Author’s response to reviews

Title: A randomised controlled crossover trial investigating the short-term effects of different types of vegetables on vascular and metabolic function in middle-aged and older adults with mildly elevated blood pressure: the VEgetableS for vaScular hEaLth (VESSEL) study protocol

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Author’s response to reviews:

We would like to thank the reviewers and editors for their constructive feedback. We have made substantial changes to the manuscript and believe our protocol has improved greatly. Please find below a point-by-point response highlighting the changes made in the revised manuscript to address each point raised. Please see revised manuscript for additional changes made that are not related to reviewers comments but have been made to improve the manuscript.

Reviewer #1, comment 1:
What are the total kcals provided for each intervention? Are calories the same between both interventions?

Response: As shown below, the energy content of each soup is approximately matched.

Cruciferous veg soup: ~579 kJ per day, ~139 Kcal per day
Control veg soup: ~612 kJ per day, ~146 Kcal per day

Reviewer #1, comment 2:
The authors state: "During the study, it is expected that there will be a background vegetable intake of approximately 2-3 servings per day during the "usual diet" washout as well as during the 1-week baseline periods where standard lunch and dinner meals will be provided by the study investigators. Therefore, the additional 4 serves of vegetables that the soups provide will shift vegetable intake to above the recommended 5-6 servings per day"
Is there any actual data on total amount of vegetables consumed during intervention? Potentially, if participants are receiving required vegetable intake during the intervention, their ad libitum intake may actually decrease, in which case, they would not be consuming the recommended servings of vegetables per day.

Response: The standard lunch and dinner meals provided contain 0.5-2 servings (40-150 g) of non-cruiferous vegetables per meal. The combinations of meals provided to participants allow for the consumption of approximately 1-4 backgrounds servings (75-300 g) of vegetables daily. Therefore, participants will consume a total of 5-8 servings (375-600 g) of vegetables daily during the intervention. As we will be providing the lunch and dinner meals to participants, we will be able to quantify the vegetable servings consumed. However, as participants will select their frozen meals based on their preferences, this may vary slightly between participants. Nevertheless, total vegetable intake will be quantified and should be similar between interventions due to the crossover nature of the trial.

Lines 190-193: “It is expected that participants will have a background vegetable intake of approximately 1-4 servings (~75-300 g) per day during the intervention periods. Therefore, the additional 4 servings of vegetables that the soups provide will shift vegetable intake to at or above the recommended 5-6 servings (375-450 g) per day.”

Lines 199-206: “To limit background diet variation, participants will consume frozen pre-prepared lunch and dinner meals provided by study investigators. These meals will provide approximately 1-4 servings (75-450 g) of background vegetables per day. Slight variation in
vegetable intake between participants may occur, as participants will be able to select meals based on personal preference. With the exception of the intervention soups, participants will be asked to consume the same foods during both intervention periods. Participants will also be asked to replicate exactly what they ate in the 24 hours prior to each clinic visit. This will allow us to directly compare the two vegetable types in both intervention periods.”

Reviewer #1, comment 3:
"To match the energy intake from the soups provided during the intervention period, during the 1-week baseline period two slices of multigrain bread with a small amount of margarine will be consumed with lunch and dinner meals." Does this equate to the same macronutrient and fiber intake? Rather than simply matching total caloric intake.

Response: The study has been modified to remove the baseline weeks and extend the length of the intervention. The soups will be approximately matched in macronutrients.

Cruciferous veg soup: 579 kJ/d energy, 18.8 g/d carbohydrates, 10.4 g/d protein, 0.6 g/d fat, 9.1 g/d fibre
Control veg soup: 612 kJ/d energy, 27.3 g/d carbohydrates, 5.0 g/d protein, 0.3 g/d fat, 5.9 g/d fibre

Reviewer #1, comment 4:
Were dietary records asked to be recorded daily, weekly?

Response: Participants will be asked to record daily dietary intake throughout the baseline and intervention periods.

Lines 219-220: “Food and nutrient intake will be assessed daily throughout the intervention periods using dietary records.”

Reviewer #1, comment 5:
Were participants asked to consume all of the soup provided to them? Did they have to return food containers whether empty or with food remaining? This would have been another tool to determine compliance.

Response: Participants will be asked to consume all of the soup provided and will be asked to consume the soup prior to the standard meal to ensure the soup will be eaten before filling up with the standard meal. Participants will also be asked to return the soup containers as a measure of compliance. This has been included in the manuscript.

Lines 222-223: “We will also use self-reported soup intake diaries and ask participants to return their empty soup containers as a measure of soup compliance.”

Reviewer #1, comment 6:
In methods:
- Measurement of vegetable metabolites (carotenoids, S-methyl cysteine sulfoxide) not described.
Response: Carotenoids will be measured using high performance liquid chromatography and S-methyl cysteine sulfoxide will be measured using liquid chromatography-mass spectrometry (LC-MS)/MS. This has been added and highlighted in the manuscript:

Lines 224-233: “We will measure plasma carotenoids using high performance liquid chromatography (26, 27). Plasma carotenoids will be measured as a biomarker of vegetable intake (28). It is expected that total plasma carotenoids will be increased substantially after both the CRV and OCV interventions. However, the carotenoid profiles will vary slightly due to the differences in individual carotenoids present in the different vegetables consumed. S-methyl cysteine sulfoxide concentration in plasma samples will be used as a biomarker of CRV intervention and will be measured using liquid chromatography-mass spectrometry (LC-MS)/MS techniques as previously described (29). It is expected that plasma S-methyl cysteine sulfoxide concentration will be increased after CRV intervention, but not OCV intervention, and is therefore a measure of CRV intervention compliance.”

Reviewer #1, comment 7:  
-If participants are not blinded to their glucose readings, this could potentially influence their compliance to diet and other food choices during the intervention periods. Is this addressed?  

Response: Changes have been made to blind participants to their glucose readings. The reader will be covered with tape such that the reading will not be visible.

Lines 355-357: “The screen of the reader will be covered such that participants will remain blinded to their glucose readings throughout the study.”

Reviewer #1, comment 8:  
-line 219: this statement seems out of place.  

Response: The sentence “All forms and questionnaires will be compiled in separate participant files for easy accessibility” has now been removed.

Reviewer #1, comment 9:  
-Methods section is disjointed. Many sections after line 219 should have been included at or near the beginning.

Response: The methods section has now been substantially revised.

Reviewer #1, comment 10:  
-line 281: Is this based on prior data? A prior study?  

Response: This has been based on previous studies. A reference has been included in the text (reference #30).
Reviewer #2, comment 1:
I highly recommend to keep blood pressure as the primary outcome and glycemic control as a secondary outcome. The co-primary outcome approach is not well justified and shifts the focus of this study. The underlying mechanisms of how cruciferous vegetables affect vascular health and glycemc response could be very different. It doesn't seem appropriate to include both of them as primary outcomes.

Response: We agree and have changed this to ensure blood pressure is the primary outcome and glycaemic control is the secondary outcome (lines 239-254).

Reviewer #2, comment 2:
Given that the anticipated low intake of vegetables of participants' background diet, (2-3 serving/day, ln 156), I would recommend to shorten the baseline period and extend the duration of intervention. Are there any justifications on the one week duration of intervention?

Response: The two 1-week baseline periods have now been removed and the two intervention periods have been extended from 1-week to 2-week periods.

Reviewer #2, comment 3:
Description of the run-in diet and intervention diet was indeed not clear. It seems like participants will be on standardized diet + bread and butter diet during the one week baseline period and standardized diet + vegetable soup during the one week intervention period. If this is the case, I would suggest to reconstruct the paragraph between line150-166. It is really not clear what is the standardized diet. I would suggest to provide a sample menu to give a better understanding on the standardized lunch and dinner. Similarly it reads like both baseline diet and intervention diet are the same Ln 136-137,

Response: As mentioned above, the intervention periods have now been extended to two weeks and the baseline periods have been removed. Therefore, there is no longer a requirement for a different diet during the baseline period. During the intervention periods, participants will consume standardised frozen lunch and dinner meals with either the cruciferous or control soups. All frozen meals will be low saturated fat and sodium and will be purchased for participants.

Reviewer #2, comment 4:
Cruciferous vegetables are not high in carotenoid with the exception of kale. In the present study, the cruciferous soup consists of broccoli, cabbage, cauliflower, and kale, in conjunction with the short intervention period, I would hesitate to use carotenoid as a biomarker for compliance. Why not use the bioactive compounds exclusive to cruciferous vegetables (ln 69-78) as biomarkers of compliance?

Response: S-methyl cysteine sulfoxide will be used as a biomarker of cruciferous vegetable intake and, as a result, as a measure of compliance with the active soup. Carotenoids will be used as a biomarker of background vegetable intake, including that of the control soup. This has been rewritten for clarity:
We will measure plasma carotenoids using high performance liquid chromatography (26, 27). Plasma carotenoids will be measured as a biomarker of vegetable intake (28). It is expected that total plasma carotenoids will be increased substantially after both the CRV and OCV interventions. However, the carotenoid profiles will vary slightly due to the differences in individual carotenoids present in the different vegetables consumed. S-methyl cysteine sulfoxide concentration in plasma samples will be used as a biomarker of CRV intervention and will be measured using liquid chromatography-mass spectrometry (LC-MS)/MS techniques as previously described (29). It is expected that plasma S-methyl cysteine sulfoxide concentration will be increased after CRV intervention, but not OCV intervention, and is therefore a measure of CRV intervention compliance.”

Reviewer #2, comment 5:
How about participants retention?

Response: This study requires a high level of commitment from participants and participants are encouraged to voice any concerns regarding their ability to complete all study requirements prior to randomisation into the study. Our research group have performed more than 25 nutrition intervention trials with similar crossover design (1-12 weeks) and have found that retention is usually very high (<10% dropout).

Reviewer #2, comment 6:
ln 43, "modifiable risk factors….is a health diets", heath diet is not a RF, either change to "modifiable factor" or "modifiable risk factors…is a low quality diet"

Response: This has been changed to “most important modifiable risk factors for cardiovascular disease is an unhealthy diet” (lines 64-65).

Reviewer #2, comment 7:
Page 3, paragraph 2, I would suggest to summarize the association between consumption of vegetables and cardiometabolic health in one or two sentences instead of list the association with each disease.

Response: This section has now been modified.

Lines 67-69: “Like cardiovascular disease, the prevalence of type 2 diabetes is increasing worldwide (4). Adults with type 2 diabetes have a two- to three-fold increased risk of experiencing a heart attack or stroke (5).”

Reviewer #2, comment 8:
Page 4, paragraph 1, the aim of the study is to examine the effects of cruciferous vegetables on cardiometabolic health, indeed, it is the effects of a dietary patterns rather than individual nutrient. I would recommend to remove paragraph 1 or summarize it in one or two sentences.

Response: This paragraph has now been condensed; however, we have still briefly discussed potential nutrients involved as we believe noting potential mechanisms is important.
Reviewer #2, comment 9:
Ln 102 "and at least one additional risk factor for T2D and CVD…” Are there specific reasons to choose these RFs? These risk factors largely represent risk factors of metabolic syndrome (except total cholesterol).

Response: We have made changes to the inclusion and exclusion criteria to increase the likelihood of recruiting enough participants within the given timeframe of a PhD project.

Lines 151-176:
“Eligibility criteria
Participants must provide written informed consent before any physical assessments occur (see Appendix 2 for sample Participant Information and Consent Form).
Inclusion criteria
We will recruit ambulant community-dwelling men and women aged between 50 and 75 years who have mildly elevated blood pressure (systolic blood pressure 120-160 mmHg, inclusive, and diastolic blood pressure &lt;100 mmHg).

Exclusion criteria
Volunteers will be excluded from participation based on the following criteria: body mass index &lt;18.5 or ≥40 kg/m2; systolic blood pressure &gt;160 mmHg or &lt;120 mm Hg; diastolic blood pressure &gt;100 mmHg; use of &gt;2 antihypertensive medications or irregular use of nitric oxide donors, organic nitrates and nitrates, and sildenafil and related drugs; diagnosed diabetes or fasting blood glucose &gt;6.5 mmol/L; fasting total cholesterol &gt;8 mmol/L; current or recent (&lt;12 months) smoking; adhesive allergy; regular aspirin use; medication use for thrombosis or anticoagulants (Warfarin); history of cardiovascular or peripheral vascular disease (myocardial infarction, stroke, transient ischaemic attack, amputation due to arterial insufficiency, any form of arterial revascularisation, history of exertional angina or claudication); psychiatric illness or other major illnesses, such as cancer; alcohol intake &gt;100 g per week; current or recent (within previous 6 months) significant weight loss or gain (&gt;6% of body weight) or actively trying to lose weight; pre-menopausal women; inability to attend clinic/office visits; use of antibiotics (within previous 2 months); use of antibacterial mouthwash and not willing to cease for trial duration; reported participation in night shift work during the study period; and inability or unwillingness to follow the study protocol. Volunteers with specific dietary requirements, allergies, or intolerances (e.g. following a low FODMAP diet) that will interfere with their ability to follow the dietary requirements of the study will also be excluded.”

Reviewer #2, comment 10:
Ln 107, what are the reasons excluding participants with BMI &gt;40 and those who regular use of antibacterial mouthwash (Ln117-8)?

Response: We are excluding those with a BMI &gt;40 kg/m2 as these individuals are often at a much higher risk of multiple chronic diseases. Furthermore, it is often difficult to obtain blood samples, which is a major requirement for the study. We have now slightly changed the exclusion criteria around regular use of antibacterial mouthwash. Individuals that regularly use antibacterial mouthwash and are not willing to cease using it for the trial duration will be excluded. The use of antibacterial mouthwash interrupts the
dietary nitrate-nitrite-nitric oxide pathway, impacting blood pressure (Bondonno et al. Antibacterial mouthwash blunts oral nitrate reduction and increases blood pressure in treated hypertensive men and women. American Journal of Hypertension. 2015;28(5)572-575). Therefore, those not willing to cease using antibacterial mouthwash will be excluded to mitigate external influences on blood pressure.

Reviewer #2, comment 11:
Ln 168 How many days of dietary record are collected i.e. random single day collection or multiple consecutive days? When will these dietary records be collected at baseline, during intervention? Please be specific.

Response: Dietary records will be collected daily during each intervention period. This has been clarified.

Lines 219-222: “Food and nutrient intake will be assessed daily throughout the intervention periods using dietary records. Participants will be asked to record their entire food and beverage intake each day using the application “Research Food Diary” (Xyris Software, Brisbane, Australia) or via a paper-based record.”

Reviewer #2, comment 12:
How about data on participant's habitual diet?

Response: Each participant completes a validated food frequency questionnaire at their first baseline visit to provide data on the participant’s habitual diet over the past 12 months.

Lines 430-432: “…2) CCV-FFQ to determine overall habitual dietary intake, a self-administered semi-quantitative dietary questionnaire developed by the Cancer Council of Victoria, Australia (40)…”

Reviewer #2, comment 13:
1) Ln 251 what are the purposes of collecting bacteria samples?

Response: The oral bacterial samples are collected to later evaluate the microbial DNA present on the dorsal surface of the tongue. Oral nitrate-reducing bacteria are responsible for the conversion of nitrate to nitrite via the nitrate-nitrite-nitric oxide pathway.