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## The Effect of Dietary Nitrate and Vitamin C on Endothelial Function, Oxidative Stress and Blood Lipids in Untreated Hypercholesterolemic Subjects: A Randomized Double-Blind Crossover Study

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## Randomized Control Trials

# The effect of dietary nitrate and vitamin C on endothelial function, oxidative stress and blood lipids in untreated hypercholesterolemic subjects: A randomized double-blind crossover study



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## SUMMARY

**Background:** Vitamin C may enhance nitric oxide (NO) production through stepwise reduction of dietary nitrate (NO<sub>3</sub>) to nitrite (NO<sub>2</sub>) to NO. The combined effect of vitamin C and NO<sub>3</sub> supplementation is relatively unexplored in untreated hypercholesterolemia.

**Aims:** We aimed to examine whether co-administration of vitamin C and nitrate for 4-weeks would improve endothelial function (primary outcome), plasma NO metabolites, oxidative stress, and blood lipids (secondary outcomes).

**Methods:** Subjects 50–70 years of age with low density lipoprotein (LDL) > 130 mg/dL and RHI ≤ 2 were enrolled in this randomized double-blind crossover study. Subjects were assigned to two 4-week supplementation treatments starting with 70 ml of concentrated beetroot juice (CBJ) with 1000 mg of vitamin C (NC) or CBJ with matched placebo (N), then switched to alternate treatment following 2-week washout. The change in reactive hyperemia index (RHI), sum of plasma NO metabolites (NO<sub>2</sub> + NO<sub>3</sub> (NOx)), oxidized LDL (oxLDL), and serum lipids were assessed at baseline and at 4-weeks of each treatment period.

**Results:** Eighteen subjects (11M:7F) completed all study visits. No significant treatment differences were observed in RHI change (N: 0.21 ± 0.12; NC: 0.20 ± 0.17; p = 0.99). Secondary analysis revealed that a subgroup of NC subjects who started with a baseline RHI of <1.67 (threshold value for ED) had greater improvements in RHI compared to subjects with RHI >1.67 (1.23 ± 0.15 to 1.96 ± 0.19; n = 8 vs. 1.75 ± 0.11 to 1.43 ± 0.10; n = 8; p = 0.02). Compared to N, NC experienced a significant increase in plasma NOx (N: 94.2 ± 15.5 μmol/L; NC: 128.7 ± 29.1 μmol/L; p = 0.01). Although there was no significant difference in oxLDL change between treatments (N: -1.08 ± 9.8 U/L; NC: -6.07 ± 9.14 U/L; p = 0.19), NC elicited significant reductions in LDL (N: 2.2 ± 2; NC: -10.7 ± 23; p = 0.049), triglycerides (N: 14.6 ± 43; NC: -43.7 ± 45; p = 0.03), and no change in serum high density lipoprotein. Within treatment group comparisons showed that only NC reduced oxLDL significantly from baseline to 4 weeks (p = 0.01).

**Conclusions:** No between intervention differences were observed in RHI. RHI only improved in NC subjects with ED at intervention baseline. Four weeks of NC enriched the NO pool and promoted reduction of blood lipids and oxidative stress in subjects with hypercholesterolemia. These preliminary findings highlight a supplementation strategy that may reduce the progression of atherosclerotic disease and deserves further attention in studies using flow mediated dilation methods.

**Clinical trial registration:** [www.clinicaltrials.gov](http://www.clinicaltrials.gov) (NCT04283630).

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## 1. Introduction

Dietary inorganic nitrate supplementation leads to increased nitric oxide (NO) generation and has received attention for its potential role in reversing endothelial dysfunction (ED) [1–3]. Low serum and tissue NO status is a hallmark of ED that is associated

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### Abbreviations

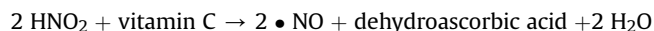
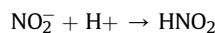
AS	atherosclerosis
CBJ	concentrated beetroot juice
CVD	cardiovascular disease
CVRFs	cardiovascular risk factors
ED	endothelial dysfunction
eNOS	endothelium nitric oxide synthase
HDL	high density lipoprotein cholesterol
LDL	low density lipoprotein cholesterol
N	dietary nitrate supplementation alone
NC	dietary nitrate and vitamin C supplementation
NO	nitric oxide
NO <sub>2</sub>	nitrite
NO <sub>3</sub>	nitrate
NOx	sum of nitric oxide metabolites
oxLDL	oxidized low density lipoprotein
RH-PAT	reactive hyperemia peripheral artery tonometry
RHI	reactive hyperemia index
TC	total cholesterol
TG	triglycerides

with most cardiovascular risk factors (CVRFs) and cardiovascular events [4–6]. NO is a vasodilator and anti-atherogenic molecule that maintains vascular homeostasis and modulates *in vitro* lipid peroxidation reactions [7]. NO presents in circulation from two pathways but direct *in vivo* measurement of NO cannot be measured due to its short half-life (milliseconds) [8–10]. NO is rapidly auto-oxidized to nitrate and nitrite in circulation when it is synthesized from its primary endogenous precursor (arginine) by endothelium nitric oxide synthase (eNOS) enzyme [11]. In the presence of CVRFs, dietary nitrate provides an exogenous source and alternate pathway of NO generation when eNOS is compromised [11]. Thus, in both pathways, nitrite and nitrate act as stable end products that reflect total systemic NO concentrations [10] and their plasma concentrations can be used as surrogate measures of vascular NO bioavailability [4]. Combined measurements of NO metabolic pathway products (nitrate + nitrite) are termed as NOx [12].

There is a growing interest in examining the utility of dietary nitrate on the management and reversal of ED and attenuating oxidative stress biomarkers [13,14]. Oxidative stress is a key mediator contributing to the promotion of ED and atherosclerosis (AS) in subjects with CVRFs, and may be counteracted with strategies designed to preserve endothelial NO [15–17]. Promising findings from preclinical studies reported that inorganic nitrate supplementation was able to reduce well-established markers of oxidative stress, such as malondialdehyde and pro-inflammatory cytokines [18,19]. To our knowledge, few clinical trials have investigated the effect of inorganic nitrate supplementation on different markers of oxidative stress [1,20,21]. One of these studies reported a trend in oxLDL reduction in untreated hypercholesterolemic subjects following 6 mmol of dietary nitrate supplementation [1]. Borsa et al. [12] reported that systemic NO was negatively correlated with circulating oxLDL in hyperlipidemic elderly patients. Further studies are needed to better understand the use of dietary nitrate in relation to circulatory oxLDL in hypercholesterolemia and to further elucidate the dietary nitrate benefit on oxidative stress associated ED [1].

Vitamin C plays a role in enhancing the exogenous NO pathway through the stepwise reduction of dietary nitrate to nitrite and then NO [22]. Under hypoxic and acidic condition in the blood and gastric lumen, the presence of vitamin C may augment the second

reduction step of nitrite to NO [23]. 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 conversion of acidified nitrite to NO, thus, increasing the NO pool [23–27].



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 combined supplementation effect on cardiovascular health among patients with CVRFs. Recently, Ashor et al. (2019) [22] demonstrated a significant synergistic effect following a single co-administered dose of vitamin C with dietary nitrate in healthy individuals. Results showed improvements in arterial stiffness as measured by pulse wave amplitude, 3-nitrotyrosine concentrations, and blood pressure compared to vitamin C or nitrate given separately. However, this combination did not improve endothelial function in all groups as measured by post-occlusion reactive hyperemia. Studies of longer duration designed to provide daily combined dosing of these supplements interventions have yet to be conducted.

We aimed to examine whether co-administration of vitamin C and nitrate for 4-weeks would improve markers of endothelial function (primary outcome), plasma NO metabolites, oxidative stress, and blood lipids (secondary outcomes). On the basis of their complementary mode of action to augment NO and improve endothelial function, we hypothesized that daily co-administration of inorganic NO<sub>3</sub> and vitamin C for 4-weeks would elicit a greater improvement in endothelial function as a result of changes in reactive hyperemia index (RHI) score and other blood biomarkers such as NO metabolites, oxLDL, and lipid profile compared to nitrate alone.

## 2. Materials and methods

### 2.1. Subjects

Male and female non-smokers between the age of 50 and 70 with a body mass index (BMI) of 18.5–34.9 kg/m<sup>2</sup> and high LDL concentrations (>130 mg/dL) were enrolled. This age range was chosen to target individuals more prone to hypercholesterolemia and endothelial abnormalities [28]. The BMI range was selected to examine whether our intervention would have different effects on different BMI classes.

Subjects were excluded if receiving lipid lowering therapy, multivitamin supplements exceeding recommended dietary allowance (RDA), or on hormone replacement therapy, clonidine, proton pump inhibitors (PPI), or antibiotic medications. Moreover, subjects who consumed greater than 6 servings of alcohol per week, performed 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. Subjects with a documented history of CVD, diabetes mellitus, chronic inflammatory disease, celiac disease, uncontrolled hypertension, hepatic or renal disease, and active cancer were not included in the study. Final enrollment decisions were contingent upon having an RHI ≤2.

This 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. This study was registered with [ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT04283630) (NCT04283630).

## 2.2. Study design and randomization

This was a 10-week double-blind, placebo-controlled crossover study. Randomization was conducted by the Investigational Drug Service (IDS) at University of Kentucky. IDS prepared a randomization list treatment sequence. As subjects enrolled, they received their intervention order and blinded supplement assignment. Vitamin C and its placebo packaging were identified by the letter “A” or “B” and therefore blinded to subjects and investigators. The blinded supplements were then provided to the study coordinator with the treatment allocation in a sealed envelope. The preparation and labeling of the vitamin C and its placebo were performed in such a way that both capsules were identical in shape, color, and size to ensure adequate blinding of both subjects and investigators.

## 2.3. Study protocol

### 2.3.1. Screening visits

At screening visit 1 a phone screening or a face-to face interview was performed to gauge subject’s interest followed by assessing BMI and screening for inclusion and exclusion criteria. At screening visit 2 a non-invasive technique called Reactive Hyperemia Peripheral Artery Tonometry (RH-PAT) was employed to only include subjects with RHI score  $\leq 2$ . RH-PAT is intended for detection of ED since the decline of endothelium-derived NO bioavailability is reflected by impaired RH-PAT vascular responses [29,30]. The main advantages of this system are that it is operator independent, less expensive, provides automated analysis, and easily performed [30]. In addition, validation studies reported that lower RHI is associated with several cardiovascular risk factors [31–34].

### 2.3.2. Study visits

Subjects completed 4 visits as described in Fig. 1. Each visit includes anthropometric measures, blood pressure, vascular testing, and blood collection. Vascular testing was defined as measures of RHI ratio (primary outcome). Venous blood samples were collected at baseline and endpoint visits of each intervention period following an overnight fast. Plasma was used to measure nitrate

and nitrite, vitamin C, oxLDL, total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) concentrations. Our washout length was chosen based on previous work showing that a 2-week washout returned NO metabolites and vitamin C to baseline concentrations [35,36].

Prior to each visit, 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 h [35]; 2) refrain from taking anti-hypertensive agents and long-acting vasoactive medications including steroids, beta-blockers, antacids, anticoagulants, calcium channel blockers, angiotensin converting enzyme inhibitors (ACE-inhibitors), and aspirin for 24 h; 3) abstain from alcoholic beverages, coffee, high fat foods, chewing gum; 4) abstain from strenuous exercise for 24 h [1]; and 5) fast for 12 h prior to study visit [32].

Throughout the intervention periods, subjects were instructed to avoid high vitamin C foods and maintain their usual dietary patterns throughout the duration of the washout period. At the beginning of the study, subjects were provided with a list of high vitamin C foods and vitamin C fortified foods to avoid during both intervention periods. Dietary intake was assessed via 24-hr dietary recall. Three 24-hour 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.

## 2.4. Endothelial function measurement

Reactive hyperemia peripheral artery tonometry (RH-PAT) was used to assess endothelial function in peripheral arteries. RH-PAT is a non-invasive technique comprised of two finger plethysmographic probes connected to the EndoPAT-2000 device (Itamar Medical, Caesarea, Israel) to measure changes in arterial pulsatile volume induced by reactive hyperemia. EndoPAT tests occurred at approximately 10 AM at 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 [32].

RH-PAT examination included three stages: pre occlusion, occlusion, and post occlusion. A manual blood pressure cuff was placed on the upper arm of the non-dominant hand (occlusion arm) to initiate PAT measurement. Then, two plethysmography probes were placed on both index fingers of each hand and inflated. Subjects were asked to stay as still as possible during the entire

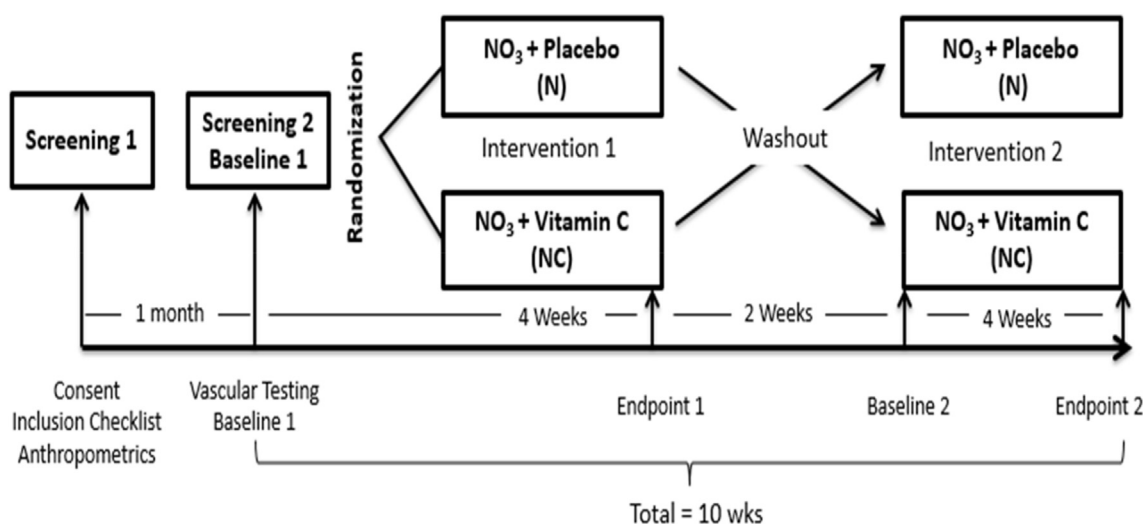


Fig. 1. Schematic representation of the study design. NO<sub>3</sub>: inorganic nitrate from concentrated beetroot juice dietary supplement.

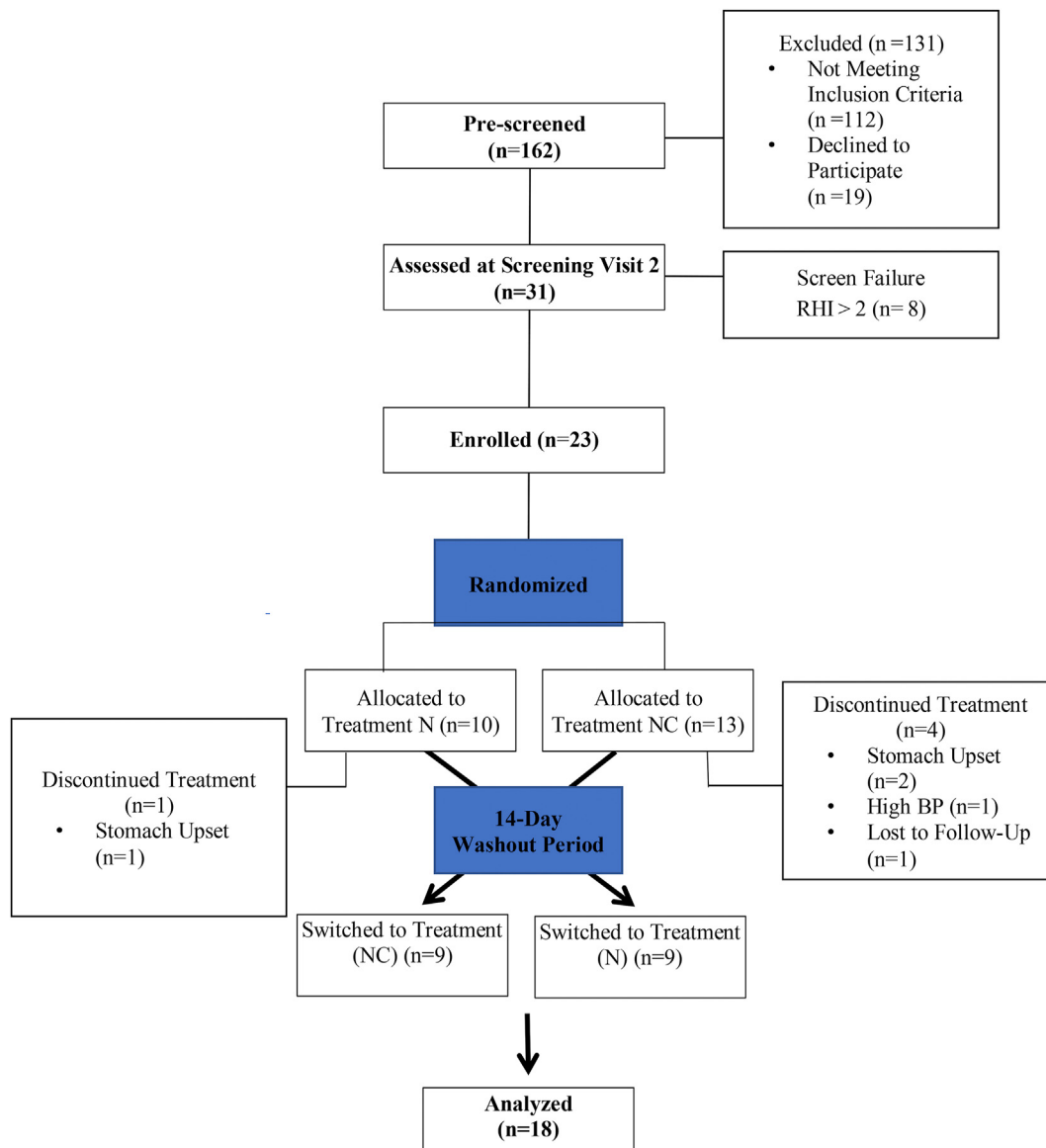


Fig. 2. Consort diagram of the progress through the phases of a crossover randomized trial of two treatment groups.

testing period. Following a 10 min rest period, the blood pressure cuff was inflated manually to suprasystolic pressures by adding at least 60 mmHG above systolic BP for five minutes. The cuff was then deflated quickly and the EndoPAT device continued recording pulse wave response for five minutes [29,37]. The RHI value was calculated automatically by dividing post-occlusion pulse wave amplitude (PWA) to the pre-occlusion value of PWA of the same arm, normalized to the control arm, and then multiplied by baseline correction factor [29,37].

2.5. Plasma biomarkers

Fasting blood samples were drawn by trained staff at the University of Kentucky's CCTS immediately following vascular testing. Plasma samples were stored at -80 °C for analysis at a later time. Plasma nitrate and nitrite were analyzed using Parameter™ Assay Kit KGE001 (R&D Systems, Minneapolis MN, USA). The result was read using the Griess Reaction @ 540–570 nM [38]. Plasma vitamin C concentrations were analyzed using high performance liquid chromatography (HPLC, ESA Inc.). Plasma oxidized LDL was

measured using ELISA kit (Mercodia kit, Uppsala, Sweden) based on the monoclonal antibody 4E6 [39]. 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 [40].

2.6. Dietary supplementation

The nitrate supplement was a commercially available beetroot juice beverage (Sport Beet IT shot, Heartbeet Ltd). Sport Beet IT was a concentrated beetroot juice (CBJ) (70 ml) that delivered an average of 400–450 mg (~6.4 mmol) dose of inorganic nitrate per the 70 ml serving [2]. The dietary nitrate dose was chosen due to documented safety and efficacy in previous studies that have shown improvements in vascular health or a reduction in blood pressure with no signs of toxicity [41–43]. Vitamin C (Nature Made®), and the placebo (corn starch) were provided as supplement capsules based on the randomization order. The selected dose of

vitamin C (1000 mg) was based on observations from a previous meta-analysis and systematic review reporting a minimum of 1000 mg of vitamin C per day elicited an improvement in endothelial function [44]. 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 capsules (1000 mg of vitamin C or placebo) one hour later. Supplement adherence was assessed by counting unused capsules and CBJ bottles at each intervention endpoint.

### 2.7. Statistical analysis

Using the nQuery Advisor program, sample size calculations were based on the work of Kwak et al. (2012) [45]. 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. Distribution of continuous variables were examined by Shapiro–Wilk test.

For within group comparisons, paired t-tests (for normally distributed data) and Wilcoxon signed rank-test (for non-normally distributed data) were employed using data at baseline and 4-weeks for two interventional periods separately. Changes in measures over the 4-week period were assessed by calculating the difference from baseline to 4 week in each treatment (change = endpoint–baseline). For between group comparisons, the treatment effect ( $\Delta$ ) was evaluated by a two-sided one-sample t-test based on the mean of the differences between the two changes for each pair (change during NC–change during N) [46].

Additionally, based on work by Welk et al. [47], nonparametric Wilcoxon tests were used to verify whether there were any carry-over effects from one treatment period to another. An independent sample t-test was used to assess the changes in RHI between high-responders and low-responders regardless of treatment group. Linear mixed models were used to assess potential interaction effects of variables such as BMI and BP medication, sex, and age with dependent variables. Pearson and Spearman correlations were used to assess the relationship between dependent variables. Statistical analyses was performed using Statistical Package for Social Sciences (SPSS) software (version 22, IBM, Armonk, NY). Data are presented as means  $\pm$  standard error of the mean (SEM).

## 3. Results

### 3.1. Subjects

Of the 23 subjects who enrolled, 18 completed both interventions and all study visits (Fig. 2). Further explanation of subject attrition is found in Fig. 2. All baseline characteristics are shown in Table 1. The interventions were generally well-tolerated with no reports of severe adverse effects. Analyses of three dietary recalls at each treatment period indicated no significant differences in dietary nutrient intake or dietary patterns (data not shown).

### 3.2. Adherence and subject response to supplementation

Based on returned CBJ and capsule bottles and supplement diaries, more than 99% of CBJ and 98% of vitamin C distributed in both interventional periods were reported to be ingested.

### 3.3. Nitrate, nitrite concentrations

Significant differences were observed in the mean change of plasma nitrate ( $\Delta = 50.02\uparrow$ ,  $p < 0.01$ ) and nitrite ( $\Delta = -0.03\downarrow$ ,  $p = 0.03$ ) between both treatment groups (Table 2). Within treatment group comparisons showed that endpoint plasma nitrate

**Table 1**  
Baseline characteristics.

Variables	Treatment N (n = 9)	Treatment NC (n = 9)	Total (n = 18)
Male sex, n (%)	3 (33%)	4 (44%)	7 (39%)
Age (years)	58 $\pm$ 1	59 $\pm$ 2	59 $\pm$ 1
BMI (kg/m <sup>2</sup> )	29.6 $\pm$ 1	27.1 $\pm$ 1.0	28.3 $\pm$ 0.8
Subjects on BP medications n (%)	3 (33.3%)	3 (33.3%)	6 (33.3%)
Plasma vitamin C (mg/dL)	0.73 $\pm$ 0.2	0.88 $\pm$ 0.2	0.81 $\pm$ 0.1
Plasma NO <sub>3</sub> ( $\mu$ mol/L)	13.4 $\pm$ 1.8	18.5 $\pm$ 2.8	16.0 $\pm$ 1.7
Plasma NO <sub>2</sub> ( $\mu$ mol/L)	0.44 $\pm$ 0.1	0.46 $\pm$ 0.1	0.45 $\pm$ 0.1
Plasma NOx ( $\mu$ mol/L)	14.4 $\pm$ 1.8	18.9 $\pm$ 2.8	16.7 $\pm$ 1.7
RHI	1.55 $\pm$ 0.1	1.66 $\pm$ 0.1	1.60 $\pm$ 0.1
Plasma oxLDL (U/L)	71.7 $\pm$ 2.9	70.1 $\pm$ 4.2	75.9 $\pm$ 2.9
Plasma TC (mg/dL)	235.4 $\pm$ 6.6	249.1 $\pm$ 16.1	242.3 $\pm$ 8.6
Plasma LDL (mg/dL)	135.3 $\pm$ 6.1	167.9 $\pm$ 13.7	160.6 $\pm$ 7.5
Plasma HDL (mg/dL)	55.1 $\pm$ 2.2	54 $\pm$ 4.0	54.56 $\pm$ 2.2
Plasma TG (mg/dL)	119.2 $\pm$ 16	136 $\pm$ 18	127.6 $\pm$ 11.9

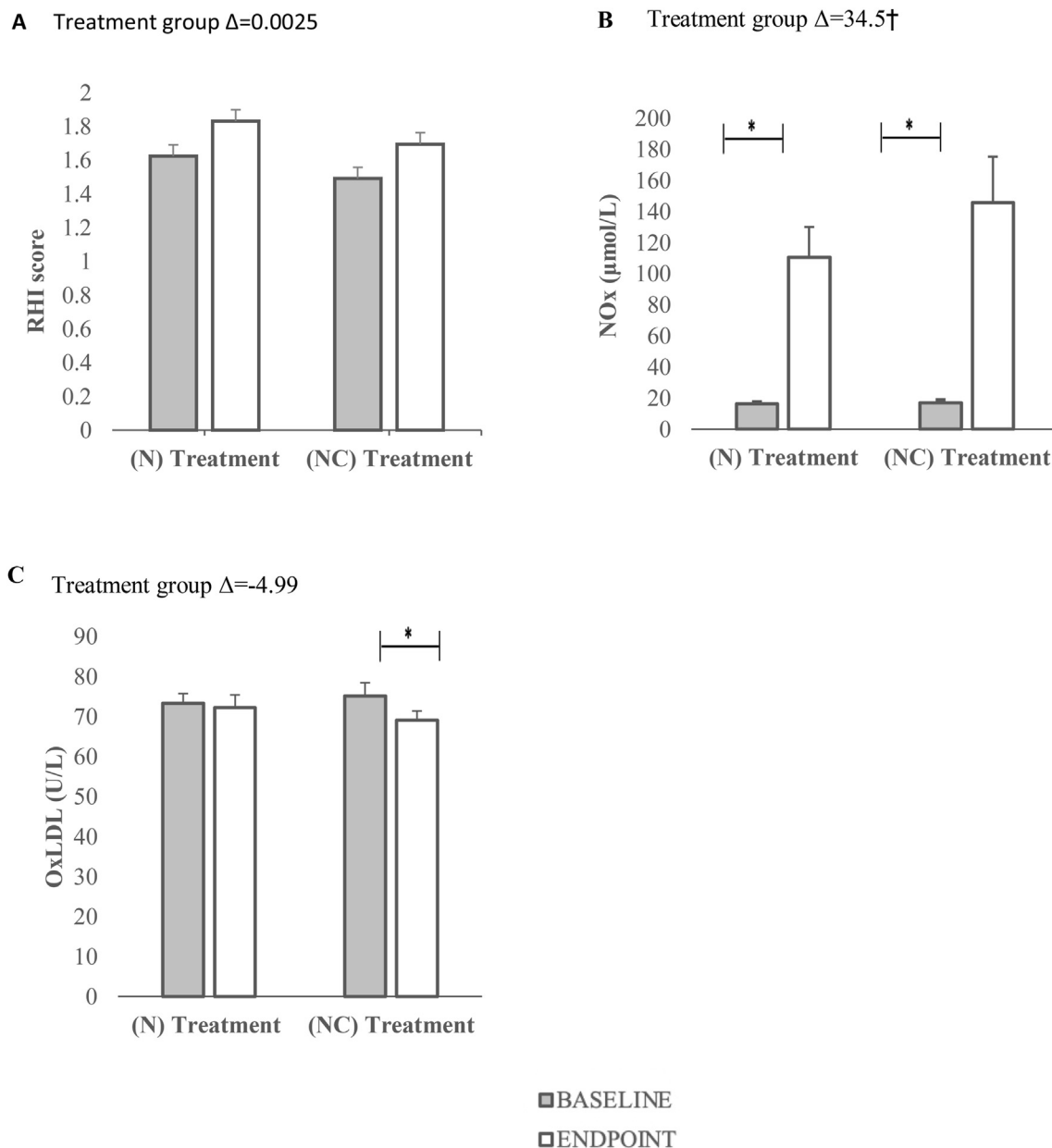
BMI: body mass index; BP: blood pressure; NO<sub>3</sub>: nitrate; NO<sub>2</sub>: nitrite; NOx: nitric oxide metabolic pathway products (nitrate (NO<sub>3</sub>) + nitrite (NO<sub>2</sub>)); RHI: reactive hyperemia index; TC: total cholesterol; LDL: low-density lipoprotein; HDL: high-density lipoprotein; TG: Triglycerides. Quantitative variables were described as the mean  $\pm$  SEM. Data were analyzed using independent t-test ( $p > 0.05$ ) and values were not significantly different between treatments at baseline.

**Table 2**  
Response to 4-week supplementation with N and NC.

Value	Time point	N	NC
Plasma NO <sub>3</sub> ( $\mu$ mol/L)	Baseline	15.6 $\pm$ 1.3	16.6 $\pm$ 1.9
	Endpoint	94.2 $\pm$ 15.6	145.3 $\pm$ 29.6
	Change 1	78.6 $\pm$ 15.4*	128.7 $\pm$ 29.1*
	Change 2 ( $\Delta$ )	50.02 $\uparrow$	
Plasma NO <sub>2</sub> ( $\mu$ mol/L)	Baseline	0.42 $\pm$ 0.05	0.45 $\pm$ 0.06
	Endpoint	0.49 $\pm$ 0.05	0.49 $\pm$ 0.06
	Change 1	0.07 $\pm$ 0.02*	0.04 $\pm$ 0.03
	Change 2 ( $\Delta$ )	-0.03 $\downarrow$	
Plasma Vitamin C (mg/dL)	Baseline	0.81 $\pm$ 0.10	0.77 $\pm$ 0.10
	Endpoint	0.66 $\pm$ 0.09	1.2 $\pm$ 0.13
	Change 1	-0.15 $\pm$ 0.12	$\pm$ 0.16*
	Change 2 ( $\Delta$ )	0.58 $\uparrow$	
Plasma NO <sub>3</sub> ( $\mu$ mol/L)	Baseline	15.6 $\pm$ 1.3	16.6 $\pm$ 1.9
	Endpoint	94.2 $\pm$ 15.6	145.3 $\pm$ 29.6
	Change 1	78.6 $\pm$ 15.4*	128.7 $\pm$ 29.1*
	Change 2 ( $\Delta$ )	50.02 $\uparrow$	
Plasma NO <sub>2</sub> ( $\mu$ mol/L)	Baseline	0.42 $\pm$ 0.05	0.45 $\pm$ 0.06
	Endpoint	0.49 $\pm$ 0.05	0.49 $\pm$ 0.06
	Change 1	0.07 $\pm$ 0.02*	0.04 $\pm$ 0.03
	Change 2 ( $\Delta$ )	-0.03 $\downarrow$	
Plasma Vitamin C (mg/dL)	Baseline	0.81 $\pm$ 0.10	0.77 $\pm$ 0.10
	Endpoint	0.66 $\pm$ 0.09	1.2 $\pm$ 0.13
	Change 1	-0.15 $\pm$ 0.12	$\pm$ 0.16*
	Change 2 ( $\Delta$ )	0.58 $\uparrow$	

NO<sub>3</sub>: nitrate; NO<sub>2</sub>: nitrite. Change 1: the mean difference between baseline and endpoint within treatment group. Values are reported as means  $\pm$  SEM. \*(p-value  $\leq 0.05$ , Paired t-test). Change 2: the mean difference between treatments. Data for delta absolute value were analyzed using one sample t-test,  $\uparrow p < 0.05$ .

concentrations were significantly increased compared to baseline ( $p < 0.001$ ) (Table 2). Likewise, the endpoint plasma nitrite concentrations were significantly increased after N ( $p = 0.006$ ) but did not increase after 4 weeks of NC ( $p = 0.23$ ) (Table 2). The change of plasma NOx status between treatments was significant (N: 94.24  $\pm$  19.5  $\mu$ mol/L; NC: 128.7  $\pm$  29.1  $\mu$ mol/L;  $p = 0.01$ ). Within treatment group comparisons revealed that N and NC endpoint NOx measures significantly increased over time ( $p < 0.001$ ) (Fig. 3). Secondary analysis indicated that post-supplementation changes in plasma NOx concentration correlated significantly with changes in plasma vitamin C following NC treatment ( $r = 0.49$ ,  $p = 0.04$ ) with no correlation seen following N treatment ( $r = 0.29$ ,  $p = 0.26$ ).



**Fig. 3.** Functional and circulatory markers in response to N and NC interventions. RHI score (n = 16) (A), NOx: nitric oxide metabolites (n = 18) (B), oxLDL: plasma oxidized low-density lipoprotein (n = 18) (C). (N): inorganic nitrate and vitamin C placebo or (NC): inorganic nitrate and active vitamin C (1000 mg). Data were analyzed using Paired t-test for RHI and oxLDL, and Wilcoxon-test for plasma NOx, \*p ≤ 0.05. Δ = mean change between treatments. Data were analyzed using one sample t-test for delta absolute value, †p < 0.05.

### 3.4. Vitamin C concentrations

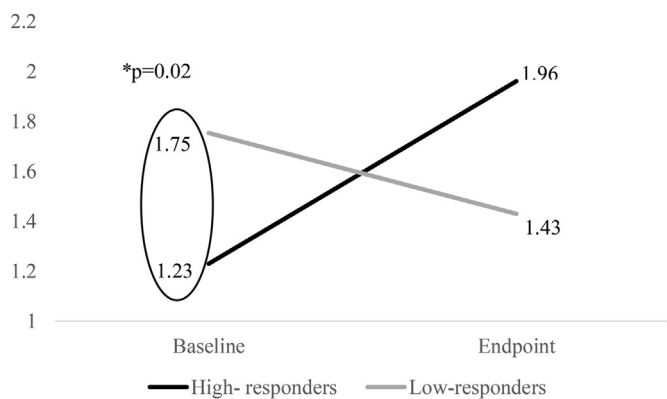
There was a significant difference in plasma vitamin C change between the treatments ( $\Delta = 0.58 \ddagger$ ,  $p < 0.001$ ). Within treatment group comparisons showed that plasma vitamin C concentrations increased significantly over time following 4-week administration of NC (Table 2).

### 3.5. Endothelial function

For our primary outcome, there were no treatment group differences in RHI change (N:  $0.21 \pm 0.12$ ; NC:  $0.20 \pm 0.17$ ;  $p > 0.05$ ). No evidence of carryover effect was observed from one treatment

course to the other ( $p = 0.64$ ). Within treatment group comparisons, revealed that RHI scores over time did not change significantly in either treatment group (NC:  $p = 0.24$ ; N:  $p = 0.09$ ) (Fig. 3).

Secondary analysis revealed that some subjects responded greater than others to NC treatment. Subjects were grouped into “high-responders” and “low-responders” based on their RHI response to NC treatment. High-responders (n = 8) demonstrated significant increases in RHI in response to NC ( $1.23 \pm 0.15$  to  $1.96 \pm 0.19$ ;  $p < 0.01$ ), while the low-responders (n = 8) had a significant decrease in RHI ( $1.75 \pm 0.11$  to  $1.43 \pm 0.10$ ;  $p < 0.01$ ). Significant differences were observed between RHI baseline scores in high-responders and low-responders ( $p = 0.02$ ) (Fig. 4). No notable interactions were detected among demographics and



**Fig. 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. 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,  $*p \leq 0.05$ .

baseline characteristics between the two treatment groups (sequence 1: N-NC and sequence 2: NC-N) in terms of age, BMI, gender, RHI, number of subjects on BP medication, vitamin C status, and NO metabolite concentrations. Two subjects who started NC treatment were excluded from RHI analysis due to poor RHI signals at endpoint.

### 3.6. Oxidized LDL and blood lipids

Compared to N, there was no significant differences observed in the change of plasma oxLDL following NC (N:  $-1.08 \pm 2.3$  U/L; NC:  $-6.07 \pm 2.1$  U/L;  $p = 0.19$ ). Within treatment group comparisons revealed that NC endpoint oxLDL was significantly lower than baseline ( $p = 0.01$ ) (Fig. 3). However, N endpoint oxLDL showed no change over time ( $p = 0.64$ ).

Fasting lipid profile analysis indicated that the mean change in lipids was significantly different following NC and N treatments for LDL (N:  $2.2 \pm 3.5$ ; NC:  $-10.67 \pm 5.4$ ;  $p = 0.049$ ) and TC (N:  $0.17 \pm 5.3$ ; NC:  $-16.06 \pm 6.1$ ;  $p = 0.05$ ). Serum HDL was unaltered between treatment groups (N:  $0.05 \pm 0.8$ ; NC:  $0.33 \pm 1.5$ ;  $p = 0.81$ ). No treatment carryover was evident in LDL ( $p = 0.78$ ), TC ( $p = 0.73$ ), and HDL ( $p = 0.93$ ) analyses. However, there was a significant carryover effect in TG ( $p = 0.03$ ). As a result of this effect, we only compared period one data for triglyceride changes between the two treatments. We demonstrated a significant difference in the mean change of TG between treatments (N:  $14.6 \pm 14.2$ ; NC:  $-43.7 \pm 14.9$ ;  $p = 0.01$ ). Within treatment group comparisons showed no change in TC ( $p = 0.97$ ), LDL ( $p = 0.52$ ), TG ( $p = 0.33$ ), and HDL ( $p = 0.95$ ) from pre-to post following N treatment. In contrast, there were significant decreases in TC ( $p = 0.02$ ) and TG ( $p = 0.02$ ) with a trend towards a reduction in LDL concentrations from pre-to post following NC ( $p = 0.06$ ) (Fig. 5). HDL did not change by time after NC treatment ( $p = 0.83$ ) (Fig. 5). Secondary, exploratory analysis demonstrated that changes in plasma oxLDL correlated significantly with changes in plasma LDL following both NC and N treatments ( $p < 0.01$ ).

## 4. Discussion

### 4.1. Summary of the main findings

NC did not improve RHI but significantly increased NOx and reduced blood lipids. Secondary analyses included subgroup and

within groups comparisons. RHI improved only in a subgroup of subjects with RHI values indicative of ED. Within treatment group comparisons revealed that oxLDL was reduced over time only in the NC intervention.

### 4.2. Reactive hyperemia index

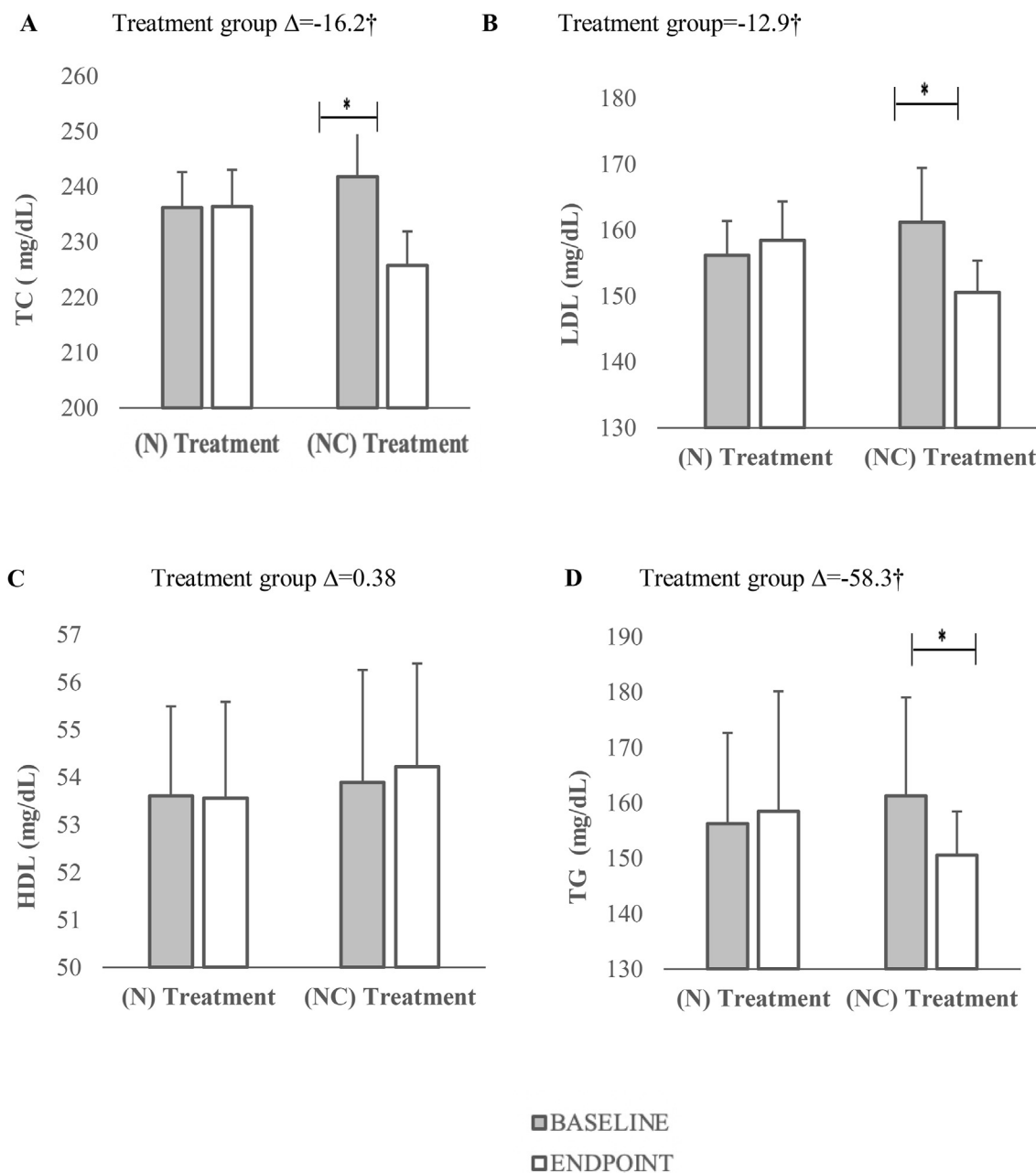
There were no significant differences in RHI between treatment interventions. This is contrary to previous data that showed a positive improvement in coronary endothelial function measured by flow mediated dilation (FMD) following 4–6 weeks administration of inorganic NO<sub>3</sub> in subjects with CVRFs [1,2,30,48,49]. However, previous work by Ashor et al. has shown no effects of nitrate combined with vitamin C on endothelial function using laser Doppler measurements [22]. Comparisons between our findings and the findings of others should be interpreted carefully since these studies used different techniques to measure endothelial function. The absence of a significant change in RHI in the present study may have many other explanations. First, the hyperemic response in microcirculation beds is partly dependent on NO [50] and other non-endothelium-dependent regulators [51,52] while FMD response in conduit arteries is mainly NO mediated [53]. Thus, it has been suggested that RH-PAT may not be sensitive enough to detect the influence of lifestyle modifications that target NO pathway on microvascular beds [34,37,51]. An additional explanation may be that the baseline RHI values of some subjects were greater than 1.67. An RHI value below 1.67 was established by EndoPAT as the threshold value to define subjects with coronary ED [54]. Therefore, subjects with RHI baseline scores greater than this cut point value may be less responsive to improvements in endothelial function. In our secondary subgroup analyses of RHI, it was shown that the eight “high responder” subjects who showed an individual increase in RHI following NC treatment (~60% improvements from baseline) had low 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. These observations add novel findings and add to the work from others that noticed substantial RHI improvements following dietary interventions in subjects with ED [55,56].

While this was the first study to examine the relationship between RHI and inorganic nitrate supplementation with and without vitamin C supplementation, we were not able to verify if all subjects took vitamin C exactly one hour following CBJ consumption. Proper timing of vitamin C ingestion 1 h after nitrate dosing is thought to be 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 [57]. We acknowledge that our findings may also suggest that subgroup responders adhered with the timing of vitamin C ingestion more than low responders.

### 4.3. Circulatory biomarkers

As expected, the 4-week NC intervention yielded significant differences in NO<sub>3</sub> and NO<sub>2</sub> concentrations and reflect increased capacity for NO production compared to N. The approximate 2-fold increase in plasma NOx in NC compared to N support align with previous studies suggesting that vitamin C acts as a potent reducing agent to support nitrite reduction from the exogenous NO pathway [24–28]. These findings are not aligned with recent crossover study of Ashor et al. [22] who found no modifying effect of vitamin C on plasma NO metabolites following acute dose of potassium nitrate (5.1–8.7 mmol) ingestion combined with vitamin C (20 mg/kg) in healthy individuals. This disparity could be due to the shorter duration of their study and the differences in the population





**Fig. 5.** Serum lipid profile response to N and NC. 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). (N): inorganic nitrate and vitamin C placebo or (NC): inorganic nitrate and active vitamin C (1000 mg). Data were analyzed using Paired t-test for all lipid profile parameters, \*p < 0.05. Δ = mean change between treatments. Data were analyzed using one sample t-test for the absolute delta value, †p < 0.05.

studied. In addition, our 8.8 fold increase in plasma nitrate following NC was greater than Velmurugan and colleagues [1] who examined a similar nitrate dosage (~6 mmol) alone and reported a 7.5 fold nitrate increase in untreated hypercholesterolemia patients.

In the present study, we did not observe between intervention differences in oxLDL. Within group analysis of the N intervention offer no evidence of oxLDL reduction and are consistent with previous work by Velmurugan et al. (2015) [1] who found no nitrate induced changes in oxLDL in untreated hypercholesterolemic adults. In contrast, we observed a significant within group reduction in oxLDL (-6.07 U/L) as a result of the NC intervention. These findings are in accordance with recent work reporting a synergistic

effect of a single combined dose of nitrate and vitamin C on the oxidative stress marker 3-nitrotyrosine [22]. As LDL peroxidation and reduced NO synthesis are the two characteristic features of ED, and share significant antagonistic roles in overall vascular health, it may be that any observed benefit of NC on oxLDL may be driven by NOx changes interacting with oxidants on the vascular wall [16]. This speculation requires further study and greater statistical power as the capacity of both bioactive substances as donors of NO may have a direct effect on attenuating the lipid peroxidation reaction [12].

Moreover, we observed the NC intervention resulted in substantial reductions in TC (-16.1 mg/dL or 6.6%), LDL (-10.7 mg/dL or 6.6%), and TG (-43 mg/dL or 32%). These findings are of

particular interest because of both the magnitude of change observed and how they compare with a recent meta-analysis of vitamin C treatment in hypercholesterolemic adults [58]. In this meta-analysis, thirteen trials found that TC, LDL, and TG decreased by 10.76 mg/dL (4.5%), 7.9 mg/dL (5%), and 20.1 mg/dL (8.8%), respectively. In the present study, nitrate appeared to modify and improve the lipid lowering effect of vitamin C. Our NC findings can also be compared to a previous study [59] that combined a 30 day inorganic nitrate administration along with the antioxidant hawthorn berry to reduce TG concentrations. The TG lowering effect (−32%) seen in our work was similar to the percentage of TG reduction (27%) shown by Zand et al. [59]. Collectively, the improvements in blood lipids from our work and others suggest that NO<sub>3</sub> supplementation with the addition of vitamin C (or other nutraceutical antioxidants), may be a valuable dietary approach to combat dyslipidemia which could positively impact cardiovascular health [60,61]. The exact mechanism of action to explain the serum lipids findings remains to be determined, but may be partially explained by complementary antioxidant effects of both supplements that work in concert to decrease oxidative stress [58,62–64].

#### 4.4. Strengths and limitations

This work has many strengths including study design with strict inclusion and exclusion criteria and high supplement adherence. Furthermore, the significant increase of plasma NO metabolites from baseline with or without vitamin C confirmed excellent adherence with CBJ and agree with previous findings that total systemic NO bioavailability is strongly influenced by changes in dietary nitrate in subjects with CVRFs [1,65]. There are also limitations to consider when interpreting results. The major limitation was the small sample size that may have limited our ability to observe significant RHI differences between treatments or potential interactions between the dependent and independent variables. Although we observed differences between N and NC in most dependent variables, comparison interpretations should be made cautiously. A number of exploratory analyses of secondary outcome variables were conducted for which the study was not powered. Another limitation is that not all the subjects enrolled the study had RHI <1.67. In addition, this study was not designed to examine the effect of vitamin C alone, therefore, it is unknown if systemic NO accumulation from NC has a direct effect on lipids and oxidative biomarkers or if the direct beneficial effect is explained by vitamin C supplementation.

#### 5. Conclusion

Four weeks of NC intervention improved endothelial function as measured by RHI only in subjects with low RHI at baseline. The provision of dietary nitrate supplementation is a natural, cost effective strategy that promotes augmentation in systemic NO synthesis, and reduction in oxLDL and blood lipids when co-supplemented with vitamin C. Larger randomized trials with a four-arm design that includes a vitamin C arm are needed for a comprehensive assessment of the effect of NC on restoring endothelial function using FMD and to confirm the oxidative stress and lipid modulating properties of NC.

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#### Statement of authorship

R.B takes primary responsibility for this work. R.B had full access to the data and confirms the accuracy of the data analysis. R.B drafted the manuscript and R.B and D.T conceived, designed the study as well as interpreted data and critically edited the manuscript. R.B and M.S acquired the data. R.J contributed to the data analysis. All authors have read and approved the final manuscript.

#### Conflict of interest

The authors have no conflicts of interest.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.clinu.2020.10.012>.

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