Professoriate Webinar September 2019
Introduction to Academic Leadership- Academic Leadership Center February 2018

Workshop in Visual Communication-University of Kentucky October 2018
Teaching Assistant Workshop Series-Center for Learning and Teaching
at University of Kentucky October 2018
Innovation in Education/Utilizing Dietetics Simulation for Clinical Experience
– Food and Nutrition Conference & Expo FENCE October 2017
Time Management Workshop – UK Human Resources May 2017
Clinical Lipid update course- National Lipids Academy March 2016

IX. TRAINING WORK EXPERIENCES

KENTUCKY CLINIC
Lexington, Kentucky
Pediatric cystic fibrosis clinic 2014 (50 hours)
Pediatric BMI Clinic 2013 (30 hours)

MARKEY CANCER CENTER
Lexington, Kentucky
Outpatient Clinic 2014 (50 hours)

X. STUDENTS MENTORED 2016-2019

Mentored 4 graduate students- Reham Hasanain, Ola Takrooni, Nilima Mishra, and Basma Alwahaib. Provided clinical trials' consenting experience, data entry for clinical trials, and other research related activities.

XI. TEACHING SPECIFIC COURSES AND COUNSELING TAKEN UNIVERSITY OF KENTUCKY

GS610-College Teaching
EDP605-Introduction to Counseling

XII. OTHER PROJECT ACTIVITIES AND CONFERENCE CERTIFICATES

Performed two educational videos to increase nutrition knowledge of the Carolyn's Crown and Glory Beauty Salon's customers about My Plate and Nutrition Myths 2013-2014
Several Certificate of attendance reginal conferences related to nutrition in Jeddah, Saudi Arabia.

XIII. MEMBERSHIP IN PROFESSIONAL SOCIETIES:

Academy of Nutrition and Dietetics
American Heart Association
American Society for Nutrition