ORIGINAL CONTRIBUTION

Inter-individual differences in the blood pressure lowering effects of dietary nitrate: a randomised double-blind placebo-controlled replicate crossover trial

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Abstract

Purpose Dietary nitrate supplementation increases nitric oxide (NO) bioavailability and reduces blood pressure (BP). Inter-individual differences in these responses are suspected but have not been investigated using robust designs, e.g., replicate crossover, and appropriate statistical models. We examined the within-individual consistency of the effects of dietary nitrate supplementation on NO biomarkers and BP, and quantified inter-individual response differences.

Methods Fifteen healthy males visited the laboratory four times. On two visits, participants consumed 140 ml nitrate-rich beetroot juice ($\sim$14.0 mmol nitrate) and, on the other two visits, they consumed 140 ml nitrate-depleted beetroot juice ($\sim$0.03 mmol nitrate). Plasma nitrate and nitrite concentrations were measured 2.5 h post-supplementation. BP was measured pre- and 2.5 h post-supplementation. Between-replicate correlations were quantified for the placebo-adjusted post-supplementation plasma nitrate and nitrite concentrations and pre-to-post changes in BP. Within-participant linear mixed models and a meta-analytic approach estimated participant-by-condition treatment response variability.

Results Nitrate-rich beetroot juice supplementation elevated plasma nitrate and nitrite concentrations and reduced systolic (mean:-7 mmHg, 95%CI: -3 to -11 mmHg) and diastolic (mean:-6 mmHg, 95%CI: -2 to -9 mmHg) BP *versus* placebo. The participant-by-condition interaction response variability from the mixed model was $\pm$7 mmHg (95%CI: 3 to 9 mmHg) for systolic BP and consistent with the treatment effect heterogeneity t=$\pm$7 mmHg (95%CI: 5 to 12 mmHg) derived from the meta-analytic approach. The between-replicate correlations were moderate-to-large for plasma nitrate, nitrite and systolic BP (r=0.55 to 0.91).

Conclusions The effects of dietary nitrate supplementation on NO biomarkers and systolic BP varied significantly from participant to participant. The causes of this inter-individual variation deserve further investigation. Trial registration: https://clinicaltrials.gov/study/NCT05514821.

Keywords Dietary nitrate · Beetroot juice · Blood pressure · Hypertension · Individual differences · Inter-individual variation

Introduction

High blood pressure (BP) is a leading cause of morbidity and mortality worldwide [1], and the identification of effective strategies to lower BP remains a major research and public health priority [2, 3]. Consumption of a healthy diet can lower BP and is recommended as a first line treatment for low grade hypertension and as an adjunct to BP-lowering pharmacotherapy for more severe hypertension [4]. Various dietary approaches have been demonstrated to help lower BP, including consumption of dietary compounds/foods with potential anti-hypertensive properties such as dietary inorganic nitrate [5–7].

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For most people, the major dietary sources of inorganic nitrate are vegetables such as lettuce, spinach and beetroot [8, 9], and this nitrate serves as a precursor for nitric oxide (NO)– a gaseous signalling molecule with vasodilatory properties [10, 11]. Consumption of nitrate-rich vegetables or vegetable juices (e.g [6, 12–14]), and nitrate salts (e.g [5, 15, 16]), has been shown to elevate NO biomarkers (e.g., plasma nitrate and nitrite concentrations) and lower BP both acutely (within hours of supplementation) and chronically (over several weeks/months) [7]. However, individual differences in the response to nitrate ingestion have been suggested, with some researchers hypothesising the existence of individuals deemed 'responders' and 'non-responders' to nitrate supplementation [17–21]. If confirmed, this notion could have important implications for the development of personalised recommendations around nitrate intake. For example, if it is possible to identify the individuals most likely to benefit from nitrate supplementation, then these individuals could be targeted for nitrate-based interventions in future trials and public health initiatives.

Identification of meaningful inter-individual differences in response to nutritional interventions is not straightforward. Notably, with conventional parallel-arm or crossover trials, it is not possible to determine whether any apparent difference between participants in the changes from pre- to post-intervention are due to genuine treatment response heterogeneity or are a consequence of random within-subject variability and/or measurement error [22]. Without repeated administration of trials, a typical crossover design does not allow formal estimation of variance attributable to the participant-by-treatment interaction [23, 24]. Only by quantifying this interaction can treatment response heterogeneity be detected properly. As a form of n-of-1 trial, a replicate crossover trial constitutes a pragmatic research design for quantifying treatment response heterogeneity [22, 23, 25]. This research design involves repeated administration of the intervention and control/placebo arms of a trial protocol in randomised order on at least two occasions [22]. This allows quantification of treatment response heterogeneity using, for example, a within-participant covariate-adjusted linear mixed model for estimation of any participant-by-treatment interaction effects [22, 23, 25].

We aimed to quantify the magnitude of inter-individual variability in the effects of dietary nitrate supplementation on the outcomes of NO biomarkers and BP using a replicate crossover design. We also aimed to examine the consistency of these responses on repeated occasions. We hypothesized that there is 'true' inter-individual variability (i.e., exceeding random within-subject variability) in the effects of dietary nitrate supplementation on NO biomarkers and BP, and that these responses would be consistent on two occasions.

Methods

The protocol for this study was registered prospectively on ClinicalTrials.gov (NCT05514821). The study was approved by the Faculty of Medical Sciences Research Ethics Committee at Newcastle University (2345/23609).

Participants

Healthy male participants were recruited from the general population via posters, university email lists, and social media to take part in this study (see Supplementary Fig. 1 for CONSORT flow chart). Participants were required to be non-smokers who were not currently taking any medication or using any dietary supplements, had no history of cardiovascular, metabolic, or gastrointestinal diseases, and were not currently using antibacterial mouthwash.

Design

Participants attended the laboratory on 5 separate occasions (data collection period: October 2022 to September 2023). On the first visit, participants provided written informed consent and underwent screening to determine eligibility to participate. A pre-screening questionnaire was completed, and body mass and stature were measured. Subsequently, participants completed four experimental visits (two nitrate and two placebo) in a randomised order (Fig. 1). A randomised sequence schedule (see Supplementary Text 1) was created using http://www.randomization.com) for our replicate crossover experimental research design [25]. Experimental visits were separated by ∼7 days (minimum 3 days, maximum 14 days). Participants were asked to record their diet in the 24 h prior to the first visit and to replicate this as closely as possible prior to each subsequent visit. Participants were asked to abstain from intensive exercise and alcohol in the 24 h prior to each visit and were instructed to avoid consumption of any food or drink except for plain water on the morning on the experimental visits.

Experimental visits

Participants arrived at the laboratory between 8 and 9 am and rested, seated in quiet room for 10 min. Subsequently, systolic and diastolic BP of the brachial artery was measured using an automated sphygmomanometer following best-practice guidelines [26]. Four measurements were taken, with the mean of the final three measurements used for subsequent analyses. Participants then received a standardised breakfast including 140 ml concentrated nitrate-rich (∼14.0 mmol nitrate) or nitrate-depleted (∼0.03 mmol nitrate) beetroot juice and a bowl of porridge (60 g oats, 200 ml whole
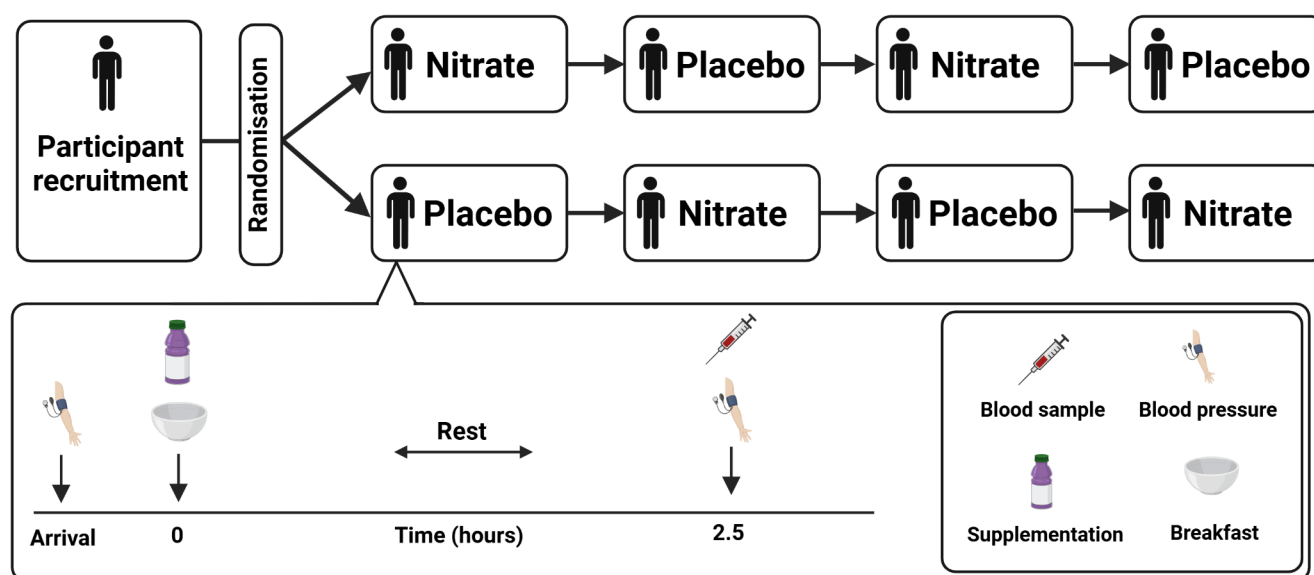
Fig. 1 A schematic of the replicate crossover study design with example randomisation schedules. Participants completed four experimental trials in a randomised order, two involving supplementation with nitrate-rich beetroot juice (~14.0 mmol nitrate) and two involving milk). We used commercially available beetroot juice supplements (Beet It Sport, James White Drinks Ltd., Ipswich, UK). All supplements were from the same batch, with each batch undergoing homogenisation during manufacturing to maximise consistency in the nitrate concentrations. We analysed a single bottle each of the 'active' and placebo supplement using the same chemiluminescence approach outlined below to provide indicative nitrate concentrations. Supplements were administered double blind. Participants then rested, seated in a quiet room for 2.5 h during which time they were permitted to carry out non-stimulating activities (e.g., reading). Blood pressure measurements were then repeated, and a blood sample was collected via venepuncture of an antecubital vein into two, 4 ml lithium heparin containing tubes (Vacutainer, Becton Dickinson, Plymouth, UK). Samples were immediately centrifuged at 3000 rpm and 4 °C for 10 min, plasma was extracted and frozen at -80°C. Participants were then free to leave the laboratory.

supplementation with nitrate-depleted beetroot juice (placebo, ~0.03 mmol nitrate). Blood pressure was measured pre- and 2.5 h post-supplementation and a blood sample was collected 2.5 h post-supplementation at each experimental visit

Biochemical analyses

Measurements of plasma nitrate and nitrite concentrations were conducted using ozone-based chemiluminescence [27]. For the measurement of plasma nitrate concentration, vanadium reagent (24 mg of vanadium tri-chloride and 3 ml of 1 M hydrochloric acid) and 100 μL of anti-foaming agent were placed into a glass purge vessel infused with nitrogen and heated to 95 °C. This purge vessel was connected to a NO analyser (Sievers NOA 280i, Analytix, UK). A standard curve was produced by injecting 25 μL of nitrate solutions (100 μM, 50 μM, 25 μM, 214 12.5 μM, and 6.25 μM) and a control sample containing deionised water. The area under the curve (AUC) for the control sample was subtracted from those for the nitrate solutions to account for nitrate in the water used for dilutions. Plasma samples were thawed in a water bath at 37 °C for 3 min and de-proteinised using zinc sulphate/sodium hydroxide solution (200 μL of plasma, 400 μL of zinc sulphate in deionised water at 10% w/v and 400 μL of 0.5 M sodium hydroxide). The samples were then vortexed for 30 s before being spun at 4000 rpm for 5 min. Subsequently, 15–25 μL of the sample was injected into the purge vessel in duplicate. The concentration of NO produced was then measured by the NO analyser. The AUC was calculated using Origin software (version 7) and normalised using the Y value from the calibration curve.

For the measurement of plasma nitrite concentrations, 2.5 ml glacial acetic acid, 0.5 ml of 18 Ω deionised water, 25 mg sodium iodide, and 100 μL of an anti-foaming agent were placed into the glass purge vessel and heated to 50 °C. A standard curve was produced by injecting 100 μL of nitrite solutions (1000 nM, 500 nM, 250 nM, 125 nM, and 62.5 nM) and a control sample of deionised water. The AUC for the latter was subtracted from those for the nitrite solutions to account for nitrite in the water used for dilutions. Following this, plasma samples were thawed in a water bath and 100 μL of the sample was injected into the purge vessel in duplicate. The nitrite concentration was determined via the AUC, as described for nitrate analysis.

Statistical analysis

We adopted a pragmatic approach to sample size considerations [28, 29]. Given the onerous nature of the four-condition replicate design and procedures, we recruited 15 participants, which is a sample size similar to previous replicate crossover trials in nutrition research [30, 31]. As detailed below, the between-replicate correlation coefficient is an indicator of individual stability of response. Using GPower (version 3.1), we estimated the minimum statistically significant between-replicate correlation coefficient to be 0.44 for a sample size of 15. The 90% confidence interval (CI) for this correlation coefficient is estimated to be 0.01 to 0.74. A one-tailed directional hypothesis (90% CI) is relevant here because the null hypothesis (H_o) is that $r = 0$ OR < 0, i.e., if the correlation is either zero or negative, this would lead to the same conclusion (non-rejection of H_o) of no consistent responses being present [32].

The analysis protocol comprised a four-step approach consistent with previous research [30, 31, 33] and more recent advances [34] relevant to the elaboration of continuous data from a replicate crossover trial designed experiment. Primary outcome measures were systolic and diastolic BP. Secondary outcome measures were plasma nitrate and plasma nitrite. First, we estimated Pearson's product moment correlation coefficients between the first and second response replicates for each outcome to assess the within-person stability of the replicated placebo-adjusted supplementation effect [22] - a high correlation between the two repeated responses indicating a relatively stable individual response. For this correlation analysis of consistency of response, the first supplementation condition of each participant was paired to their respective first placebo condition in their individual sequence and the placebo-adjusted supplementation effect was computed for response 1 (supplementation 1 *minus* placebo 1 for BP changes). This process was replicated for the second condition pairs to calculate response 2 (supplementation 2 *minus* placebo 2).

Second, an overall "naive" estimate of the true individual difference standard deviation (SD_{IR}) for the supplementation response was calculations according to methods reported in [23]. The SD_{IR} is the SD of the individual differences in the supplementation response (SD_S) adjusted for the standard deviation in the placebo conditions (SD_P), respectively [23]. A positive SD_{IR} indicates that the variability in supplementation response is greater than any random within-subject variability. This calculation is considered a naive estimation of the SD_{IR} because it is not derived from a statistical model on the raw data and was originally formulated for parallel arm, rather than crossover studies.

Third, and in view of the issues above with the naive estimate of SDir, we used the PROC MIXED procedure in SAS OnDemand for Academics (SAS Institute) to derive the SD_{IR}, in line with previous studies [23]. Distinct within-participant covariate-adjusted linear mixed models were used to quantify inter-individual differences in NO biomarkers and blood pressure responses specified as participant-by-condition interaction terms, with the variance-covariance matrix structure set to *variance components* [22, 25, 35]. Each model included the outcome (response) variable, condition, period (condition sequence) and the period-by-condition interaction included as fixed effects, with participant and the participant-by-condition interaction modelled as random effects (Supplemental File 1). The variance for the participant by condition interaction indicates the degree of response heterogeneity and the P-value for this interaction represents whether the variance can be considered statistically significant from zero (no response heterogeneity). The adequacy of the modelled covariance parameter estimates was assessed via formal residual diagnostics procedures [36, 37]. Effects were reported as estimated marginal means alongside relevant measures of dispersion (SD) and uncertainty (95% CI). Using data from prognostic studies in cardiovascular medicine as a guide, we defined a $\Delta = 2$ mmHg reduction in systolic and diastolic BP as the minimal clinically important difference (MCID) when interpreting the meaningfulness of interindividual response differences [38, 39].

Fourth, according to Senn's meta-analytical approach, we calculated a standard error from within-subjects variance using appropriate degrees of freedom to derive per-participant replicate-averaged treatment effects over the completed trial cycles [34]. Using the *metagen()* function available in the *meta* package [40], we conducted a random-effects meta-analysis with Hartung-Knapp adjustment [41] to summarise individual-participant replicate-averaged treatment effects, respective sampling errors and the tau-statistic (t) value that describes the between-participant treatment effect response variability across the distribution of true treatment effects [42, 43]. As Senn [34] explained, it is more appropriate to use a pooled variance in the meta-analysis, resulting in the same SE being applied to each person. The τ-statistic was reported alongside the 95%CI [44]. Weighted raw replicate-averaged treatment effect differences were presented with the respective 95% prediction interval (95% PI) [45, 46]. Meta-analyses were conducted in R (version 3.6.3, R Foundation for Statistical Computing); see Supplementary file 1 for the associated R Markdown file. Additional exploratory analyses [47] examined relationships between plasma nitrite and nitrate with BP variables as presented in detail in Supplementary Text 2.

Results

Fifteen healthy male participants with mean (SD) age of 27 (5) years and BMI of 24.0 (4.1) kg/m^2 took part in this study.

Plasma nitrate concentration

There was a strong positive correlation ($r = 0.91$, 90%CI: 0.78 to 0.96) between the two sets of placebo-adjusted responses to nitrate supplementation for plasma nitrate concentration (Fig. 2A). The within-trial SD for plasma nitrate concentration was substantially greater for the nitrate supplementation *versus* placebo conditions (Table 1). The model-based treatment-by-condition interaction response variability was ±45 µm (95%CI, 22 to 60 µm). Linear mixed models revealed a main effect of condition ($p < 0.001$), with a larger mean plasma nitrate concentration of 224 µm (95%CI: 199 to 250 µm) in the nitrate supplementation *versus* placebo condition. When averaged over the two replicates, the placebo-controlled post-supplementation mean plasma nitrate concentration ranged from 163 to 300 µm between the participants (Fig. 3A). The meta-analytic approach-estimated between-participant replicate-averaged treatment effect nitrate response heterogeneity (t) was ±43 µm (95%CI: 30 to 69 µm).

Plasma nitrite concentration

There was a moderate positive correlation ($r = 0.55$, 90%CI: 0.14 to 0.80) between the two sets of placebo-adjusted
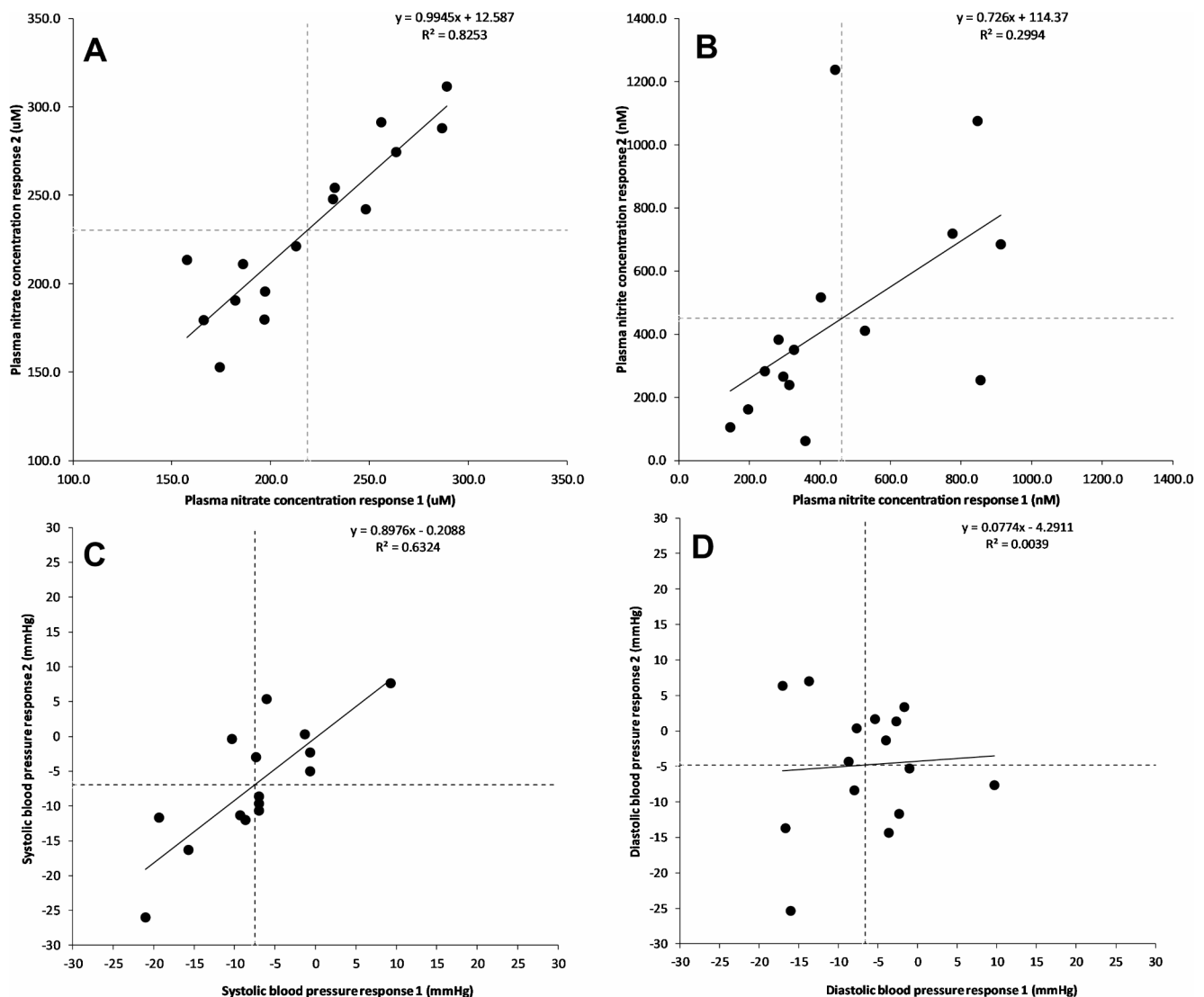

Fig. 2 Individual panels present the relationship between the placebo-adjusted plasma nitrate concentrations (uM, panel **A**), plasma nitrite concentrations (nM, panel **B**), systolic BP (mmHg, panel **C**), and diastolic BP (mmHg, panel **D**) on the two occasions. "Response 1" reflects data for the first pair of conditions (nitrate 1 minus placebo 1) and "response 2" for the second pair of conditions (nitrate 2 minus placebo 2). The dashed vertical and horizontal lines reflect the mean responses

Table 1 Estimated marginal means and SEs for primary outcome measures in the supplementation and placebo conditions with the true individual differences SD

Outcome	Mean (SE)		Main effect of condition	Estimate 1[1]	Estimate 2[2]	
	Supplementation	Placebo	Mean difference (95% CI)	Individual differences SD	Individual differences SD (95% CI)	P value (int)
Plasma nitrate concentration (uM)						
	239 (12)	15 (2)	224 (199 to 250)	44	45 (22 to 60)	0.0104
Plasma nitrite concentration (nM)						
	570 (85)	109 (12)	461 (269 to 653)	308	324 (125 to 441)	0.0214
Systolic blood pressure (mmHg)						
	-7 (2)	1 (1)	-7 (-11 to -3)	6	7 (3 to 9)	0.0196
Diastolic blood pressure (mmHg)						
	-5 (2)	1 (1)	-6 (-9 to -2)	2	3 (-4 to 6)	0.5107

Data for the analysis involved 60 experimental conditions in $n = 15$ males. Systolic and diastolic blood pressure values were obtained immediately prior to, and 2.5 h following, nitrate or placebo supplementation and data reflect the change in pre-to-post intervention values. Blood samples were obtained 2.5 h post supplementation only, therefore data for plasma nitrate and nitrite concentrations reflect post-supplementation values only

[1]Estimate 1: A naive estimate of the individual differences SD using the simple equation of $SD_{IR} = \sqrt{SD_S^2 - SD_P^2}$ where SD_{IR} is the SD of the true individual response, and SD_S and SD_P are the SDs of the primary outcome measures in the supplementation and placebo conditions, respectively [23]

[2]Estimate 2: Individual differences SD estimated using a random effects within-subjects statistical model [25]. The SD was calculated from the participant-by-condition interaction term modelled as a random effect and the P value is for this interaction term

SE, standard error; SD, standard deviation; CI, confidence interval; *int*, participant-by-condition interaction
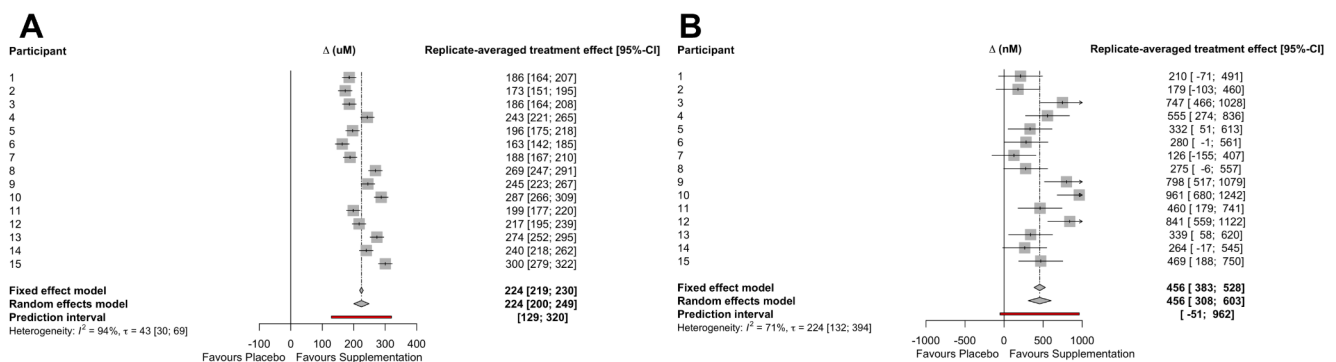
Fig. 3 Forest plots for the plasma nitrate (**A**) and nitrite (**B**) summary effects and between-participant (t) replicated-averaged treatment effect heterogeneity. t, denotes tau-statistic; CI, denotes confidence interval

responses to dietary nitrate supplementation for plasma nitrite concentration (Fig. 2B). The within trial SD for plasma nitrite concentration was substantially greater for the nitrate supplementation versus placebo conditions (Table 1), with the model-based treatment-by-condition interaction response variability of ±324 (95%CI: 125 to 441 nM). Linear mixed models revealed a significant main effect of condition ($p < 0.001$), with the mean plasma nitrite concentration 461 nM higher (95%CI, 269 to 653 nM) in the nitrate supplementation versus placebo condition. When averaged over the two replicates, the placebo-controlled post-supplementation mean plasma nitrite concentration ranged from

126 to 961 nM between the participants (Fig. 3B). The meta-analytic approach-estimated between-participant replicate-averaged treatment effect nitrite response heterogeneity (t) was ±224 nM (95%CI: 132 to 394 nM).

Systolic BP

There was a strong positive correlation ($r = 0.80$, 90%CI: 0.55 to 0.92) between the two sets of placebo-adjusted responses to dietary nitrate supplementation for systolic BP (Fig. 2C). The within trial SD for systolic BP was substantially greater for the nitrate supplementation *versus* placebo
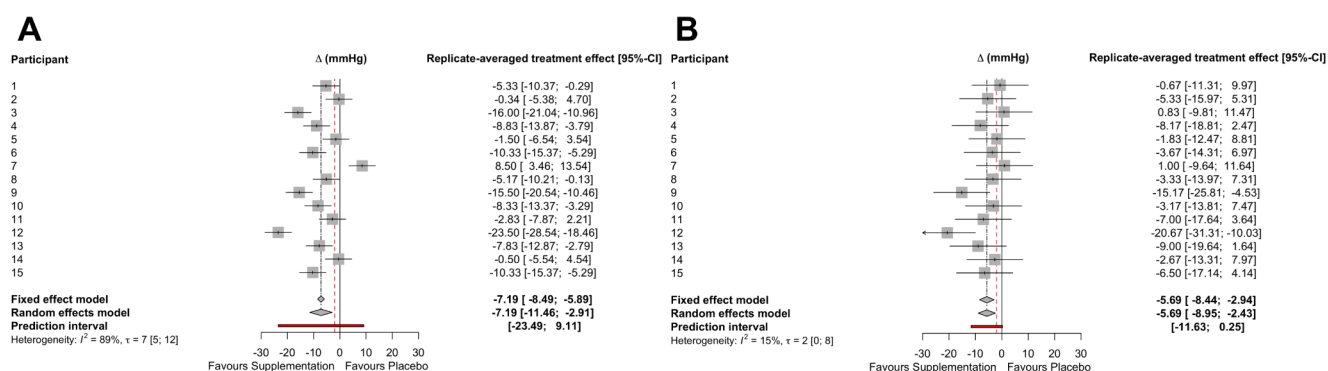
Fig. 4 Forest plots for systolic BP (**A**) and diastolic BP (**B**) summary effects and between-participant (t) replicated-averaged treatment effect heterogeneity. The red dashed line indicates the target difference reduction of 2 mmHg. t, denotes tau-statistic; CI, denotes confidence interval

conditions, and the model-based treatment-by-condition interaction response variability was ±7 mmHg (95%CI: 3 to 9 mmHg; Table 1). Linear mixed models revealed a main effect of condition ($p=0.001$), with a mean reduction in systolic BP that was 7 mmHg (95%CI: 3 to 11 mmHg) greater in the nitrate supplementation *versus* the placebo condition (Supplementary Table 1). When averaged over the two replicates, the placebo-controlled nitrate supplementation response ranged from a 9 mmHg increase to a 24-mmHg reduction between the participants (Fig. 4A). The meta-analytic approach revealed the upper confidence limit for the between-participant replicate-averaged treatment effect heterogeneity of t=±7 mmHg (95%CI: 5 to 12 mmHg) surpassed the clinically relevant target reduction of 2 mmHg for 8 participants (Fig. 4A).

Diastolic BP

Correlations between the two sets of placebo-adjusted responses to dietary nitrate supplementation for diastolic BP were small and non-significant ($r=0.06$, 90% CI: -0.39 to 0.49, Fig. 2D). The within trial SD for diastolic BP was similar for the dietary nitrate versus placebo conditions, with the model-based treatment-by-condition interaction response variability of ±3 mmHg (95%CI: -4 to 6 mmHg; Table 1). Linear mixed models revealed a significant main effect of condition ($p=0.003$), with a mean reduction in diastolic BP that was 6 mmHg (95%CI: 2 to 9 mmHg) greater in the nitrate supplementation *versus* placebo condition (Supplementary Table 1). When averaged over the two replicates, the placebo-controlled nitrate supplementation response ranged from a 1 mmHg increase to a 21-mmHg reduction between the participants (Fig. 4B). The meta-analytic approach revealed the upper confidence limit for the between-participant replicate-averaged treatment effect heterogeneity of t=±2 mmHg (95%CI: 0 to 8 mmHg) surpassed the clinically relevant target reduction of 2 mmHg for 2 participants (Fig. 4B).

Correlations amongst outcome variables

There was a small, non-significant positive correlation ($r=0.37$, 90%CI: -0.09 to 0.70, $p=0.09$) between the placebo-adjusted plasma nitrate and nitrite concentrations following dietary nitrate supplementation. In addition, there were small, non-significant negative correlations between the placebo-adjusted plasma nitrate concentrations and the change in both systolic ($r=-0.20$, 90%CI: -0.59 to 2.63, $p=0.23$) and diastolic ($r=-0.26$, 90%CI: -0.63 to 0.21, $p=0.174$) BP following nitrate supplementation. There was a moderate, significant negative correlation between the placebo-adjusted plasma nitrite concentrations and the change in both systolic ($r=-0.76$, 90%CI: -0.90 to -0.47, $p<0.001$) and diastolic ($r=-0.51$, 90%CI: -0.77 to -0.10, $p=0.03$) BP following nitrate supplementation. The slope of the regression line between the placebo-adjusted plasma nitrite concentration and the change in systolic ($y=-0.016+1.791$) and diastolic ($y=-0.007+3.319$) BP was −0.016 (95%CI: -0.022 to -0.010) and −0.007 (95%CI: -0.016 to 0.001), respectively. Supplementary Table 2 illustrates the expected BP changes for a given plasma nitrite concentration.

Discussion

This is the first study on the topic of dietary nitrate supplementation to follow a replicate crossover design and associated expert guidance for data analysis (22, 25, 34). We detected inter-individual differences in the effects of dietary nitrate supplementation on plasma nitrate and nitrite concentrations and systolic BP that were distinguishable from random within-subject variability. In addition, our data suggest that the effects of nitrate supplementation on plasma nitrate and nitrite concentrations, and systolic BP are consistent within individuals when measured on at least two occasions.

We observed a mean reduction in systolic BP following dietary nitrate supplementation of 7 mmHg. A novel finding of our study is that the degree of systolic BP reduction following nitrate supplementation, i.e., the treatment response was highly variable between individuals, and greater than the within-participant trial-to-trial random variation. Our study, therefore, supports the notion that there may be genuine 'responders' and 'non-reponders' (or, more accurately, higher and lower responders) to dietary nitrate supplementation [17–20]. Application of a recently described meta-analytic approach for replicate crossover trial examination revealed that the upper confidence interval for the control-adjusted BP reduction following nitrate supplementation exceeded the MCID of 2 mmHg for approximately half (8/15) of our participants. This suggests that these individuals are likely to experience BP reductions with nitrate supplementation of a magnitude which could potentially contribute to mitigating CVD incidence and mortality [38, 53].

Nitrate supplementation also significantly reduced diastolic BP, with a mean reduction of 6 mmHg. However, the between-replicate correlation for diastolic BP was low. This suggests that the effects of nitrate on diastolic BP are not as clear (relative to the random within-subjects variability) compared with systolic BP. This may help explain why reductions in diastolic BP are less frequently observed in the extant literature [7]. The participant-by-condition interaction was also small, suggesting inconsistent inter-individual variability in the effects of nitrate supplementation on diastolic BP. It is currently unclear why there appears to be repeatable interindividual differences in the effects of nitrate supplementation on systolic, but not diastolic, BP. It is possible that there are measurement or experimental issues that could obscure true inter-individual differences in diastolic BP responses, but more research is needed to explicate this phenomenon.

The potential for evidence-based personalised recommendations around dietary nitrate intake rests on formal identification of individual participants who will benefit most from consumption of this compound [54]. This could be achieved by identifying participant characteristics that are associated with the level of BP response to dietary nitrate. At the group level, previous data suggests that nitrate supplementation may be more effective at lowering BP in males versus females (although more studies in females are needed to confirm this hypothesis) [15, 55] and in younger versus older adults [56, 57]. All participants in our study were healthy young males which emphasises the importances of factors other than age, sex and health in determining inter-individual responses to nitrate supplementation. Following consumption, dietary nitrate is absorbed in the upper gastrointestinal tract, increasing plasma nitrate concentrations

[10]. Whilst most of the ingested nitrate is excreted in urine, approximately 25% is returned to the oral cavity via the salivary glands [58] where it is reduced to nitrite by oral bacteria [59]. This nitrite is swallowed and partly converted to NO and other nitrogen oxides in the stomach. Some nitrite also reaches systemic circulation, where it can be reduced to NO in various tissue [10]. Investigation of factors that may explain inter-individual variations in response to nitrate supplementation could focus on key steps within the gastrointestinal tract (including actions of oral bacteria involved in the regulation of NO bioavailability [21, 60, 61] and stomach pH which influences non-enzymatic conversation of nitrate to NO and other reactive nitrogen oxides [62]). Interestingly, recent data from Willmott et al. [63] suggests that individuals with a greater oral nitrate reducing capacity achieve a larger reduction in diastolic BP after nitrate supplementation. In addition, research should further explore the impact of genetic variants (or other factors) that alter the biological activity of the proteins e.g., nitric oxide synthases (NOS) involved in production of NO from both nitrate and other dietary sources (e.g., L-arginine/L-citrulline). Hobbs et al. [17] found that nitrate was more effective at lowering diastolic BP in T carriers (compared with GG carriers) of the Glu298Asp polymorphism in the gene (*NOS3*) encoding eNOS [17]. To date, there has been no systematic investigation of these and other factors that may be causally responsible for inter-individual differences in the response to nitrate supplementation using appropriate designs such as replicate crossover studies. This is a priority for future research with potential to be an important exemplar for precision nutrition.

Strengths of this study include the adoption of a replicate crossover study design and the use of appropriate statistical approaches for quantifying between-participant outcome response variability. This extends previous studies that have explored the impact of nitrate on biological markers and/or BP in traditional crossover or parallel group designs [7] or with repeat administration of nitrate *but not* control/placebo arms [21], such that the participant-by-condition interaction could not be estimated. Undeniably, the repeated administration of treatment and placebo for derivation of the person by treatment interaction complicates the statistical analysis, but this is a necessary complication for appropriate study of this topic. Unfortunately, previous researchers have arrived at erroneous claims about treatment response hetergeneity on the basis of simple, but compromised, responder counting and simply observing individual changes solely from the treatment group in a trial [23]. Potential limitations include only recruiting healthy young males as participants. While this may represent a limitation in generalisation of our findings to the general population, this design feature was a distinct advantage in revealing evidence of

true inter-individual variations in physiological response to nitrate supplementation that cannot be explained by sex or age differences alone. Whilst lowering BP in this group may be less of a public health priority than individuals with hypertension, lowering BP in individuals who are not hypertensive down to a least ~ 115/75 mmHg could reduce risk of vascular death [53]. A further limitation is the relatively low sample size ($n = 15$), although both the crossover (within-subjects) and replicate aspects of our design increased statistical power (relative to a parallel arm study) for detection of mean treatment effects. Our design and sample size also enabled the detection of statistically significant individual differences in responses for all outcome variables apart from diastolic BP. We cannot rule out the possibility that response heterogeneity in diatolic BP would be detected with a larger sample size, more reliable measurements and/or a greater number of replicates in the design. Repeated administration of treatments is the key aspect for detecting, confidently, treatment response heterogeneity but laboratory-based and somewhat invasive replicate crossover studies like ours are difficult to recruit for. Additionally, we studied acute responses so it remains to be established whether there are similar inter-individual differences in response to nitrate supplementation over longer intervention periods. Longer-term interventions might also clarify whether the benefits of nitrate consumption on CVD risk proposed in observational studies [64] occurs with prolonged dietary nitrate supplementation, and whether benefits are restricted to certain population sub-groups/individuals.

This study revealed evidence of substantial inter-individual differences in the physiological responses (NO biomarkers and systolic BP) to dietary nitrate supplementation in healthy young males. This provides proof-of-concept as a basis for further investigations of the magnitude, durability and pervasiveness of these inter-individal responses across diverse populations and of the (biological) factors responsible for the observed intra-individual variation. These findings open up the exciting possibility of personalised recommendations for dietary nitrate intake for optimal management of BP and related health outcomes.

Declarations

References

1. Zhou B, Perel P, Mensah GA, Ezzati M (2021) Global epidemiology, health burden and effective interventions for elevated blood pressure and hypertension. Nat Rev Cardiol 18:785–802. https://doi.org/10.1038/s41569-021-00559-8

2. Olsen MH, Angell SY, Asma S et al (2016) A call to action and a lifecourse strategy to address the global burden of raised blood pressure on current and future generations: the Lancet Commission on hypertension. Lancet 388:2665–2712. https://doi.org/10.1016/S0140-6736(16)31134-5

3. Bundy JD, Li C, Stuchlik P et al (2017) Systolic blood pressure reduction and risk of Cardiovascular Disease and Mortality: a systematic review and network Meta-analysis. JAMA Cardiol 2:775–781. https://doi.org/10.1001/jamacardio.2017.1421

4. Williams B, Mancia G, Spiering W et al (2018) 2018 ESC/ESH guidelines for the management of arterial hypertension: the Task Force for the management of arterial hypertension of the European Society of Cardiology and the European Society of Hypertension. J Hypertens 36:1953. https://doi.org/10.1097/HJH.0000000000001940

5. Larsen FJ, Ekblom B, Sahlin K et al (2006) Effects of Dietary Nitrate on blood pressure in healthy volunteers. N Engl J Med 355:2792–2793. https://doi.org/10.1056/NEJMc062800

6. Webb AJ, Patel N, Loukogeorgakis S et al (2008) Acute blood pressure lowering, vasoprotective and anti-platelet properties of dietary nitrate via bioconversion to Nitrite. Hypertension

51:784–790. https://doi.org/10.1161/HYPERTENSIONAHA.107.103523

7. Jackson JK, Patterson AJ, MacDonald-Wicks LK et al (2018) The role of inorganic nitrate and nitrite in cardiovascular disease risk factors: a systematic review and meta-analysis of human evidence. Nutr Rev 76:348–371. https://doi.org/10.1093/nutrit/nuy005

8. Hord NG, Tang Y, Bryan NS (2009) Food sources of nitrates and nitrites: the physiologic context for potential health benefits. Am J Clin Nutr 90:1–10. https://doi.org/10.3945/ajcn.2008.27131

9. Griffiths A, Alhulaefi S, Hayes EJ et al (2023) Exploring the advantages and disadvantages of a whole Foods Approach for elevating Dietary Nitrate intake: have researchers concentrated too much on Beetroot Juice? Appl Sci 13:7319. https://doi.org/10.3390/app13127319

10. Lundberg JO, Weitzberg E, Gladwin MT (2008) The nitrate–nitrite–nitric oxide pathway in physiology and therapeutics. Nat Rev Drug Discov 7:156–167. https://doi.org/10.1038/nrd2466

11. Siervo M, Scialò F, Shannon OM et al (2018) Does dietary nitrate say NO to cardiovascular ageing? Current evidence and implications for research. Proc Nutr Soc 77:112–123. https://doi.org/10.1017/S0029665118000058

12. Vanhatalo A, Bailey SJ, Blackwell JR et al (2010) Acute and chronic effects of dietary nitrate supplementation on blood pressure and the physiological responses to moderate-intensity and incremental exercise. AJP Regul Integr Comp Physiol 299:R1121–R1131. https://doi.org/10.1152/ajpregu.00206.2010

13. Jonvik KL, Nyakayiru J, Pinckaers PJ et al (2016) Nitrate-rich vegetables increase plasma nitrate and nitrite concentrations and lower blood pressure in healthy adults. https://doi.org/10.3945/jn.116.229807. J Nutr jn229807

14. Siervo M, Shannon O, Kandhari N et al (2020) Nitrate-Rich Beetroot Juice reduces blood pressure in Tanzanian adults with elevated blood pressure: a double-blind randomized controlled feasibility trial. J Nutr 150:2460–2468. https://doi.org/10.1093/jn/nxaa170

15. Kapil V, Milsom AB, Okorie M et al (2010) Inorganic Nitrate supplementation lowers blood pressure in humans: role for Nitrite-derived NO. Hypertension 56:274–281. https://doi.org/10.1161/HYPERTENSIONAHA.110.153536

16. Rammos C, Hendgen-Cotta UB, Sobierajski J et al (2014) Dietary nitrate reverses vascular dysfunction in older adults with moderately increased cardiovascular risk. J Am Coll Cardiol 63:1584–1585. https://doi.org/10.1016/j.jacc.2013.08.691

17. Hobbs DA, George TW, Lovegrove JA (2014) Differential effect of beetroot bread on postprandial DBP according to Glu298Asp polymorphism in the eNOS gene: a pilot study. J Hum Hypertens 28:726–730. https://doi.org/10.1038/jhh.2014.16

18. Coggan AR, Broadstreet SR, Mikhalkova D et al (2018) Dietary nitrate-induced increases in human muscle power: high versus low responders. Physiol Rep 6:e13575. https://doi.org/10.14814/phy2.13575

19. Wylie LJ, Kelly J, Bailey SJ et al (2013) Beetroot juice and exercise: pharmacodynamic and dose-response relationships. J Appl Physiol 115:325–336. https://doi.org/10.1152/japplphysiol.00372.2013

20. James PE, Willis GR, Allen JD et al (2015) Nitrate pharmacokinetics: taking note of the difference. Nitric Oxide. https://doi.org/10.1016/j.niox.2015.04.006

21. Liddle L, Burleigh MC, Monaghan C et al (2019) Variability in nitrate-reducing oral bacteria and nitric oxide metabolites in biological fluids following dietary nitrate administration: an assessment of the critical difference. Nitric Oxide 83:1–10. https://doi.org/10.1016/j.niox.2018.12.003

22. Senn S (2016) Mastering variation: variance components and personalised medicine. Stat Med 35:966–977. https://doi.org/10.1002/sim.6739

23. Atkinson G, Batterham AM (2015) True and false interindividual differences in the physiological response to an intervention. Exp Physiol 100:577–588. https://doi.org/10.1113/EP085070

24. Senn S (2019) Sample size considerations for n-of-1 trials. Stat Methods Med Res 28:372–383. https://doi.org/10.1177/0962280217726801

25. Senn S, Rolfe K, Julious SA (2011) Investigating variability in patient response to treatment– a case study from a replicate crossover study. Stat Methods Med Res 20:657–666. https://doi.org/10.1177/0962280210379174

26. Muntner P, Shimbo D, Carey RM et al (2019) Measurement of blood pressure in humans: A Scientific Statement from the American Heart Association. Hypertension 73:e35–e66. https://doi.org/10.1161/HYP.0000000000000087

27. Rogers SC, Khalatbari A, Gapper PW et al (2005) Detection of human red blood cell-bound nitric oxide. J Biol Chem 280:26720–26728. https://doi.org/10.1074/jbc.M501179200

28. Bacchetti P (2010) Current sample size conventions: flaws, harms, and alternatives. BMC Med 8:17. https://doi.org/10.1186/1741-7015-8-17

29. Lakens D (2022) Sample size justification. Collabra Psychol 33267

30. Goltz FR, Thackray AE, Atkinson G et al (2019) True interindividual variability exists in postprandial appetite responses in healthy men but is not moderated by the FTO Genotype. J Nutr 149:1159–1169. https://doi.org/10.1093/jn/nxz062

31. Goltz FR, Thackray AE, King JA et al (2018) Interindividual responses of appetite to Acute Exercise: a replicated crossover study. Med Sci Sports Exerc 50:758–768. https://doi.org/10.1249/MSS.0000000000001504

32. Atkinson G, Nevill AM (2001) Selected issues in the design and analysis of sport performance research. J Sports Sci 19:811–827. https://doi.org/10.1080/026404101317015447

33. Shen T, Thackray AE, King JA et al (2024) Are there interindividual responses of Cardiovascular Disease risk markers to Acute Exercise? A replicate crossover trial. Med Sci Sports Exerc 56:63–72. https://doi.org/10.1249/MSS.0000000000003283

34. Senn S (2024) The analysis of continuous data from n-of-1 trials using paired cycles: a simple tutorial. Trials 25:128. https://doi.org/10.1186/s13063-024-07964-7

35. Araujo A, Julious S, Senn S (2016) Understanding variation in sets of N-of-1 trials. PLoS ONE 11:e0167167. https://doi.org/10.1371/journal.pone.0167167

36. Schabenberger O, SAS Users Group International Conference (2004) Mixed model influence diagnostics. In: In: Proceedings of the twenty-ninth annual; 2004 May 9–12: Cary (The United States): SAS Institute. pp 189–29

37. West BT, Galecki AT (2012) An overview of current Software procedures for Fitting Linear mixed models. Am Stat 65:274–282. https://doi.org/10.1198/tas.2011.11077

38. Stamler J, Rose G, Stamler R et al (1989) INTERSALT study findings. Public health and medical care implications. Hypertens Dallas Tex 1979 14(570–577). https://doi.org/10.1161/01.hyp.14.5.570

39. Turnbull F, Blood Pressure Lowering Treatment Trialists' Collaboration (2003) Effects of different blood-pressure-lowering regimens on major cardiovascular events: results of prospectively-designed overviews of randomised trials. Lancet Lond Engl 362:1527–1535. https://doi.org/10.1016/s0140-6736(03)14739-3

40. Schwarzer G (2007) meta: an R package for meta-analysis. R News 40–45

41. IntHout J, Ioannidis JP, Borm GF (2014) The Hartung-Knapp-Sidik-Jonkman method for random effects meta-analysis is straightforward and considerably outperforms the standard DerSimonian-Laird method. BMC Med Res Methodol 14:25. https://doi.org/10.1186/1471-2288-14-25

42. Langan D, Higgins JPT, Jackson D et al (2019) A comparison of heterogeneity variance estimators in simulated random-effects meta-analyses. Res Synth Methods 10:83–98. https://doi.org/10.1002/jrsm.1316

43. Veroniki AA, Jackson D, Viechtbauer W et al (2016) Methods to estimate the between-study variance and its uncertainty in meta-analysis. Res Synth Methods 7:55–79. https://doi.org/10.1002/jrsm.1164

44. Viechtbauer W (2007) Confidence intervals for the amount of heterogeneity in meta-analysis. Stat Med 26:37–52. https://doi.org/10.1002/sim.2514

45. Borenstein M, Hedges LV, Higgins JPT, Rothstein HR (2010) A basic introduction to fixed-effect and random-effects models for meta-analysis. Res Synth Methods 1:97–111. https://doi.org/10.1002/jrsm.12

46. Borenstein M (2024) Avoiding common mistakes in meta-analysis: understanding the distinct roles of Q, I-squared, tau-squared, and the prediction interval in reporting heterogeneity. Res Synth Methods 15:354–368. https://doi.org/10.1002/jrsm.1678

47. Williams R (2012) Using the Margins Command to Estimate and interpret adjusted predictions and marginal effects. Stata J 12:308–331. https://doi.org/10.1177/1536867X1201200209

48. IntHout J, Ioannidis JPA, Rovers MM, Goeman JJ (2016) Plea for routinely presenting prediction intervals in meta-analysis. BMJ Open 6:e010247. https://doi.org/10.1136/bmjopen-2015-010247

49. Riley RD, Higgins JPT, Deeks JJ (2011) Interpretation of random effects meta-analyses. BMJ 342:d549. https://doi.org/10.1136/bmj.d549

50. Velmurugan S, Gan JM, Rathod KS et al (2016) Dietary nitrate improves vascular function in patients with hypercholesterolemia: a randomized, double-blind, placebo-controlled study123. Am J Clin Nutr 103:25–38. https://doi.org/10.3945/ajcn.115.116244

51. Kapil V, Khambata RS, Robertson A et al (2015) Dietary nitrate provides sustained blood pressure lowering in hypertensive patients: a randomized, phase 2, double-blind, placebo-controlled study. Hypertens Dallas Tex 1979 65:320–327. https://doi.org/10.1161/HYPERTENSIONAHA.114.04675

52. Babateen AM, Shannon OM, O'Brien GM et al (2023) Moderate doses of dietary nitrate elicit greater effects on blood pressure and endothelial function than a high dose: a 13-week pilot study. Nutr Metab Cardiovasc Dis NMCD S. https://doi.org/10.1016/j.numecd.2023.02.024. 0939-4753(23)00084–4

53. Lewington S, Clarke R, Qizilbash N et al (2002) Age-specific relevance of usual blood pressure to vascular mortality: a meta-analysis of individual data for one million adults in 61 prospective studies. Lancet Lond Engl 360:1903–1913. https://doi.org/10.1016/s0140-6736(02)11911-8

54. Shannon OM, Easton C, Shepherd AI et al (2021) Dietary nitrate and population health: a narrative review of the translational potential of existing laboratory studies. BMC Sports Sci Med Rehabil 13:65. https://doi.org/10.1186/s13102-021-00292-2

55. Wickham KA, Spriet LL (2019) No longer beeting around the bush: a review of potential sex differences with dietary nitrate supplementation 1. Appl Physiol Nutr Metab Physiol Appl Nutr Metab 44:915–924. https://doi.org/10.1139/apnm-2019-0063

56. Capper T, Clifford T, Taylor G et al (2022) Ageing modifies acute resting blood pressure responses to incremental consumption of dietary nitrate: a randomised, cross-over clinical trial. Br J Nutr 1–12. https://doi.org/10.1017/S0007114522001337

57. Shannon OM, Clifford T, Seals DR et al (2022) Nitric oxide, aging and aerobic exercise: sedentary individuals to Master's athletes. Nitric Oxide 125–126:31–39. https://doi.org/10.1016/j.niox.2022.06.002

58. Qin L, Liu X, Sun Q et al (2012) Sialin (SLC17A5) functions as a nitrate transporter in the plasma membrane. Proc Natl Acad Sci U S A 109:13434–13439. https://doi.org/10.1073/pnas.1116633109

59. Duncan C, Dougall H, Johnston P et al (1995) Chemical generation of nitric oxide in the mouth from the enterosalivary circulation of dietary nitrate. Nat Med 1:546–551

60. Burleigh MC, Liddle L, Monaghan C et al (2018) Salivary nitrite production is elevated in individuals with a higher abundance of oral nitrate-reducing bacteria. Free Radic Biol Med 120:80–88. https://doi.org/10.1016/j.freeradbiomed.2018.03.023

61. Burleigh M, Liddle L, Muggeridge DJ et al (2019) Dietary nitrate supplementation alters the oral microbiome but does not improve the vascular responses to an acute nitrate dose. Nitric Oxide Biol Chem 89:54–63. https://doi.org/10.1016/j.niox.2019.04.010

62. Sanches-Lopes JM, Ferreira GC, Pinheiro LC et al (2020) Consistent gastric pH-dependent effects of suppressors of gastric acid secretion on the antihypertensive responses to oral nitrite. Biochem Pharmacol 177:113940. https://doi.org/10.1016/j.bcp.2020.113940

63. Willmott T, Ormesher L, McBain AJ et al (2023) Altered oral nitrate reduction and bacterial profiles in Hypertensive Women Predict blood pressure lowering following Acute Dietary Nitrate supplementation. Hypertension 80:2397–2406. https://doi.org/10.1161/HYPERTENSIONAHA.123.21263

64. Tan L, Stagg L, Hanlon E et al (2024) Associations between Vegetable Nitrate Intake and Cardiovascular Disease Risk and Mortality: a systematic review. Nutrients 16:1511. https://doi.org/10.3390/nu16101511

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