A Nitrate-Rich Vegetable Intervention Elevates Plasma Nitrate and Nitrite Concentrations and Reduces Blood Pressure in Healthy Young Adults

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ABSTRACT

Background Emerging evidence suggests that increasing dietary nitrate intake may be an effective approach to reduce blood pressure. Beetroot juice is often used to supplement dietary nitrate, whereas nitrate intake levels from habitual diet are low. An increase in the habitual intake of nitrate-rich vegetables may represent an alternative to nitrate supplementation. However, the effectiveness and acceptability of a nitrate–rich-vegetables diet remain to be established.
Objective The aim was to investigate the effect and feasibility of two different intervention strategies to increase dietary nitrate intake, on plasma nitrate/nitrite concentrations and blood pressure.
Design A randomized, crossover trial was used.
Participants Participants were healthy men and women (both n=15; age: 24±6 years) from the Netherlands.
Intervention Participants were instructed to consume ~400 mg nitrate at lunch, provided through nitrate-rich vegetables and dietary counseling, or beetroot juice supplementation. Both interventions lasted 1 week, with 1-week washout (January to April 2017).
Main outcome Plasma nitrate and nitrite concentrations and resting systolic and diastolic blood pressure were measured in an overnight fasted state (before and after intervention) and ~2.5 hours after lunch (before and throughout intervention on day 1, 4, and 7).
Statistical analysis Two-factor (time × treatment) repeated-measures analyses of variance were performed.
Results Mean plasma nitrate concentrations increased with both interventions, with a larger increase in beetroot juice vs nitrate-rich vegetables, both in a fasted state and ~2.5 hours after lunch (day 1, beetroot juice: 2.31±0.56 mg/dL [373±90 μmol/L] vs nitrate-rich vegetables: 1.71±0.83 mg/dL [277±134 μmol/L]; $P<0.001$). Likewise, mean plasma nitrite concentrations increased with both interventions, but were higher after lunch in beetroot juice than in nitrate-rich vegetables (day 1: 2.58±1.52 μg/dL [560±331 nmol/L] vs 2.15±1.21 μg/dL [468±263 nmol/L]; $P=0.020$). Fasting mean systolic and diastolic blood pressure did not change, but mean systolic and diastolic blood pressure assessed ~2.5 hours after lunch were significantly reduced throughout both intervention periods ($P<0.05$), with no differences between beetroot juice and nitrate-rich vegetables (day 1, systolic blood pressure: −5.1±9.5 mm Hg and diastolic blood pressure: −5.3±8.9 mm Hg).
Conclusion Short-term consumption of dietary nitrate in the form of nitrate-rich vegetables represents an effective means to increase plasma nitrate and nitrite concentrations, and reduces blood pressure to the same extent as beetroot juice supplementation.
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VEGETABLES ARE CONSIDERED ESSENTIAL SOURCES of dietary fiber, vitamins, minerals and antioxidants.[1] Prospective epidemiological studies have indicated that green leafy vegetables (eg, spinach, arugula, lettuce, and bok choy) are among the vegetables most protective against cardiovascular diseases.[2,3] These vegetables share the common characteristic of high nitrate content, and together they account for ~75% to 80% of the average daily nitrate intake.[4,5] The role of dietary nitrate as a biologically active nitric oxide donor, has gained interest, because of its proposed cardioprotective and ergogenic properties. As recently reviewed, dietary nitrate supplementation has been shown to result in both acute and chronic reductions in resting blood pressure[6,7] and improved revascularization in chronic ischemia.[8] Furthermore, nitrate can induce beneficial effects on sports and/or functional performance.[9] Previous work aiming for these beneficial effects of increased dietary nitrate intake has mainly focused on the use of (concentrated) nitrate-rich beetroot juice or sodium or potassium nitrate.[8,10-19] Of interest, recent work seems to indicate that these effects may also be attained through the ingestion of (blended) nitrate-rich vegetables, either upon a single dose,[20-23] or upon receiving nitrate-rich meals over a few days up to 4 weeks.[24-29] However the acceptability and feasibility of a high nitrate diet remain to be further investigated. Moreover, it is currently unknown whether the effects are similar to those obtained with beetroot juice, especially when nitrate dose is kept constant.

Despite the increasing interest in the benefits of nitrate-rich vegetables, general vegetable consumption is below the recommended intake, although large variations exist.[30,31] Because dietary nitrate intake is strongly related to vegetable intake, there is also large variation in nitrate intake. Indeed, individual daily nitrate consumption is estimated to range from <20 mg/day to >400 mg/day in healthy adults, with both mean and median intakes of ~100 mg/day.[4,32,33] From recent studies investigating the health and ergogenic properties of dietary nitrate, it is evident that at least 300 to 600 mg (4.8 to 9.8 mmol) nitrate is needed to induce its cardiovascular[34,35] and performance-enhancing effects.[35-37] Accordingly, the average daily nitrate intake from habitual diet is substantially lower than the dose needed to attain beneficial effects.[5] In the present study, a dietary approach was applied in which participants received dietary counseling to prepare their own nitrate-rich meals to assess the feasibility of reaching a daily nitrate intake of ~400 mg at lunch. Furthermore, it was assessed whether this dietary approach would elicit similar effects as a more traditional nitrate supplementation regimen applying the same nitrate dose (400 mg). Therefore, this study directly compared the effects of a 1-week dietary approach with a standard 1-week supplementation protocol (one bottle of concentrated beetroot juice per day) on dietary nitrate intake, plasma nitrate and nitrite concentrations, and blood pressure. Despite recent attention for its beneficial effects, dietary nitrate is currently still considered a contaminant due to previously suggested links with the formation of potentially carcinogenic nitrosamines.[38] Hence, to provide some insight into the potential detrimental effects of a high nitrate intake, urinary nitrosamine formation was also assessed. This is the first study to directly compare the effects of these two different, self-administered, 1-week dietary nitrate intervention strategies within a single study design under free-living conditions, using a randomized, crossover trial.

RESEARCH SNAPSHOT

Research Question: Is there a difference in the effects of a 1-week nitrate-rich vegetable intervention compared with a standard 1-week supplementation regimen on dietary nitrate intake, plasma nitrate and nitrite concentrations, blood pressure, and urinary nitrosamine formation.

Key Findings: In this study, healthy men and women (both n=15) were instructed to ingest ~400 mg nitrate, provided through nitrate-rich vegetables or beetroot juice, in a randomized, crossover experiment. The daily consumption of nitrate-rich vegetables was observed to be an acceptable strategy to increase plasma nitrate and nitrite concentrations and decreased systolic and diastolic blood pressure to the same extent as beetroot juice supplementation ($P<0.05$).

METHODS

Participants

A total of 30 healthy, recreationally active (>2 hours/week exercise activities) male and female adults (both n=15, age: 24±6 years) participated in the study. Based on a medical screening questionnaire for eligibility, participants were nonsmoking, were not diagnosed with any disease (eg, cardiovascular, metabolic, orthopedic, neurological malignancies, and genetic disorders), and were not using any prescribed medication. Recruitment was conducted during winter 2016 in the Netherlands using university e-mail listservs, newsletters, flyers, and newspaper ads. Participants completed an initial online screening questionnaire followed by a brief telephone interview to determine eligibility. During December 2016 to February 2017, eligible participants were invited to attend an orientation session to complete a consent form and baseline measurements, including body weight (to the nearest 0.1 kg), height (to the nearest 0.1 cm), and blood pressure. Study staff revealed randomization (determined using a computer-based random number generator) to determine the order of intervention. This study was approved by the Medical Ethical Committee of the Maastricht University Medical Centre and conforms to the principles for use of human subjects and tissue outlined in the Declaration of Helsinki. All participants were informed of the nature and possible risks of the experimental procedures, before their written informed consent was obtained.

Study Design

Habitual dietary intake was recorded for 1-wwwk before all participants randomly followed two 1-week intervention periods in a randomized crossover manner, in which they were instructed to consume ~400 mg (6.5 mmol) of dietary nitrate at lunch in the form of concentrated red beetroot juice or ~250 g nitrate-rich vegetables. A full week of washout separated the two intervention periods.

Experimental Protocol

After inclusion, participants commenced a 1-week registration period (before intervention) in which they tracked their habitual dietary intake through food diaries. Subsequently, the day before starting the first interventional period (D0), participants were asked to collect their urine for a 24-hour period. In addition, they reported to the laboratory ~2.5 hours after lunch, where blood pressure was determined and a blood sample was collected. Participants then returned home with a standardized dinner (based on a fixed amount of energy per kilogram bodyweight) that they consumed during that evening. Participants were requested to report to the laboratory the following day, in an overnight fasted state (D1). Blood pressure was measured and a fasting blood sample was collected. Subsequently, participants returned home, marking the start of the intervention period. On the same day (D1), participants again reported to the laboratory ~2.5 hours after lunch. Again, blood pressure was assessed and a blood sample was collected. These afternoon measurements were repeated on day 4 and 7 of the intervention period. Finally, on day 8 (ie, the day after the last intervention day), measurements were again performed in the morning, in an overnight fasted state (ie, blood pressure, blood sample and handing in 24-hour urine collection). All measurements and analyses were performed by researchers/technicians who were blinded to treatment allocation and time points.

Dietary Interventions

Participants were informed about the possible effect of dietary nitrate, but were not aware of the possible differences between the two interventions. Participants were instructed to consume beetroot juice or nitrate-rich vegetables, in a crossover fashion, not altering their normal diet in any other way. To be able to compare the effects of beetroot juice vs nitrate-rich vegetables properly, participants consumed products daily during lunch (12:00 to 12:30 PM), during both intervention weeks. For the nitrate-rich vegetables intervention, participants were informed how they could prepare nitrate-rich meals at lunch. They were provided with a brochure detailing recipes and instructions how to cook (short cooking time) and store (sealed in the fridge) the vegetables. In addition, they were provided with a bag (at day 1 and day 4) with a sufficient amount of fresh nitrate-rich vegetables such as salad, lettuce, arugula, and beetroot (Table 1). Quantities were calculated so that participants would be able to consume up to ~250 g vegetables, providing ~400 mg nitrate daily at lunch, based on average nitrate content reported previously.[4] Nitrate content of vegetables is influenced by environmental factors (eg, humidity, temperature, water content, and exposure to sunlight) as well as food preparation (eg, washing, peeling, and cooking).[39] To control for this as much as possible, participants were provided with fresh vegetables twice a week, and the entire trial was completed within a 4-month period. To stimulate variation in dietary intake, participants were instructed not to consume a chosen meal more than two times. For the beetroot juice intervention, participants were provided with a sufficient amount (70 mL) of red beetroot juice (Beet-it Sport; James White Drinks Ltd) and were instructed to daily consume one bottle during lunch. Compliance was confirmed by the return of empty bottles and via the completion of food diaries during both intervention weeks. Habitual dietary nitrate intake was not restricted in this study.

Physical Activity and Dietary Records

All participants were instructed to refrain from any sort of heavy physical labor 24 hour before all test days and were requested to record their exercise pattern using an exercise diary and to maintain their normal exercise pattern throughout the study to avoid potential effects on blood

Table 1. Nitrate-rich vegetables and recipes[a] provided during the 1-week nitrate-rich vegetables intervention and their estimated dietary nitrate content[4] in a randomized crossover trial on the effects of dietary nitrate among 30 healthy young adults

Vegetable (raw)	Nitrate (mg/100 g)
Arugula (rocket salad)	420
Bok choy	325
Rhubarb	294
Lettuce	205
Beetroot	188
Spinach	180
Zucchini	68
Green beans	53
Recipes	**Nitrate (mg/portion)**
Red beet soup	350 mg
- 5 g oil for baking	
- 125 g fresh raw beetroot	
- 10 g onions	
- 50 g chicken/beef	
- 125 mL water	
- 5 g broth	
Spinach pizza	445 mg
- 1 pizza crust 22 cm (eg, from cooling or deep-fried)	
- 5 g oil for baking	
- 5 g garlic	
- 120 g chicken fillet (cut into thin strips)	
- 30 g mushrooms in slices	
- 70 g red pepper in small pieces	
- 200 g spinach	
- 50 mL tomato puree	
- 15 g lettuce (eg, arugula)	
- 30 g zucchini	
- 30 g cheese (eg, mozzarella, feta)	
Smoothie	330 mg
- 120 g bok choy	
- 150 mL apple juice	
- 20 g ginger	
- 1 small tablespoon of sesame seeds	
- 60 g rhubarb compote ('rhubarb quark')	
Rhubarb quark	200 mg
- 1 portion of rhubarb compote:	
115 g rhubarb stems	
20 g honey	
25 mL water	
- 150 g low-fat (soy) quark	

[a]The recipes shown are examples of the seven recipes that were provided to participants.

pressure.[40] Participants were also asked to avoid caffeine and alcohol intake during the 12 and 24 hours preceding a test day, respectively.[14,41-43] To prevent any attenuation in the reduction of nitrate to nitrite by commensal bacteria in the oral cavity, participants were asked to refrain from using any antibacterial mouthwash/toothpaste, chewing gum, and tongue-scraping during their participation in this study.[44]

Participants filled in 21 food diaries over the total study period and were instructed to make a daily note of the exact amount and time of ingestion of the investigational products to measure compliance. All food diaries were checked for completeness and reviewed with the participants, by a registered dietitian nutritionist especially trained for this task using standardized procedures, including a standardized weight and portion book. Food diaries were analyzed using Compl-eat, a web-based program built by Wageningen University.[45] Compl-eat is based on the 5-step multiple-pass method, a validated technique to increase accuracy.[46] The program includes a wide selection of foods commonly used in a Dutch food pattern[47] and has previously been used to assess dietary nitrate intake, with nitrate contents of food included in the program's software.[4]

Analysis of Plasma Nitrate and Nitrite Concentrations

A total of 7.5 mL blood was drawn at each time point. Each sample was collected in lithium-heparin containing tubes, for analysis of nitrite and nitrate. To minimize any nitrate/nitrite conversion, the tubes were immediately centrifuged at 4°C, and 3,000 RPM (1000 g) for 5 minutes after which the remaining plasma (~3.5 mL) was aliquoted (600 μL aliquots), and frozen in liquid nitrogen and stored at −80°C until analysis. Analysis of plasma nitrate and nitrite concentrations was performed using the chemiluminescence technique, which has been described in previous studies.[13,35,48] Units for nitrate and nitrite were converted from International System of Units to conventional units.[49]

Blood Pressure

After a 10-minute seated rest period, blood pressure was taken four times, right sided and in seated position, using an automated cuff (Omron Healthcare Inc), with the last three measurements averaged to obtain mean systolic (SBP) and mean diastolic blood pressure (DBP).

Analysis of Urinary Nitrosamines

Urinary *N*-nitroso compounds (ATNC) concentrations were determined using chemiluminescence detection.[50] Urinary samples were treated with preservation solution (0.1 M N-ethylmaleimide and 0.01 M diethylenetriaminepentaacetic acid (DTPA)) and then incubated with 50 g/L sulfamic acid for 1 to 5 minutes. Samples were injected into the purge vessel (60°C) containing 10 to 15 mL reduction solution (11.11 g/L potassium iodide and 5.55 g/L iodine in 40 mL water and 140 mL glacial acetic acid). Preservation solution was added to preserve the nitrosation state of thiols by alkylating free thiol groups and scavenging metal ions, which can cause a release of nitric oxide from nitroso-thiols. Tri-iodide reduction solution releases nitric oxide from nitrite, nitrosothiols, nitrosamines, iron-nitrosylhemoglobin, and nitrosohemoglobin. ATNC contribution to the total chemiluminescence detection signal was determined by subtracting the nitrite response from the total response. When ATNC was undetectable, 0.0 μmol/L was used for statistical analyses.

Statistical Analysis

A power calculation was performed to detect a difference in the increase in plasma nitrate concentration between interventions. Based on data from a previous study, the standard deviation of the between-treatment difference in plasma nitrate was expected to be approximately 123 μM.[20] Furthermore, previous work from others,[35,51] indicates that a difference in the increase in plasma nitrate levels of ~50 to 130 μM can result in relevant differences in functional outcome. Based on these findings, the average value of 90 μM was taken as a relevant difference to be detected between the two interventions. The sample size was calculated with a power of 95%, a significance level of 0.05, and a dropout rate of 10%. The final number of participants to be included after screening was 30. All statistical analyses were performed using SPSS version 23.0.[52] The data were checked for normality using the Shapiro-Wilk test. Plasma nitrate and nitrite concentrations, and resting blood pressure were analyzed using a 2-factor (time × treatment) repeated-measures analyses of variance. Separate analyses were performed for fasting values (D1 vs D8), and for values assessed 2.5 hours after lunch (D0, D1, D4, and D7). To further specify any interactions or main effects, post hoc tests were performed using Bonferroni corrections for primary variables (plasma nitrate and nitrite concentrations). Nonparametric tests (Wilcoxon, Kruskal-Wallis and Friedman tests) were used for nonnormally distributed continuous variables; that is, all dietary intake data and urinary ATNC. Although this study was not specifically aimed at detecting any sex effects on the response to dietary nitrate consumption, the individual plasma nitrate and nitrite graphs appeared different between men and women upon visual inspection. Therefore, as a secondary analysis, sex was included as a between-subject factor in the analyses of variance described above, and these explorative data are reported in the Results section. All data are presented as mean±standard deviation, or median (interquartile range [IQR]) for nonparametric data. Statistical significance was set at $P<0.05$.

RESULTS

The total group of 30 recreationally active individuals had an average age of 24±6 years and a body mass index of 22.6±2.4. Baseline characteristics are reported in Table 2. Body weight and body mass index did not change throughout the course of either intervention (data not shown). Ingestion of the beetroot juice as well as the nitrate-rich vegetables were well tolerated by all participants, and no adverse events were reported.

Dietary Intake

A complete overview of reported dietary intake before and during the interventions, including differences between men and women, is provided in Table 3. Before the experimental phase, median daily vegetable and dietary nitrate intake was 189 g/day (IQR 134 to 304 g/day) and 130 mg/day (IQR, 90 to 203 mg/day), respectively. One week of increased nitrate-rich vegetables consumption resulted in higher vegetable intake

Table 2. Baseline characteristics of a cohort of 30 healthy men (n=15) and women (n=15) participating in a randomized crossover trial on the effects of dietary nitrate

Characteristic	All	Men	Women
	←mean±standard deviation→		
Age (y)	24±6	26±7	23±5
Height (m)	1.77±0.07	1.81±0.05	1.72±0.08*
Weight (kg)	70.4±7.7	74.5±4.9	66.3±8.0*
Body mass index	22.6±2.4	22.8±2.0	22.5±2.3
Systolic blood pressure (mm Hg)	121±13	129±11	114±10*
Diastolic blood pressure (mm Hg)	66±6	66±5	66±7

*Significantly different from men at $P<0.05$.

(median 384 g/day, IQR 334 to 454 g/day; $P<0.001$), whereas vegetable intake did not change with beetroot juice (median 177 g/day, IQR 115 to 278 g/day) (Figure 1). Daily nitrate intake increased with both interventions ($P<0.001$) but was slightly lower with nitrate-rich vegetables (median 555 mg/day, IQR 495 to 642 mg/day) vs beetroot juice (median 683 mg/day, IQR 632 to 751 mg/day; $P=0.001$) (Figure 1). Food diaries suggested good compliance, with 100% of beetroot juice supplements ingested, and a median daily intake of 241 g (IQR 197 to 331 g) of the ∼250 g targeted nitrate-rich vegetables at lunchtime.

Plasma Nitrate Concentrations

Fasting plasma nitrate concentrations (D1) averaged 0.35±0.17 mg/dL (57±28 μmol/L) before intervention. A time × treatment interaction ($P<0.001$) was observed, after which post hoc analyses showed that fasting plasma nitrate concentration had increased with both interventions (D8; both P values <0.001), with a larger increase in beetroot juice compared with nitrate-rich vegetables (up to 0.78±0.26 mg/dL [126±42 μmol/L] vs 0.52±0.22 mg/dL [84±36 μmol/L], respectively; $P<0.001$). Plasma nitrate concentrations after lunch (D0) were 0.40±0.32 mg/dL (66±52 μmol/L) before intervention. Again, a significant time × treatment interaction ($P<0.001$) was observed, with post hoc analyses showing that plasma nitrate concentrations after lunch had increased with both interventions (all P values <0.001), but were higher following beetroot juice compared with nitrate-rich vegetables consumption (eg, D1: 2.31±0.56 mg/dL [373±90 μmol/L] vs 1.72±0.83 mg/dL [277±134 μmol/L], respectively; $P<0.001$). Similar findings were observed for plasma nitrate concentrations at D4 and D7 (Figure 2).

Plasma Nitrite Concentrations

Fasting plasma nitrite concentrations (D1) averaged 0.96±0.32 μg/dL (208±69 nmol/L) before intervention and increased with both interventions (D8; main effect of time $P=0.012$), with no differences between beetroot juice and nitrate-rich vegetables consumption (1.14±0.41 μg/dL [247±88 nmol/L] vs 1.03±0.30 μg/dL [224±66 nmol/L]; time × treatment interaction $P=0.095$). Plasma nitrite concentrations after lunch (D0) were 1.04±0.39 μg/dL (227±86 nmol/L) before intervention, and increased with both interventions (main effect of time $P<0.001$), with no differences between interventions (time × treatment interaction $P=0.44$). Yet, plasma nitrite concentrations after lunch were on average higher in beetroot juice compared with nitrate-rich vegetables (main effect of treatment $P=0.020$) (eg, D1; 2.57±1.52 μg/dL [560±331 nmol/L] vs 2.15±1.21 μg/dL [468±263 nmol/L], respectively). Similar findings were observed for plasma nitrite concentrations at D4 and D7 (Figure 2).

Blood Pressure

Fasting SBP was 116±10 mm Hg and did not change with both interventions (D8: beetroot juice: 115±8 mm Hg, nitrate-rich vegetables: 116±9 mm Hg; main effect of time $P=0.86$, time × treatment interaction $P=0.23$). In contrast, SBP assessed ∼2.5 hours after lunch (D0) was 121±13 mm Hg before intervention and was significantly reduced with both interventions (main effect of time $P=0.001$), with no differences between beetroot juice and nitrate-rich vegetables (time × treatment interaction $P=0.12$). Post hoc analyses showed that SBP 2.5 hours after lunch was significantly reduced with both interventions at D1 (−5.1±9.5 mm Hg and −5.3±8.9 mm Hg for beetroot juice and nitrate-rich vegetables, respectively; effect of time $P=0.002$), as well as at D4 and D7 (Figure 3).

Fasting DBP was 64±8 mm Hg and did not change with both interventions (D8) (beetroot juice: 65±7, nitrate-rich vegetables: 64±9 mm Hg; main effect of time $P=0.31$, time × treatment interaction $P=0.96$). In contrast, DBP assessed ∼2.5 hours after lunch (D0) was 66±6 mm Hg before intervention, and was significantly reduced (main effect of time $P=0.013$), with no differences between beetroot juice and nitrate-rich vegetables (time × treatment interaction $P=0.23$). Post hoc comparisons revealed a significant reduction in DBP with both interventions for D1 and D7, but not D4 (Figure 3).

Urinary Nitrosamine

Median apparent total urinary ATNC was undetectable in 27 of 30 participants before intervention, 19 of 30 participants in the nitrate-rich vegetables intervention, and 4 of 30 participants in the beetroot juice intervention. Using values of 0.0 μmol/L for undetectable samples, median urinary ATNC was 0.0 μmol/L (IQR 0.0 to 0.0 μmol/L) and significantly increased with 1 week of increased nitrate-rich vegetables consumption (median 0.0 μmol/L, IQR, 0.0 to 0.7 μmol/L; $P=0.003$), as well as 1 week of beetroot juice supplementation (median, 1.1 μmol/L, IQR, 0.4-3.1 μmol/L; $P<0.001$). Furthermore, median values were higher in beetroot juice vs nitrate-rich vegetables ($P=0.004$).

Sex

When sex was included as a between-subject variable, plasma nitrate concentrations after lunch did not differ between men and women before intervention, nor with both interventions (main effect of sex $P=0.25$, no significant interactions between sex and time/treatment; data not shown). Fasting plasma nitrate was higher in women vs men before

Table 3. Reported daily dietary intake before (habitual diet) and during intervention of 30 healthy individuals (15 men and 15 women) participating in a randomized crossover trial on the effects of dietary nitrate

	Habitual diet			Beetroot juice intervention			Nitrate-rich vegetables intervention		
Nutrient[a]	**All**	**Men**	**Women**	**All**	**Men**	**Women**	**All**	**Men**	**Women**
	←———— *median (interquartile range)* ————→								
Vegetable intake (g)	189 (134-304)	173 (129-303)	214 (156-342)	177 (115-278)	149 (115-234)	245 (102-498)	384 (334-454)[b]	361 (306-422)[b]	420 (353-475)[b]
Nitrate intake (mg)	130 (90-203)	129 (90-205)	130 (87-202)	683 (632-751)[c]	686 (643-744)[c]	645 (612-765)[d]	555 (495-642)[d]	547 (495-605)[d]	596 (494-746)[d]
Energy (kcal)	2,208 (1,762-2,730)	2,671 (2,664-2,917)[e]	1,777 (,1616-2,127)	2,001 (1,602-2,907)	2,613 (1,909-3,051)[e]	1,845 (1,477-2,047)	2,285 (1,963-2,960)[f]	2,281 (2,231-3,060)[e]	2,010 (1,835-2,271)[f]
									8.4 (7.7-9.5)
Carbohydrate g	237 (196-314)	304 (226-339)[e]	206 (166-237)	220 (186-309)	284 (215-358)[e]	211 (160-220)	274 (226-350)[b]	345 (287-369)[e]	230 (220-262)[f]
% energy g/kg	44 (40-49)	44 (41-47)	45 (38-51)	45 (42-48)	46 (44-50)	45 (42-48)	48 (45-52)[d]	47 (45-52)	48 (46-53)
	3.5 (2.7-4.4)	4.1 (3.1-4.9)	3.2 (2.5-4.4)	3.2 (2.6-4.3)	3.9 (2.9-4.9)	3.1 (2.5-3.8)	4.1 (3.2-4.8)[b]	4.6 (4.0-4.9)	3.6 (3.1-4.9)[f]
Fat g	89 (61-101)	99 (89-114)[e]	69 (56-90)	73 (59-105)	97 (61-116)[e]	63 (57-80)	80 (72-96)	89 (79-110)[e]	76 (68.0-81.0)
% energy	34 (30-38)	34 (33-39)	34 (29-38)	33 (29-37)	33 (29-37)	33 (31-37)	31 (28-34)	31 (27-33)	31 (30-35)
Protein g	86 (63-106)	100 (92-111)[e]	65 (60-86)	77 (67-114)	103 (81-124)[e]	73 (66-77)	95 (71-128)[d]	124 (98-130)[e]	79 (64-92)
g/kg	1.2 (1.0-1.4)	1.4 (1.1-1.5)[e]	1.0 (0.9-1.2)	1.2 (0.9-1.6)	1.4 (1.1-1.7)	1.1 (0.8-1.4)	1.5 (1.1-1.7)[d]	1.7 (1.4-1.8)[e]	1.3 (0.9-1.5)
Dietary fiber (g)	29 (20-37)	29 (22-37)	29 (10-37)	27 (18-35)	28 (20-38)	27 (17-32)	34 (27-41)[b]	35 (30-43)[b]	31 (23-40)
Vitamin C (mg)	113 (62-151)	114 (79-147)	90 (55-157)	94 (59-150)	102 (80-144)	71 (44-165)	112 (100-148)	121 (109-140)	118 (80-159)
Sodium (g)	2.2 (1.7-2.5)	2.3 (2.1-3.4)[e]	1.9 (1.7-2.2)	2.1 (1.7-2.6)	2.5 (1.9-3.4)[e]	2.0 (1.7-2.1)	2.3 (2.1-3.1)[f]	3.0 (2.3-3.7)[de]	2.2 (1.8-2.4)[d]

[a]Sata represent median (interquartile range) intake levels, based on the mean per person from seven food diaries, not including vitamin and mineral supplements.
[b]Significantly different from habitual diet and beetroot juice ($P<0.017$).
[c]Significantly different from habitual and nitrate-rich diet ($P<0.01$).
[d]Significantly different from habitual diet ($P<0.017$) based on Friedman test.
[e]Significantly different between men and women ($P<0.05$).
[f]Significantly different from beetroot juice ($P<0.017$).

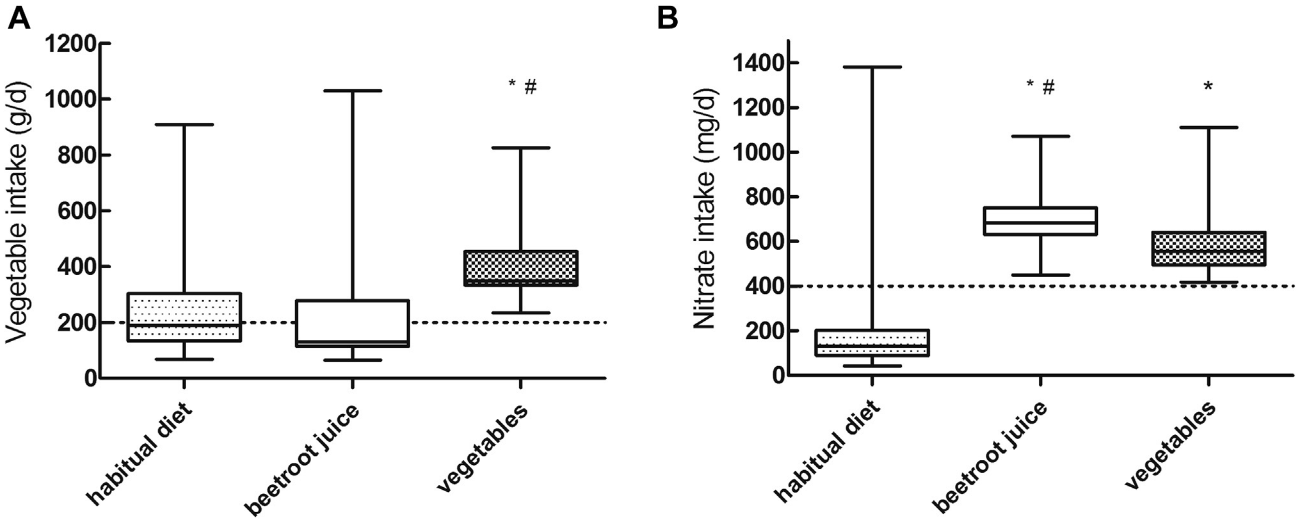

Figure 1. Daily vegetable (Panel A) and nitrate (Panel B) consumption in habitual diet (before intervention), during 1-week of beetroot juice supplementation, and during 1-week of increased nitrate-rich vegetable consumption. Boxes represent interquartile (25th to 75th percentile) ranges, whiskers represent the maximal and minimal values, and the horizontal line indicates the median. The dotted lines represent the recommended vegetable intake from the 2015 Dutch dietary guidelines of 200 g vegetables per day[30] and the targeted nitrate of the current study 400 mg dietary nitrate. *Significantly different ($P<0.05$) from before intervention. #Significantly different ($P<0.05$) between interventions.

(64±35 vs 48±15 μmol/L) and remained higher after both interventions (main effect of sex; $P=0.029$), but treatment effects were not different between women and men (no interactions between sex and time/treatment; data not shown).

Differences in plasma nitrite after lunch between beetroot juice and nitrate-rich vegetables were driven by the women (treatment × sex interaction $P=0.013$). Post hoc analyses showed that women had higher plasma nitrite concentrations at D1, D4, and D7 for beetroot juice vs nitrate-rich vegetables ($P<0.01$). In contrast, no differences between beetroot juice and nitrate-rich vegetables were observed in men ($P>0.50$; data not shown). Fasting plasma nitrite concentrations were not different between women and men. However, a time × sex interaction ($P=0.011$) was observed, with post hoc analyses showing that the increase in fasting plasma nitrite concentrations with both interventions was driven by women (main effect of time $P=0.002$), whereas no changes were observed in fasting plasma nitrite concentrations in men (main effect of time $P=0.80$; data not shown).

SBP was lower in women vs men before intervention and remained lower throughout both interventions (main effect of sex $P=0.001$) whereas DBP was not different (main effect of sex $P=0.33$) (Table 2). The changes in SBP and DBP with

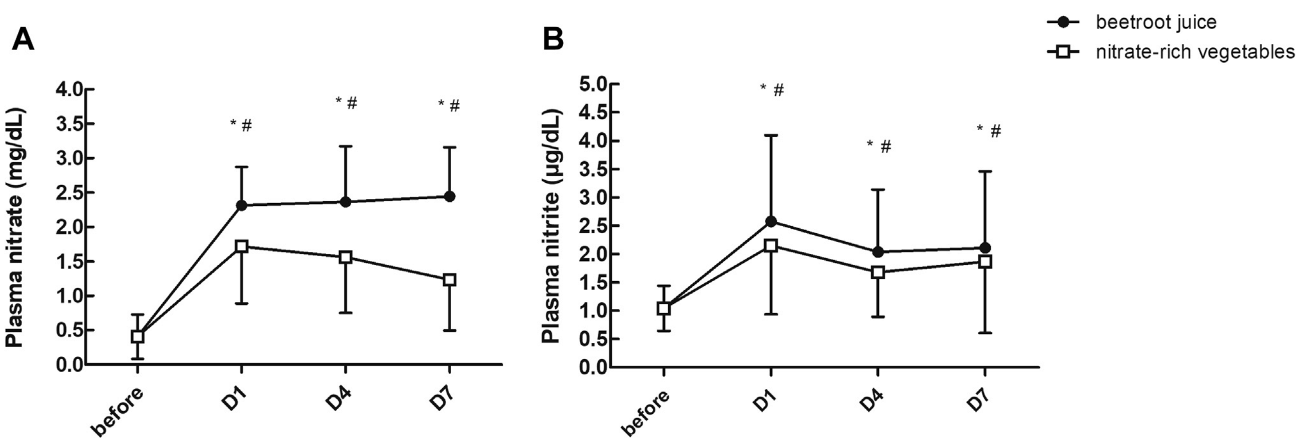

Figure 2. Plasma nitrate (Panel A) and nitrite (Panel B) concentrations (mean±standard deviation) in 30 healthy adults consuming beetroot juice (□) and nitrate-rich vegetables (●) during day 1 (D1), day 4 (D4), and day 7 (D7) of the intervention. [a]To convert mg/dL nitrate to μmol/L, multiply mg/dL by 0.0062. To convert μmol/L nitrate to mg/dL, multiply μmol/L by 161.3. Nitrate of 4.0 mg/dL=0.0248 μmol/L. To convert μg/dL nitrite to nmol/L, multiply μg/dL by 0.0046. To convert nmol/L nitrite to μg/dL, multiply nmol/L by 217.4. Nitrite of 5.0 μg/dL=0.023 nmol/L.[49] *Significantly different ($P<0.05$) from before intervention (D0). #Significantly different ($P<0.05$) between interventions.

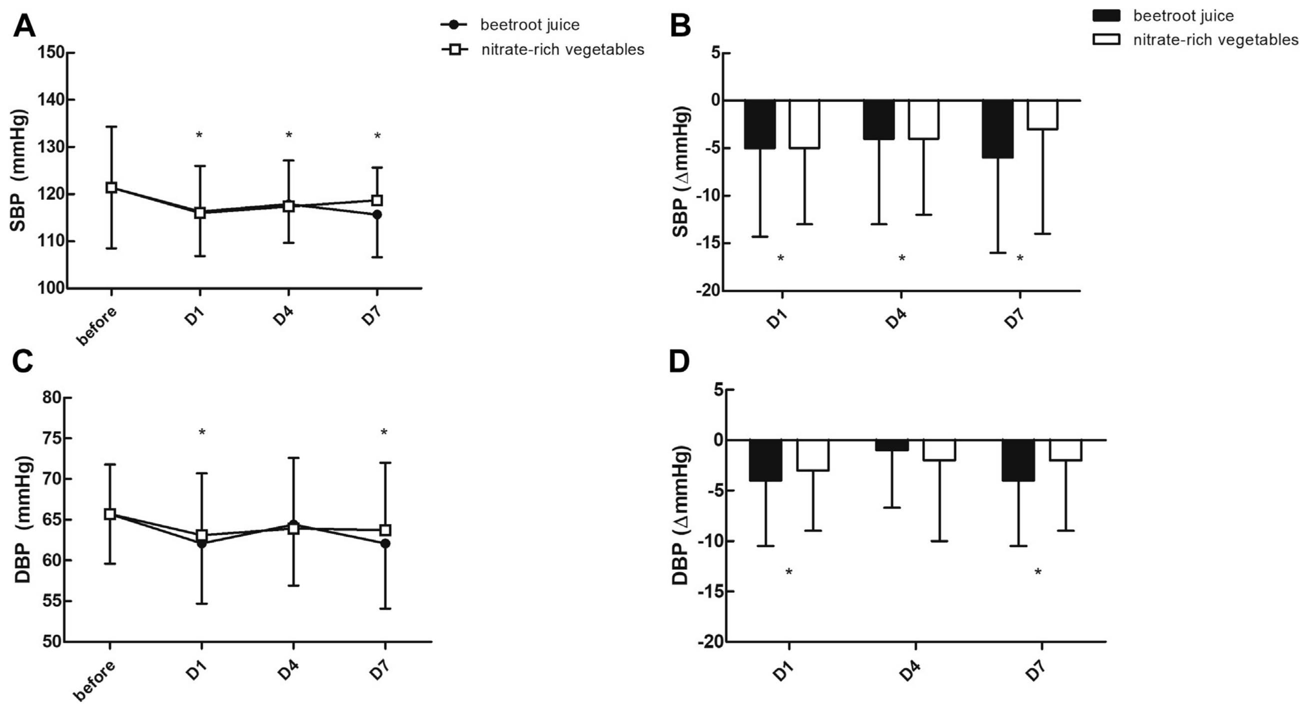

Figure 3. Changes (mean±standard deviation) in systolic blood pressure (SBP) and diastolic blood pressure (DBP) in 30 healthy adults consuming beetroot juice (□) and nitrate-rich vegetables (●) during day 1 (D1), day 4 (D4), and day 7 (D7) of the intervention. Panel A: SBP before and during intervention (D0). Panel B: decrease in SBP when compared with D0. Panel C: DBP before and during intervention. Panel D: Decrease in DBP when compared with D0. *Significantly different ($P<0.05$) from D0.

both interventions were not different between women and men (no significant interactions between sex and time/treatment; data not shown).

DISCUSSION

The present study demonstrates that the daily consumption of ~400 mg dietary nitrate in the form of nitrate-rich vegetables is an acceptable and feasible strategy to significantly increase plasma nitrate and nitrite concentrations and decrease systolic and diastolic blood pressure. Although increased nitrate intake results in higher ATNC levels, the increase is minor and values remain well within normal physiological range. In directly comparing two self-administered intervention strategies, the current study shows for the first time that the reductions in blood pressure after 1 week of beetroot juice supplementation can be obtained to the same extent through increased consumption of nitrate-rich vegetables in the diet.

Before intervention, median habitual vegetable consumption was 189 g/day, and median habitual dietary nitrate intake was 130 mg/day in this cohort of healthy men and women, which is consistent with previous reports.[4,53,54] In the dietary counseling intervention, participants received coaching, as well as recipes and a sufficient amount of nitrate-rich vegetables. This enabled them to increase their daily vegetable intake up to almost 400 g of which approximately 250 g was of a variety of nitrate-rich vegetables (eg, spinach, arugula, salad, lettuce, bok choy), as part of their daily normal diet. Consequently, the recommendation of a daily intake of at least 200 g vegetables[30] was reached for each individual participant (Figure 1). Likewise, median nitrate intake was substantially increased up to 555 mg/day (IQR 495 to 642 mg/day), clearly exceeding the suggested dose of ~400 mg for all participants (Figure 1). Good compliance to the diet was observed and no (gastrointestinal) complaints were reported after the increase in vegetable consumption. These findings strongly suggest that increasing nitrate-rich vegetable intake through the habitual diet represents an acceptable strategy when aiming for the beneficial effects of increased nitrate intake.

Such an approach has the additional benefit of providing a high diversity of vitamins, trace minerals, and dietary fibers. Besides, the consumption of whole food products is superior to liquids (smoothies/juices) in the context of total energy intake because it leads to higher ratings of satiety,[55] which can be explained by the higher fiber content,[56] volume, and increased chewing.[57,58] When successfully integrated in the lifestyle (ie, dietary habits), this approach would thus be expected to have most positive influence on overall health.

Both nitrate-rich vegetables and beetroot juice consumed at lunch significantly elevated plasma nitrate and nitrite concentrations throughout the week. Plasma nitrate concentrations increased approximately sixfold and approximately fourfold after consumption of beetroot juice and nitrate-rich vegetables, respectively (Figure 2). Plasma nitrite concentrations increased approximately fourfold for both nitrate sources (Figure 2). The increase in plasma nitrate and nitrite concentrations are in agreement with previous work in healthy cohorts, supplementing ~400 mg nitrate in the form of nitrate salts,[59,60] beetroot juice,[41,61,62] or nitrate-rich vegetables.[24,25,28] The increase in plasma nitrate and nitrite concentrations was slightly larger during the beetroot juice vs nitrate-rich vegetables intervention. This is likely explained by the slightly higher total nitrate intakes for the beetroot juice vs nitrate-rich vegetables intervention, as well as a difference in the time to reach peak plasma values between beetroot juice and nitrate-rich vegetables.[4] The potentially longer time needed to reach peak plasma nitrate concentrations following ingestion of vegetables can be related to the higher fiber content and the larger volume of food ingested, likely resulting in a slower gastric emptying.[63] Fasting plasma nitrate and nitrite concentrations remained significantly elevated ~20 hours after the final nitrate dose was ingested, but the increase was less pronounced when compared to the acute response 2.5 hours after ingestion. Previous studies have shown that plasma nitrate and nitrite concentrations return to basal concentrations after cessation of high nitrate intakes[26,64,65]; therefore, daily consumption of either nitrate supplements or nitrate-rich vegetables appears to be needed to induce a persistent increase in plasma nitrate and nitrite concentrations.

The beneficial effects of dietary nitrate relate to its ability to act as an nitric oxide donor. Indeed, the increased availability of nitrate in the plasma leads to increased (oral) bioconversion into nitrite, subsequently increasing plasma nitrite concentrations, which can be further reduced into nitric oxide, a powerful vasodilator. In accordance, increased plasma nitrate and nitrite concentration have been associated with a subsequent reduction in blood pressure.[66] Despite the normal, healthy blood pressure in the current study's participants, the increased daily nitrate intake resulted in a significant reduction in both SBP (~5 mm Hg) and DBP (~3 mm Hg) (Figure 3). Similar reductions were recently reported in a meta-analysis from Siervo and colleagues[67] showing an average decrease of 4.4 mm Hg in SBP and 1.1 mm Hg DBP after 2 to 15 days of dietary nitrate supplementation through beetroot juice or nitrate salt, in healthy individuals. These findings are also consistent with recent studies supplementing nitrate-rich vegetables.[20,21,24,28] The present data extend on previous findings by showing that the blood pressure reduction persists throughout the 1-week intervention, and, more importantly, show that this beneficial effect does not differ between beetroot juice supplementation and nitrate-rich vegetable consumption.

Plasma nitrate and nitrite concentrations increased to a higher level with beetroot juice compared with nitrate-rich vegetables, whereas the change in SBP was not different between the interventions. Several factors likely play a role in explaining this finding. Firstly, it is not the increase in plasma nitrate and nitrite per se, but rather the effective bioconversion into nitric oxide and subsequent production ofcyclic guanosine monophosphate (cGMP), that causes the decrease in blood pressure.[68] It could be suggested that this bioconversion reaches a certain plateau, with further increases in plasma nitrate and nitrite not leading to greater availability of nitric oxide and/or stimulation of cGMP production. In accordance, previous work has also shown that the increase in plasma nitrite levels is not directly related to the extent of blood pressure reduction.[20] Furthermore, the present study included healthy participants with normal blood pressure, for whom it is likely that the overall capacity for blood pressure reduction is limited and may thus already be reached with lower levels of plasma nitrite. Finally, other components that are present to a larger extent in vegetables, such as antioxidants, flavonoids, anthyocianates, polyphenols, and betaine, may also increase nitric oxide bioavailability.[69,70] Although evidence is currently scarce, it is possible that this might be accomplished through enhanced bioconversion of circulating nitrate/nitrite,[71] potentially compensating for the somewhat lower plasma nitrate/nitrite levels obtained. In any case, using direct comparisons in a crossover design, the current results demonstrate for the first time that increasing dietary nitrate intake through increased vegetable consumption in the habitual diet is equally effective as supplementation with (concentrated) beetroot juice, given that the total dose ingested is large enough.

Although blood pressure reductions persisted throughout the week, conclusions regarding the long-term health effects cannot be drawn. However, based on prospective epidemiological data, it seems that the beneficial cardiovascular effects of a diet with high amounts of vegetables can, at least partly, be attributed to the high inorganic nitrate content.[3,72,73] The current data support the health benefits of a diet high in green leafy vegetables and beetroot. Nonetheless, future clinical studies are warranted to elucidate if nitrate-rich vegetables can offer a nutrition-based approach to prevention and treatment of cardiovascular disease. This also applies to potential differences between men and women. The present work was not powered to investigate any potential sex differences. However, subanalysis of the data, including sex as a between-subjects variable, indicated differences in plasma nitrite responses. Of note, these sex differences did not translate into any significant differences in the blood pressure-lowering effects after nitrate consumption. Although evidence of sex differences in dietary nitrate handling is limited, previous studies have also reported higher plasma nitrate and/or nitrite responses in women when compared with men.[20,59] Differences in plasma concentrations could partly be explained by differences in body mass and/or plasma volume.[20,59] As such, sex differences in the response to dietary nitrate consumption may represent a worthwhile direction for future research, although evidence so far does not indicate any differences in the functional benefits. Notably, the data presented would indicate that, despite the larger increases in plasma nitrite, the reduction in blood pressure may actually be attenuated in women vs men. Although this may also be related to the already lower blood pressure before intervention in women, these potential sex differences call for specifically designed studies to further elucidate the various factors that may cause differential physiological effects of dietary nitrate.

The present work clearly shows that nitrate-rich vegetables can be effectively used as dietary nitrate donors, and increasing nitrate-rich vegetable intake represents an acceptable intervention. These findings, together with previous work from others,[74-76] indicate that offering a variety of vegetables with different sensory properties and adding chopped or puréed vegetables to mixed dishes provides an effective way to increase vegetable intake. Although participants had to prepare their own lunch, the excellent adherence to the vegetable intervention was likely facilitated by the provision of specific food items, as also reported by Kwan and colleagues.[77] Whereas the dietary approach was shown feasible for this short-term study, the circumstances of the intervention (eg, provision of food without shopping) were different from what would be experienced by individuals not participating in a research project. Feasibility is therefore limited to the context of the study. Future research should be directed towards determining the long-term effectiveness and feasibility of high-nitrate diets, especially in specific patient populations such as those with high blood pressure.[78] Based on previous work, facilitating access to high-nitrate vegetables, and embedding of strategies within the (local) health care setting of patients (eg, medical prescription of food) are key issues to take into account in such studies.[79] The current findings provide an opportunity for the further development of cost-effective strategies to improve health through increased vegetable consumption of patients at a population level, viewing food as a way to prevent or treat diseases.

The safety of high dietary nitrate intakes has been much debated, particularly with respect to endogenous *N*-nitrosamine formation.[80] In line with the vast majority of dietary nitrate intervention studies, daily nitrate intake far exceeded the current acceptable daily intake[81] of 3.7 mg/kg/day in both the nitrate-rich vegetables (~8 mg/kg/d) and beetroot juice treatment (~10 mg/kg/d). Although recent insights regarding the health benefits of dietary nitrate have spiked scientific discussion on the current acceptable daily intake,[82] it is of importance to also address any potential detrimental effects. As such, this study is among the first to examine the effect of a high nitrate diet on ATNC formation. As expected, a small increase in apparent total urinary ATNC after increased nitrate consumption was observed. However, the concentrations fell well within the range normally observed in healthy individuals,[83-85] suggesting very minimal (if any) increased risk due to formation of potentially harmful compounds. Even lower levels were found after vegetable consumption compared with beetroot juice, which might be explained by higher vitamin C and other antioxidants levels in vegetables that are proven to prevent ATNC formation.[86] Unfortunately, there is currently very limited information to interpret the actual values for ATNC formation, and there is a lack of other, validated biomarkers to assess potential health risks.[87] Yet, it is important to report these data to facilitate future comparisons and potentially start building reference values that may better enable researchers to address the issue of established health benefits versus potential health risks.

In the present study, a control condition was not included, which could be viewed as a limitation because it does not account for normal blood pressure fluctuation caused by food intake. Indeed, it is known that blood pressure slightly rises during food intake and falls about 1 to 2 hours after eating.[88] The reduction in blood pressure observed after eating is caused by a decrease in total peripheral resistance because of visceral vasodilation.[89] Blood pressure reduction may be larger in elderly and individuals with hypertension (even up to ~20 mm Hg) compared with healthy young individuals (up to ~2 mm Hg),[89,90] minimizing the chance that it affected current findings, and rendering the average decline in SBP in the current study (~5 mm Hg) clinically relevant. It has already been clearly established that beetroot juice supplementation (acutely, 1 week, and up to 6 weeks) effectively increases plasma nitrate/nitrite concentrations and reduces blood pressure.[91] By including before intervention measurements after a week of habitual dietary intake, both in fasting conditions and after lunch, the blood pressure lowering effects of the beetroot juice and nitrate-rich vegetables conditions (ie, vs before intervention) could be directly compared (ie, vs each other), without further increasing the burden on participants with an additional control/placebo intervention. Indeed, this direct comparison between beetroot juice and nitrate-rich vegetables was the primary aim, also representing the main novelty of the current work. Furthermore, only clinic blood pressure measurements were included, as previous work has shown that reductions in blood pressure upon nitrate supplementation are similar between clinic, 24-hour, and home-based blood pressure assessments.[65] There are also several limitations of using self-reported dietary intake, such as estimation of portion sizes, frequency of snacking, and underreporting.[92] However, the inclusion of three times 7 dietary diary days per person and the verification of food intake by a registered dietitian nutritionist, improved the validity for evaluating dietary intake in the current study. As a final limitation of the current work, the actual nitrate intake was calculated based on indirect estimation of nitrate content of vegetables, which may vary according to environmental, agricultural, and genetic factors.[39] Future research may consider direct measurement of nitrate concentrations in vegetables, especially when knowledge on exact intake levels is necessary. However, based on the current findings, increasing habitual nitrate-rich vegetable intake is an equally effective strategy as nitrate supplementation to elicit the beneficial effects of dietary nitrate.

CONCLUSIONS

A short-term dietary counseling intervention that includes provision of nitrate-rich vegetables effectively increases dietary nitrate intake and thereby plasma nitrate and nitrite concentrations, and reduces blood pressure to the same extent as beetroot juice supplementation in healthy free-living young adults. These data support the potential health benefits of a diet high in green leafy vegetables and beetroot. Nonetheless, future studies are warranted to establish sustainability and acceptance over more prolonged time periods, and assess whether nitrate-rich vegetables can offer a nutrition-based approach in the treatment of hypertension and cardiovascular disease.

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No potential conflict was reported by the authors.

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This study was designed by C. van der Avoort, L. van Loon M. Hopman, and L. B. Verdijk; data were collected by C. van der Avoort and analyzed by C. van der Avoort L. van Loon, M. Hopman, and L. B. Verdijk; data interpretation and manuscript preparation were undertaken by C. van der Avoort, L. van Loon, M. Hopman, and L. B. Verdijk. All authors approved the final version of the manuscript.