Supplemental Table 1 CONSORT 2010 checklist of information to include when reporting a randomized trial

<table>
<thead>
<tr>
<th>Section/Topic</th>
<th>Item No</th>
<th>Checklist item</th>
<th>Reported on page No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title and abstract</td>
<td>1a</td>
<td>Identification as a randomized trial in the title</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1b</td>
<td>Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)</td>
<td>3</td>
</tr>
<tr>
<td>Introduction</td>
<td>2a</td>
<td>Scientific background and explanation of rationale</td>
<td>4-5</td>
</tr>
<tr>
<td></td>
<td>2b</td>
<td>Specific objectives or hypotheses</td>
<td>5</td>
</tr>
<tr>
<td>Methods</td>
<td>3a</td>
<td>Description of trial design (such as parallel, factorial) including allocation ratio</td>
<td>6-7</td>
</tr>
<tr>
<td></td>
<td>3b</td>
<td>Important changes to methods after trial commencement (such as eligibility criteria), with reasons</td>
<td>Not applicable</td>
</tr>
<tr>
<td>Participants</td>
<td>4a</td>
<td>Eligibility criteria for participants</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>4b</td>
<td>Settings and locations where the data were collected</td>
<td>6</td>
</tr>
<tr>
<td>Interventions</td>
<td>5</td>
<td>The interventions for each group with sufficient details to allow replication, including how and when they were actually administered</td>
<td>7</td>
</tr>
<tr>
<td>Outcomes</td>
<td>6a</td>
<td>Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed</td>
<td>7-10</td>
</tr>
<tr>
<td></td>
<td>6b</td>
<td>Any changes to trial outcomes after the trial commenced, with reasons</td>
<td>Not applicable</td>
</tr>
<tr>
<td>Sample size</td>
<td>7a</td>
<td>How sample size was determined</td>
<td>9-10</td>
</tr>
<tr>
<td></td>
<td>7b</td>
<td>When applicable, explanation of any interim analyses and stopping guidelines</td>
<td>Not applicable</td>
</tr>
<tr>
<td>Randomization</td>
<td>8a</td>
<td>Method used to generate the random allocation sequence</td>
<td>10</td>
</tr>
<tr>
<td>Sequence generation</td>
<td>8b</td>
<td>Type of randomization; details of any restriction (such as blocking and block size)</td>
<td>10</td>
</tr>
<tr>
<td>Allocation concealment mechanism</td>
<td>9</td>
<td>Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned</td>
<td>10</td>
</tr>
<tr>
<td>Online Supporting Material</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Implementation</strong> 10</td>
<td>Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td><strong>Blinding</strong> 11a</td>
<td>If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td><strong>Statistical methods</strong> 11b</td>
<td>If relevant, description of the similarity of interventions</td>
<td>Not applicable</td>
<td></td>
</tr>
<tr>
<td><strong>Results</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Participant flow</strong> 12a</td>
<td>Statistical methods used to compare groups for primary and secondary outcomes</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td><strong>Recruitment</strong> 12b</td>
<td>Methods for additional analyses, such as subgroup analyses and adjusted analyses</td>
<td>Not applicable</td>
<td></td>
</tr>
<tr>
<td><strong>Baseline data</strong> 13a</td>
<td>For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analyzed for the primary outcome</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td><strong>Numbers analyzed</strong> 13b</td>
<td>For each group, losses and exclusions after randomization, together with reasons</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td><strong>Outcomes and estimation</strong> 14a</td>
<td>Dates defining the periods of recruitment and follow-up</td>
<td>6-7</td>
<td></td>
</tr>
<tr>
<td><strong>Ancillary analyses</strong> 14b</td>
<td>Why the trial ended or was stopped</td>
<td>Not applicable</td>
<td></td>
</tr>
<tr>
<td><strong>Harms</strong> 15</td>
<td>A table showing baseline demographic and clinical characteristics for each group</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td><strong>Numbers analyzed</strong> 16</td>
<td>For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td><strong>Outcomes and estimation</strong> 17a</td>
<td>For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)</td>
<td>11-12</td>
<td></td>
</tr>
<tr>
<td><strong>Ancillary analyses</strong> 17b</td>
<td>For binary outcomes, presentation of both absolute and relative effect sizes is recommended</td>
<td>Not applicable</td>
<td></td>
</tr>
<tr>
<td><strong>Harms</strong> 18</td>
<td>Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory</td>
<td>Not applicable</td>
<td></td>
</tr>
<tr>
<td><strong>Discussion</strong> 19</td>
<td>All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)</td>
<td>Not applicable</td>
<td></td>
</tr>
<tr>
<td><strong>Limitations</strong> 20</td>
<td>Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td><strong>Generalisability</strong> 21</td>
<td>Generalisability (external validity, applicability) of the trial findings</td>
<td>13-17</td>
<td></td>
</tr>
<tr>
<td><strong>Interpretation</strong> 22</td>
<td>Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence</td>
<td>13-17</td>
<td></td>
</tr>
<tr>
<td><strong>Other information</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Registration</strong> 23</td>
<td>Registration number and name of trial registry</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>
## Online Supporting Material

<table>
<thead>
<tr>
<th>Protocol</th>
<th>24</th>
<th>Where the full trial protocol can be accessed, if available</th>
<th>Supporting information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Funding</td>
<td>25</td>
<td>Sources of funding and other support (such as supply of drugs), role of funders</td>
<td>1</td>
</tr>
</tbody>
</table>
TRIAL PROTOCOL
The consumption of green leafy vegetables has been associated with a reduced risk of coronary heart disease and stroke. Green leafy vegetables and beetroot are particularly rich inorganic nitrate, which undergoes bioconversion to nitrite and further to nitric oxide endogenously. The consumption of 500 mL beetroot juice has been shown to lower blood pressure by approximately 10 mm Hg in healthy participants (14). A number of other studies have also confirmed these findings (13). Since endothelial dysfunction and the diminished production and bioavailability are a hallmark of cardiovascular diseases, we were interested in investigating the effects of beetroot on vascular function and arterial stiffness in healthy men. Furthermore, beetroot is one of the highest nitrate accumulating vegetables (>250 mg per 100 g) and may therefore be a sustainable and economic dietary source of dietary nitrate (8). However, it is consumed by only 4.5% of adults in the UK (21). In contrast, bread products are consumed by the majority of adults (~95%) (21) and are a good source of energy (mainly from starch), dietary fiber as well as various vitamins and minerals (23). Bread products may therefore be a suitable matrix by which the beneficial effects of beetroot can be delivered in an accepted food. The aim of this study was to test the hypothesis that acute ingestion of nitrate-rich beetroot-enriched bread will improve microvascular vasodilation, arterial stiffness and reduce blood pressure in healthy men. In the proposed project the acute effects of beetroot-enriched bread ingestion compared with control white bread (no beetroot) on microvascular function in healthy men will be investigated. To this purpose parameters of postprandial microvascular function will be investigated:

- Laser Doppler imaging with iontophoresis (LDI)

Secondly, the effects of beetroot-enriched or control white bread on postprandial arterial stiffness, blood pressure and plasma nitrate and nitrite concentrations will also be investigated. To this purpose the following parameters are investigated:

- Analysis of arterial stiffness by Pulse Wave Velocity and Analysis (PWV/A)
- Analysis of blood pressure (BP)
- Analysis of plasma nitrate and nitrite concentrations.

METHODS

Laser Doppler imaging with iontophoresis. Measurements were carried out using a moorLDI2 laser Doppler imager (Moor Instruments Limited, Axminster, UK). All measurements were carried out after a 30 min of rest in the supine position in a quiet, temperature controlled room (22-24 ºC). Acetylcholine chloride (2.5 mL, 1% in 0.5% sodium chloride solution, Sigma Aldrich, Poole, Dorset, UK) and sodium nitroprusside (2.5 mL, 1% in 0.5% sodium chloride solution, Sigma Aldrich) were delivered by iontophoresis using ION6 chambers (Moor Instruments) and placed on the forearm and connected to a MIC2 controller (Moor Instruments). Twenty scans were taken over a period of approximately 15 min, with an increase in current from 0 to 20 µA. The incremental area under the flux versus time curve over the 20 scans was calculated as a measure of microvascular response to acetylcholine (Ach, endothelium-dependent vasodilation) and sodium nitroprusside (SNP, endothelium-independent vasodilation).

Pulse Wave Analysis. PWA was measured using a SphygmoCor system (AtCor Medical, Sydney, Australia) by a single trained operator. An automatic oscillometric digital BP monitor (OMRON, Tokyo, Japan) was used to measure BP before each pulse wave
measurement, according to British Hypertension Society guidelines. PWA was measured by
applanation tonometry of the radial artery with a sensitive transducer to detect waveform
traces of the peripheral waveform. Continuous measurements were taken until a report with a
quality index of more than 80 was recorded. The corresponding aortic waveform was
generated using a validated transfer function, from which mean arterial pressure (MAP),
pulse pressure (PP), heart rate (HR) and augmentation index was calculated (AIx). The AIx, a
composite measure of wave reflection and systemic arterial stiffness, was defined as the
aortic augmentation pressure divided by the PP and was expressed as a percentage. Given
that AIx depends on HR, it was corrected for a HR of 75 bpm (AIxHR75).

**Pulse Wave Velocity.** Carotid-femoral PWV, a direct measure of arterial stiffness, was
calculated from sequential recordings of pressure waveforms from the carotid and femoral
arteries using the same tonometer (SphygmoCor, AtCor Medical, Sydney, Australia). The
timing of these waveforms was referenced with that of the R wave on a simultaneously
recorded ECG. PWV was determined by calculation of the path distance between carotid and
femoral sites divided by the difference in the R wave to waveform foot times. The difference
between the carotid and femoral path lengths was estimated from the distance between each
artery location and the sternal notch. Both PWA and PWV were performed after a 15 min rest
in a quiet, temperature controlled room (22-24 ºC) in triplicate 5 min apart.

**Blood pressure.** BP was measured using a non-invasive ambulatory BP monitor (ABPM,
TM-2430; A & D Ltd) according to the manufacturer’s instructions. Ambulatory BP monitors
were fitted by one researcher at the start of each study day and participants were asked to
leave the monitors on for a period of 6 h. Participants were rested for at least 15 min before
the BP monitors were fitted. All baseline BP measurements were taken in triplicate after
which the cuff was programmed to inflate once automatically every 30 min for a total period
of 6 h.

**Plasma and urinary nitrate and nitrite.** Plasma and urine samples were analyzed for nitrite
and nitrate using ozone chemiluminescence. In brief, total nitrate and nitrite concentrations,
collectively termed ‘NOx’, was determined by the addition of plasma and urine samples to
0.1 Mol/L vanadium (II) chloride in 1 mol/L hydrochloric acid refluxing at 80 °C. The
conditions cause the reduction of nitrate, nitrite, nitrosothiols, nitrosamines, iron-
nitrosylhemoglobin and nitrosohemoglobin to NO, which is quantified by chemiluminescence
(Model 88 AM, Eco Physics). The plasma and urinary nitrite concentrations were determined
by addition of samples to 1.1% potassium iodide in glacial acetic acid refluxing at 60 °C.
These conditions cause the reduction of nitrite, nitrosothiols, nitrosamines, iron-
nitrosylhemoglobin and nitrosohemoglobin to NO, which is then quantified as detailed above.
Concentrations of nitrate were then calculated by subtraction of nitrite from NOx values.

**Serum lipids and glucose.** Plasma triacylglycerol (TAG), non-esterified fatty acids (NEFA)
and glucose concentrations were quantified using an automated clinical chemistry analyzer
(ILAB 600; Instrumentation Laboratory) with kits supplied by Alpha Laboratories and
Instrumentation Laboratory, respectively [IL Test TM Cholesterol, IL Test TM
Triacylglycerol, and IL Test TM Glucose (0018250740), IL Test TM LDL-Cholesterol
(0018255740), ILTest TM HDL-Cholesterol (0018256040).

**Plasma insulin.** Plasma insulin was determined with an enzyme immunoassay using reagents
supplied by Dako Cytomation and a GENios plate reader (Tecan Group Ltd).
**Online Supporting Material**

*Serum potassium.* Serum potassium concentrations were determined using atomic absorption with a NovAA 350 spectrophotometer (Analytikjena) as previously described (30).

**Study protocol.** The study will be performed under the direction of Prof. Julie Lovegrove (Food and Nutritional Sciences) and Dr. Trevor George (managing director of Eccentricities Ltd). Twenty-four healthy male participants will be included in a randomized 2-way cross-over study with 2 wk washout period between intervention arms. After an explanation of the study and its purpose written consent will be asked (‘Informed consent’: appendix 1). Volunteers will be required to maintain a low nitrate and betalain diet and refrain from caffeine-containing foods and drinks for 1 d prior to each study day, and to minimize their physical activity in the 24 h prior to each study day. A standardized low nitrate and betalain meal will be provided for the evening meal the night before each study day.

On study days, the volunteers will arrive in the Hugh Sinclair Unit of Human Nutrition in the Department of Food & Nutritional Sciences at the University of Reading at 7:30 on 2 separate occasions, with at least 2 wk between each visit. They will be requested to come to the Nutrition Unit in a fasted state (having not eaten or drunk anything but water from 21:00 the night before). The participants will be asked to provide a baseline urine sample and will have an ambulatory blood pressure monitor fitted. The participants will then be randomly assigned to one of the 2 intervention breads: beetroot-enriched or control white bread. At 10:00, after 4 baseline blood pressure measurements at 15 min intervals, a baseline blood sample (25 mL) and baseline vascular reactivity measurements participants were given one of 2 intervention breads. The participants will receive a standard amount (200 mL) of low nitrate water (Buxton mineral water) 2 and 4 h post juice consumption after which they will be provided with water *ab libitum* during the first study day and the participants will be given the same volume at their subsequent visits.

Postprandial blood pressure measurements will be recorded by an ambulatory blood pressure monitor every 30 min for 6 h. The participants will be asked to collect their urine at baseline and at 2, 4 and 6 h after bread consumption. A small flexible needle (cannula) will be inserted into a vein in the arm. Two blood samples will be collected before consumption of the test breads. Further blood samples will then be taken throughout the day at 30 min intervals for 3 h and 1 h intervals for a further 3 h. Vascular reactivity will be measured using LDI, and arterial stiffness will be measured using PWV and PWA at baseline, 2, 4 and 6 h after consumption of the breads.

On all study days, the volunteers will be asked to remain in the department rested, until 17:35 pm when they will be given a low nitrate standardized dinner. The blood pressure monitor will be removed before the standardized dinner. This procedure is then repeated on 2 separate occasions, when the volunteer will be requested to consume the other test bread. Baseline measurements will also include age, weight, height and a general health questionnaire. The participants will receive £150 for participation in the study.

Inclusion Criteria:
- Male
- A signed consent form
- Age 18-64 years
- Body mass index 20-30 kg/m²
- Non-smoking, healthy individuals
- Normal blood pressure at screening (< 150/90)
- Hemoglobin >125 g/L
Exclusion Criteria:
- Participants with anemia
- Sufferers of chronic illnesses
- Individuals with food allergies
- People with diabetes
- People with celiac disease
- Women
- Vegetarians
- Participants on weight reducing diets
- Participants taking dietary supplements
- Participants taking part in vigorous exercise (>3 times per week)
- Excessive alcohol consumption

**Endpoint measures.** The primary endpoint of this study is the effect of beetroot-enriched bread or control white bread (no beetroot enrichment) on microvascular function in healthy men. This will be evaluated using (description of used techniques, see above):
- Analysis of endothelium-independent and dependent vasodilation of the microvasculature by LDI.
  - Measured at baseline (t0), 2, 4 and 6 h

The secondary endpoints of this study are the effects of beetroot-enriched bread or control white bread (no beetroot enrichment) on arterial stiffness, blood pressure and plasma nitrite and nitrite concentrations. The following parameters will be evaluated (description of used techniques, see above):

- Analysis of arterial and global stiffness by PWV and PWA
  - Measured at baseline (t0), 2, 4 and 6 h
- Analysis of ambulatory BP
  - Measured at baseline (t0) and every 30 min for a total period of 6 h
- Analysis of plasma nitrate and nitrite concentrations
  - Measured at baseline (t0) and then every 30 min for 4 h and hourly for a further 3 h.

**Intervention diets.** The intervention breads were supplied by Eccentricities Limited. They consisted of either 200 g (4 medium/thick slices) of bread containing 100 g red beetroot (comprising 50% of the total weight of the dough before baking) or control white bread containing no beetroot. The breads will be served as sandwiches with 30 g Philadelphia cheese spread containing 23% fat (Kraft Foods Limited). Breads will be served as a breakfast, time 0, on mornings of study days.

**Literature cited**


Appendix 1: Information for the subject

Principal Investigators:
- Dr. Julie A. Lovegrove
- Dr. Trevor W. George
- Ditte A. Hobbs (PhD Student)

Contact details:
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- Dr. Trevor George Tel: 0118 378 7474
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Department:
Food & Nutritional Sciences
Whiteknights, PO Box 226
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RG6 6AP

Purpose of study
You have been invited to take part in a volunteer research trial at the Department of Food & Nutritional Sciences, University of Reading. The aim of this study is to determine the effects of beetroot containing products on blood pressure and vascular reactivity. Approximately 24 volunteers will take part in this study. Before you decide whether to take part in the study, please read the following information carefully. If you want to know anything about the study, which is not written here, please ask the investigators.

Study background
Previous studies have found that consuming red beetroot juice can lower blood pressure and affect vascular function. This study aims to determine whether other products containing beetroot provide the same beneficial impacts on your heart health.

Am I suitable for participation on the study?
If you are male, do not smoke and have screening measurements of blood pressure within the normal range (less than 150/90), you will be eligible for inclusion in this study. If you wish to take part, we will inform your GP, with your permission, of any abnormalities displayed in your screening details.

Please note that you will be asked to provide blood samples at screening to make sure that you are suitable for this study. If your screening details match our requirements and you agree to participate, subsequent blood samples will also be taken during the study period for further examinations.
Online Supporting Material

To be included in the study you will need to meet the following criteria:

- Male
- A signed consent form
- Age 18-64 years
- Body mass index 20-30 kg/m\(^2\)
- Non-smoking, healthy individuals
- Normal blood pressure at screening (< 150/90)
- Hemoglobin >125 g/L

You will be excluded from the study if you meet the following criteria:

- Participants with anemia
- Sufferers of chronic illnesses
- Individuals with food allergies
- People with diabetes
- People with celiac disease
- Women
- Vegetarians
- Participants on weight reducing diets
- Participants taking dietary supplements
- Participants taking part in vigorous exercise (>3 times per week)
- Excessive alcohol consumption

What will I be asked to do?
If you are available on three separate days from 7:30 to 17:35 with 2 wk between each visit and agree to take part in this study, you will be asked to fill in a brief screening questionnaire in order to determine your inclusion in this four week study.

Day before your day at the Nutrition Unit
If you are suitable and accept to take part in the trial, a start date for the first test day will be organized. During the study, you will attend the Nutrition Unit in the Department of Food & Nutritional Sciences at the University of Reading on 2 separate occasions, with 2 wk off in between each visit. You will be asked to follow the following restrictions on the day before each of your visits to the Nutrition Unit:

1) To consume a diet low in nitrate (a natural compound found in foods, such as beetroot and spinach)-containing foods for two days (A list of suitable and unsuitable foods is amended to this Information Sheet)
2) To avoid caffeine-containing foods and beverages for one day
3) Not perform any strenuous exercise within 24 h before the study or on study days (this includes visiting the gym and jogging).

Day at the Nutrition Unit
You will be requested to come to the Nutrition Unit in a fasted state (having not eaten or drunk anything but water from 21:00 the night before). The evening before each of your visits to the Nutrition Unit, you will be provided with a standardized meal to eat, which will be the same on each of your following visits.
Online Supporting Material

On the study days you will be asked to arrive in the Hugh Sinclair Unit of Human Nutrition in the Department of Food & Nutritional Sciences at the University of Reading at 7:30. You will first be fitted with an ambulatory blood pressure monitor, which you will be required to wear for a period of 6 h. The monitor will record your blood pressure every 15 min for the first hour and then hourly for 6 h. You will return the blood pressure monitor at the end of each study visit.

On each of your study days you will be given a breakfast consisting of one of two test products and at the end of the study day you will be given a standard low nitrate meal.

You will also be asked to give urine samples before breakfast and at 2, 4 and 6 h after breakfast.

A small flexible needle (cannula) will be inserted into a vein in your arm. First two blood samples of 15 mL (equivalent to 2 tablespoons) will be collected before consumption of the test breads. Further blood samples of 13 mL (equivalent to less than 1 tablespoon) will then be taken throughout the day at 30 min intervals for 4 hour and 1 hour intervals for a further 3 h. 173 mL (equivalent to 11 and half tablespoons) of blood will be taken per visit and a total of 346 mL (the approximate volume taken at a regular blood donating) of blood for the entire study.

Vascular reactivity will be assessed using another non-invasive technique called Laser Doppler Imaging (LDI). Two small chambers containing a small amount of a chemical which stimulate your blood vessels will be placed on your skin, and the response of your blood vessels close to the skin surface will be monitored. The action of the chemicals is only short term and restricted to the small area of skin where they are applied. The procedure is pain free and will take approximately 15 min per measurement.

The elasticity of the blood vessels of your arms will be measured by Pulse Wave Analysis (PWA). Sensors will be gently placed against your wrists, neck and upper thigh region to record signals of their pulse.

Laser Doppler Imaging and Pulse Wave Velocity/Analysis will be measured before breakfast, 2, 4 and 6 h after consumption of the test bread.

A standard dinner and low nitrate mineral water (Buxton mineral water) will be provided on your study day. You will be asked not to consume any food or drinks (except the water provided) between your breakfast and dinner.

You will be asked to repeat this procedure on two separate occasions at least two weeks apart.

**What is measured?**
Blood pressure (see information below), vascular reactivity by Laser Doppler Imaging (LDI) and arterial stiffness by Pulse Wave Velocity/Analysis (PWV/A) will be measured. Nitrate in the urine and blood samples will also be measured. In addition to lipids (fats), glucose and insulin levels in the blood samples.

**Are there any risks?**
Online Supporting Material

All products contain ingredients that are available commercially. The ambulatory blood pressure monitors may cause a tightening sensation when taking a reading. The Laser Doppler and Pulse Wave Velocity/Analysis are safe and pain-free techniques. Very occasionally some people may bruise after the cannula has been removed; however this will go within a few days. Some participants may experience a slight itching sensation from the solutions used during the Laser Doppler procedure, but this will subside once the measurement is recorded. Some individuals may observe a harmless pink coloration to their urine and/or feces following consumption of beetroot juice.

Data protection and confidentiality
Your records and personal data will remain confidential and will be identified by a number code. The information linking your name with the code will be known only to the investigators. All data and samples will be stored in Food and Nutritional Sciences building at the University of Reading for a maximum of 5 y. Samples will be destroyed when no longer needed.

Where the research may be published?
The results from the study will be published in scientific journals (such as Food Quality and Preference, American Journal of Clinical Nutrition, British Journal of Nutrition, Journal of Nutrition, Plos One, European Journal of Clinical Nutrition, Journal of Human Nutrition and Dietetics, and Journal of Agriculture and Food Chemistry) and group data may be presented at scientific conferences. However individual data will not be presented and your name will never be associated with any results.

Do I receive payment for participation in the study?
Yes, £150 for the completion of the entire study.

Can I withdraw from the study at any time?
Yes, participation in this study is entirely voluntary and you may leave the study at any time without giving reason.

Can I see the results of the study?
Yes, you may request feedback on the general results from the study.

Will my GP be informed of my participation in the study?
Your GP will only be informed of any screening details that show any abnormalities.

General
This study has been subject to ethical review by the University of Reading’s Research Ethics Committee and has been given a favorable ethical opinion for conduct.

This study is sponsored by Eccentricities Ltd.
## Study day timeline

<table>
<thead>
<tr>
<th>Time</th>
<th>Activity</th>
</tr>
</thead>
</table>
| 7:30 | Arrival in the Nutrition Unit  
- Weight measurement  
- Baseline urine sample  
- Baseline BP 1 |
| 9:00 | Baseline BP 2  
- PWV |
| 9:30 | Baseline BP 3  
- LDI |
| 10:00 | Cannulation  
- Baseline blood sample 1  
- Baseline BP 4 |
| 10:25 | Baseline blood sample 2 |
| 10:30 | Breakfast |
| 11:00 | Blood sample 3  
- BP 5 |
| 11:30 | Blood sample 4  
- BP 6 |
| 12:00 | Blood sample 5  
- BP 7  
- Urine 1 |
| 12:30 | Blood sample 6  
- BP 8  
- PWV 1 |
| 13:00 | Blood sample 7  
- BP 9  
- LDI 1 |
| 13:30 | Blood sample 8  
- BP 10 |
| 14:00 | Blood sample 9  
- BP 11  
- Urine 2 |
| 14:30 | Blood sample 10  
- BP 12  
- PWV 2 |
| 15:00 | BP 13  
- LDI 2 |
| 15:30 | Blood sample 11  
- BP 14 |
| 16:00 | BP 15  
- Urine 3 |
| 16:30 | Blood sample 12  
- BP 16  
- PWV 3 |
| 17:00 | BP 17  
- LDI 3 |
| 17:30 | Blood sample 13  
- BP 18 |
| 17:35 | Low nitrate meal |
## Online Supporting Material

### Appendix 2: Diet low in Nitrate and Caffeine-containing foods

#### Foods to be avoided:

- Beetroot
- Brussel sprouts
- Bok choy
- Cabbage
- Carrots
- Cauliflower
- Celery
- Chervil
- Chinese cabbage
- Cress
- Dill
- Endive
- Fennel
- Fish
- Green beans
- Home-made vegetable soups (packet soups are fine if soup must be consumed)
- Seaweeds (including laver and kelp)
- Leek
- Lettuce
- Oriental mushroom (eg. shiitake, enoki)
- Mustard greens
- Potatoes (including sweet potatoes, chips and crisps)
- Preserved meats (eg. ham, sausages, hot dogs, bacon, etc.)
- Rocket
- Spinach
- Swede
- Turnip
- Vegetable juices
- Coffee
- Cocoa-containing foods and drinks (eg. chocolate)
- Cola drinks (e.g. Coca Cola, Pepsi, Dr Pepper, etc.)
- Energy drinks
- Tea (black, green)

As a general rule, please avoid leafy green vegetables and caffeinated foods and beverages.

#### Foods which can be eaten freely:

- Artichoke
- Asparagus
- Bread
- Broad beans
- Broccoli
- Cucumber
- Eggplant
- Fruit
- Garlic
- Common mushroom (eg. White button, chestnut, flat mushrooms)
- Onion
- Pasta, noodles, rice
- Peas
- Peppers
- Pumpkin
- Soft drinks, except those mentioned above
- Sweetcorn
- Tomatoes
- Watermelon
- Wine