Effect of nitrate supplementation on hepatic blood flow and glucose homeostasis: A double-blind, placebo controlled, randomised control trial.

Running Head: Nitrate supplementation and hepatic blood flow.

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MG, AS, JF, DW, ACS PW, NB, were involved in the conception or design of the work. AIS, JF, MG, were involved in the acquisition of data. AIS, DW, JF, NB, PW, NB, ACS, MG were involved in the analysis or interpretation of data. All authors have been involved in drafting of the work and revision for intellectually important content.

Abstract

Nitric oxide alters gastric blood flow, improves vascular function and mediates glucose uptake within the intestines and skeletal muscle. Dietary nitrate, acting as a source of nitric oxide, appears to be a potential low cost therapy that may help maintain glucose homeostasis.

In a randomised, double-blind, placebo-controlled crossover study, 31 young and older adult participants had a standardised breakfast, supplemented with either nitrate rich beetroot juice (11.91 mmol nitrate) or nitrate depleted beetroot juice as placebo (0.01 mmol nitrate). MRI was used to assess apparent diffusion coefficient (ADC), portal vein flux and velocity. Plasma glucose, incretin and C-peptide concentrations and BP were assessed. Outcome variables were measured at baseline and hourly for 3 hours.

Compared with a placebo, beetroot juice resulted in a significant elevation in plasma nitrate and plasma nitrite concentration. No differences were seen for the young or older adult cohorts between placebo and beetroot juice for ADC, or portal vein flux. There was an interaction effect in the young adults, between visits for portal vein velocity. Nitrate supplementation did not reduce plasma glucose active GLP-1, total GLP-1 or plasma C-peptide concentrations for the young or older adult cohorts.

Despite a significant elevation in plasma nitrite concentration following an acute dose of (11.91 mmol) nitrate, there was no effect on hepatic blood flow, plasma glucose, C-peptide, or incretin concentration in healthy adults.

New and Noteworthy

This is the first study investigating the effect dietary nitrate supplementation on hepatic blood flow, incretin and C-peptide concentrations in young and older adults. Despite a physiologically relevant elevation in plasma nitrite concentration following an acute dose of

50 11.9 mmol nitrate, there was no effect on hepatic blood flow, plasma glucose, C-peptide, or
51 incretin concentration. Acute supplementation of nitrate does not appear to alter hepatic
52 diffusion or modulate post-prandial glucose homeostasis.

53 *Introduction*

54 Nitric oxide is produced within the body via two independent pathways. The first pathway in
55 which nitric oxide is produced is via, nitric oxide synthase enzymes (NOS), acting on the amino
56 acid L-arginine in an O_2 dependent reaction (1). The second pathway, known as the entero-
57 salivary pathway is O_2 independent. It involves nitrate from the diet being ingested, absorbed
58 through the stomach wall and proximal small intestine (3, 19) and entering the circulation
59 where it is concentrated in the salivary glands. A reduction of nitrate to nitrite via facultative
60 anaerobic bacteria occurs within the mouth (18). Nitrite is then swallowed and some nitrite is
61 converted to nitric oxide in the acidic environment of the stomach (5) whilst some of the nitrite
62 is absorbed into the circulation and acts as a storage pool for subsequent conversion to nitric
63 oxide (48). Elevated concentrations of nitric oxide have been shown to increase gastric blood
64 flow (55) whilst acidified nitrite has been shown to protect against enteric pathogens (47).

65 Another possible mechanism exists for the conversion of nitrate to nitrite, whereby hepatic
66 xanthine oxidoreductase catalyses the reduction of nitrate to nitrite (31). This mechanism in
67 conjunction with the uptake of nitrite into the portal circulation may explain why one of the
68 highest concentrations of nitrite in any organ is found within the liver (10, 59). Subsequent
69 increases in the bioavailability of nitric oxide within the liver may be expedited by a number
70 of nitrite reductases such as; xanthine oxidoreductase (44, 50), aldehyde oxidase (39, 43),
71 cytoglobin (11, 20, 45) and deoxyhaemoglobin (16). The potential increase of nitric oxide
72 within the hepatic vasculature may lead to vasodilation of the parenchyma and lead to greater
73 surface area for glucose uptake to occur. However, with ageing there is evidence for both
74 diminished NO production and impaired vascular responses to NO (49, 52). These age related

changes may be associated with different responses to nitrate supplementation or other interventions aimed at increasing NO bioavailability.

Diets rich in vegetables have been shown to have beneficial effects on cardiovascular health (23), morbidity and mortality (33) and to reduce the risk of developing T2DM (13) and are rich in inorganic nitrate. There is growing evidence to suggest nitrate is at least in part responsible for these beneficial effects (9). Recent reports have described how nitrate supplementation can reduce systolic and diastolic blood pressure in healthy older adults (30, 34, 35) and in clinical cohorts with elevated cardiovascular risk factors (6, 36, 37, 42). Other reports have shown no effect of nitrate supplementation on systolic and diastolic blood pressure in clinical cohorts (2, 24, 58). Although in a overweight and obese group of individuals, nitrate supplementation has been shown to improve postprandial endothelial function following a mixed meal (32). Nitric oxide mediates glucose uptake from the intestines (27) and facilitates its disposal into skeletal muscle in animal models (51) and in individuals with T2DM (38). Increases in the bioavailability of nitric oxide stimulates insulin secretion (54) and increases GLUT4 translocation (46). Supplementation of nitrite in the drinking water of eNOS deficient mice (for 10 weeks) reduces glycated hemoglobin concentrations, and lowers baseline and postprandial glucose concentrations (12). A study in young adults supplemented with dietary nitrate has shown a reduction in plasma glucose concentrations post exercise compared to placebo (67). Recently, another study described a reduction in baseline plasma glucose concentrations 2.5h after supplementation with pharmacological sodium nitrate in individuals with T2DM but there was no effect on post prandial glucose concentrations following an oral glucose tolerance test (14). In contrast, Betteridge et al. (7) showed no change in glucose kinetics during exercise in a group of recreationally active individuals. Moreover, beetroot juice taken in conjunction with a 75g glucose load did not augment glucose uptake in 16 obese, insulin resistant men (21). Another potential mechanism for changes in glucose concentrations

maybe related to incretins and their insulinotropic effects. Incretin hormones are released from the small intestine in response to ingestion of food and are a key component in glucose homeostasis via their insulinotropic effect (28). Incretins mediate the uptake of glucose in the intestines in an nitric oxide dependent fashion (27) and have been shown to promote the production of nitric oxide within the portal vein (17).

Purpose / hypothesis

Aging may affect the bioavailability of nitric oxide and thus hepatic diffusion. Therefore we will assess our outcomes in a young adult and an older adult cohort. The aim of this study was to assess if inorganic nitrate modulates portal vein flux and velocity and hepatic microvascular diffusion and secondly to assess if supplementation with nitrate alters post-prandial plasma glucose, incretin and C-Peptide concentrations and blood pressure. We hypothesised that supplementation of the diet with dietary nitrate would increase blood flow to the liver and vasodilate the microvasculature causing improved postprandial glucose uptake.

Materials and Methods

Volunteers

37 individuals (17 healthy young individuals and 20 healthy older adults) provided written informed consent to participate in this double-blind, placebo-controlled, cross-over design study (see table 1 for subject characteristics). The healthy young individuals were recruited via word of mouth. The older adults were recruited via the NIHR Exeter Clinical Research Facility, Exeter 10,000 cohort. This is a database of individuals who have been pre-screened and consented to be contacted as research volunteers. The trial commenced in July 2014 and ended in April 2015. Ethical approval was obtained from the Exeter NRES Committee (14/SW/0092). This trial was registered on the ClinicalTrials.gov website (NCT02195856). Healthy young

individuals were recruited if they were aged between 18 and 35 and older adults aged between 50 and 75.

Participants were excluded if they: were unable to consent, took vasoactive medications, had uncontrolled hypertension (systolic BP> 160 mmHg), received antibiotic therapy within the preceding two weeks, took regular organic nitrate, thiazolidinidiones or nicorandil, had severe claustrophobia, were smokers (smoked within past 3 months), had an estimated glomerular filtration rate (eGFR) <30 ml/min/1.73 m^2, had a myocardial infarction or cerebro-vascular event within the preceding 3 months, had previous brain surgery, had a cardiac pacemaker, had metal fragments in the eye or larger metal objects that would interfere with data collection or analysis. Volunteers, who had medical interventions where metal implants were inserted, were assessed to determine safety in the scanner.

Experimental Overview

Screening and consent took place at the Diabetes and Vascular Research Centre at the NIHR Exeter Clinical Research Facility. Following screening checks volunteers were randomly assigned (within their respective age-group) to a double-blind crossover experimental design to consume 140 ml of nitrate rich beetroot juice (beetroot juice; containing 11.91 mmol of nitrate; Beet it, James White Drinks Ltd., Ipswich) or nitrate depleted beetroot juice (placebo; nitrate depleted beetroot juice containing 0.01 mmol of nitrate; Beet it, James White Drinks Ltd., Ipswich). The placebo production has been detailed previously (25); the final product is identical in appearance, odour, taste, colour and texture.

On the day of testing, volunteers fasted overnight (from 10pm) although water consumption was allowed to ensure they arrived in a hydrated state. Volunteers were asked to refrain from antibacterial mouthwash throughout the study and for at least 7 days prior to experimental visits. Antibacterial mouthwash has been demonstrated to reduce the concentration of oral

147 bacterial anaerobes responsible for the reduction of nitrate in the entero-salivary pathway (26).
148 Volunteers were also asked to avoid caffeine for 12 hours, alcohol and strenuous activity for
149 24 hours and nitrate rich foods on the day prior to their visits.

150 Volunteers arrived at the Exeter Magnetic Resonance Research Centre at the University of
151 Exeter. A 30 minute acclimatisation period was implemented prior to the magnetic resonance
152 imaging (MRI) scans. During this acclimatisation period a cannula was inserted in order to take
153 baseline plasma concentrations for glucose (fluoride & EDTA tubes; Sarstedt, S-Monovette,
154 Nümbrecht, Germany). Plasma nitrate and nitrite were collected (lithium heparin tubes;
155 Sarstedt, S-Monovette, Nümbrecht, Germany) and analysis was performed as previously
156 described (24). Prior to the baseline MRI scan, 5 resting, seated blood pressure (BP)
157 measurements were taken (Schiller Medical, Wissembourg, France) and an average of the final
158 3 recorded.

159 Following the baseline MRI scans the volunteers were provided with either the nitrate rich
160 beetroot juice or the placebo with two slices of toast, butter and jam. The combined quantity
161 of carbohydrate equated to 76 grams and is approximately equivalent to that that would be
162 consumed during an oral glucose tolerance test (4). Every hour, for three subsequent hours,
163 from the consumption of the beetroot juice, another set of scans were performed. Immediately
164 prior to each scan brachial artery blood pressure and venous blood samples were taken and
165 processed as previously described. A minimum 7 day washout period between the crossover
166 was employed.

167 MRI scans

168 A 1.5 T (Philips, Amsterdam, The Netherlands) magnetic resonance imaging (MRI) scanner
169 was used in order to examine changes in velocity and flux in the portal vein and microvascular
170 diffusion in the posterior right lobe of the liver.

Initial structural images were obtained to orientate the portal vein and an 8mm slice was selected perpendicular to the long axis of the vein. To determine flux and velocity a cardiac triggered velocity sensitive phase encoding imaging sequence (22) was employed which obtained image data at 20 time points throughout the cardiac cycle. Analysis of the portal vein was subsequently undertaken using a package supplied as part of the general scanner software. For each separate measurement the circumference of the vessel was manually drawn and recorded for each of the 20 time points to establish a defined region of interest (ROI). Within this ROI, flow and velocity were automatically calculated to give profiles throughout the cardiac cycle. A mean across the cardiac cycle for flux and velocity was created. Day to day repeatability for portal vein velocity and flux was assessed in 6 individuals studied on two occasions and was 16% and 13% respectively.

To examine the microvascular diffusion in the posterior right lobe of the liver, a magnetic resonance sequence sensitive to flow was employed via the application of magnetic field gradients in three orthogonal directions. Microvascular diffusion was averaged over all directions within the region of interest and is known as the apparent diffusion coefficient (ADC). Day to day repeatability was assessed in 6 individuals on two days for ADC (1 ROI: ADC = 1.15 ± 0.12, CV = 9.77%) and with multiple ROI. One ROI away from any major vessels had greater repeatability than 2, 3 and 6 sites (2 ROI: ADC = 1.11 ± 0.21, CV = 15.29%; 3 ROI: ADC = 1.10 ± 0.23, CV = 21.34%; 6 ROI: ADC = 1.09 ± 0.33, CV = 30.32%). To calculate ADC within the posterior left lobe of the liver, a ROI (typically 2500 mm^3) was manually drawn using the scanner software and the signal intensity within determined. For multiple ROI during repeatability testing, different locations within the posterior left lobe of the liver were selected. Each selection was in the same approximate location for each individual. ADC was subsequently calculated based upon the ratio of signal intensity from the

two images generated from the MR sequence employed, one of which was sensitive to flow, whereas the other had a low sensitivity to flow, where:

$ADC = -1/(b_1-b_0)\ \ Ln\ (S_0/S_1)$

S_0 is the signal intensity in the low flow sensitivity image

S_1 is the signal intensity in the flow sensitive image

b_0 is the magnetic field gradient used in the low flow sensitivity image=250 s/mm^2

b_1 is the magnetic field gradient used in the flow sensitive image=750 s/mm^2.

Incretin and C-Peptide analysis

To preserve total and active glucagon-like peptide-1 (GLP-1) for analysis, 10µl of dipeptidyl peptidase 4 inhibitor (Merck Millipore, Darmstadt, Germany) per 1ml of whole blood was injected into ice-chilled EDTA tubes (Sarstedt, S-Monovette, Nümbrecht, Germany) prior to adding the blood. Samples were immediately centrifuged at 3600 rpm for 10 minutes at 4°C, plasma was aliquoted and flash frozen with liquid nitrogen. Quantification of total and active GLP-1 were performed using an enzymatic immunoassay (Total and Active GLP-1 kits; MSD, Rockville, MD, USA). The inter-assay variation for total and active GLP-1 was 9.7% and 11.3% respectively.

C-peptide quantification was performed using a Roche E170 analyser (Roche Diagnostics, Mannheim, Germany). The assay utilised a direct electrochemiluminescence immunoassay with mouse monoclonal antibodies which were coupled to the paramagnetic particles. Venous blood samples were taken into ice chilled GEL Serum tubes (Sarstedt, S-Monovette, Nümbrecht, Germany). Samples were immediately centrifuged at 3600 rpm for 10 minutes at 4°C, aliquoted and stored at -80°C.

217 Sample size and randomisation

218 The ADC was our primary outcome. No study to date has assessed the effect of dietary nitrate
219 supplementation on liver diffusion. Therefore, no data was available to power our outcome.
220 For 90% power with an α-level set at $P = 0.05$ (two tailed), to detect a 1 SD difference, 13
221 volunteers were required to compare within group for placebo and active conditions. For 80%
222 power with an α-level set at $P = 0.05$ (two tailed), to detect a 1.05 SD difference, 16 volunteers
223 were required to compare between groups for placebo and active conditions.

224 Data and statistical analysis

225 All data were tested for normality. Where data were not normally distributed a non-parametric
226 test was performed. Data are presented as means ± standard deviation (*SD*). Statistical analyses
227 were performed on SPSS software version 21.0 (Chicago, IL, USA). Statistical difference was
228 accepted when $P < 0.05$. Statistical differences were assessed using repeated measures
229 ANOVAs. Where baseline differences were present, ANCOVAs were used with baseline as a
230 covariate. For age comparisons, group was used as a covariate. Where statistic differences
231 were present post hoc (Bonferroni corrected) analysis were performed.

232 *Results*

233 37 individuals (17 healthy young individuals and 20 healthy older adults) gave written
234 informed consent to participate. Post screening and consent 6 individuals were withdrawn from
235 the trial. 1 individual had abnormal liver function, 1 had a metal pin (in an area which would
236 interfere with data collection) and 4 had previously undiagnosed claustrophobia. 31 individuals
237 (16 healthy young individuals and 15 healthy older adults) were randomised to start in either
238 the nitrate rich beetroot arm or the placebo arm. No differences between dietary intake or
239 exercise patterns were recorded prior to both study visits. The beetroot juice was well tolerated
240 and no adverse events were reported.

Plasma nitrate *concentration*:

Supplementation with inorganic dietary nitrate caused an increase in plasma nitrate concentration compared to placebo see figure 1. The elevated plasma nitrate concentration was maintained for the entire testing period. Post hoc analysis revealed no significant differences at baseline (prior to any supplementation on the placebo and the nitrate rich juice arm of the study) for plasma nitrate concentration (young adults: mean difference; 2 ± 4.4 μM, $P = 0.64$, 95% CI -7.4, 11.5; older adults: mean difference; 1.5 ± 4.1 μM, $P = 0.72$, 95% CI -10.4, 7.4). Post hoc analysis revealed a significant increase when beetroot juice was compared with placebo at 1 hour post supplementation (young adult: mean difference; 543 ± 37 μM, $P < 0.001$, 95% CI 463, 624; older adult: mean difference; 505 ± 39 μM, $P < 0.001$, 95% CI 420, 590), 2 hours post (young adult: mean difference; 645 ± 29 μM, $P < 0.001$, 95% CI 581, 707; older adult: mean difference; 632 ± 35 μM, $P < 0.001$, 95% CI 556, 710) and 3 hours post (young adult: mean difference; 598 ± 31 μM, $P < 0.001$, 95% CI 530, 665; older adult: mean difference; 616 ± 26 μM, $P < 0.001$, 95% CI 559, 673). No statistical difference was present for the young adult group compared to the older adult group for nitrate concentration ($F_{(1, 25)} = .1$, $P = 0.75$).

Plasma nitrite *concentration*:

Supplementation with inorganic dietary nitrate caused an increase in plasma nitrite concentration compared to placebo see figure 1. This increase was faster in older adults, compared to young adults ($F_{(3, 75)} = 2.93$, $P = 0.039$). The elevated plasma nitrite concentration was maintained for the entire testing period. Post hoc analysis revealed no significant differences at baseline (prior to any supplementation on the placebo and the nitrate rich juice arm of the study) for plasma nitrite concentration (young adults: mean difference; -3.3 ± 74 nM, $P = 0.86$, 95% CI -43, 36; older adults: mean difference; 26.5 ± 78 nM, $P = 0.21$, 95% CI -17, 69). Post hoc analysis revealed a significant increase when beetroot juice was compared

with placebo at 1 hour post supplementation (young adults: mean difference; 283 ± 201 nM, P < 0.001, 95% CI 176, 391: older adults: mean difference; 471 ± 381 nM, P < 0.001, 95% CI 260, 682), 2 hours post (young adults: mean difference; 497 ± 259 nM, P < 0.001, 95% CI 353, 640: older adults: mean difference; 545 ± 325 nM, P < 0.001, 95% CI 364, 325) and 3 hours post (young adults: mean difference; 559 ± 201 nM, P < 0.001, 95% CI 442, 675: older adults: mean difference; 797 ± 525 nM, P < 0.001, 95% CI 493, 1100). There was also a significant increase at peak plasma nitrite concentration (hour 3), compared with hour 2 (mean difference; 201 ± 344 nM, P = 0.039, 95% CI 11, 392).

ADC:

There was no effect of supplementation (absolute; young adults: $F_{(1, 15)}$ = .314, P = 0.58; older adults; $F_{(1, 14)}$ = 1.65, P = 0.22; change from baseline; young adults: $F_{(1, 15)}$ = .701, P = 0.42; older adults; $F_{(1, 14)}$ = 2.91, P = 0.11) or an interaction effect (time by supplement) when comparing supplementation with inorganic dietary nitrate on hepatic diffusion compared to placebo (absolute; young adults: $F_{(3, 45)}$ = 0.25, P = 0.74; older adults; $F_{(3, 42)}$ = 1.3, P = 0.28; change from baseline; $F_{(2, 30)}$ = 0.13, P = 0.67; older adults; $F_{(2, 28)}$ = 0.45, P = 0.64). See figure 2 for details.

Portal Vein Flux:

There was a baseline difference for the older adults cohort (older adults: placebo; 14.6 ± 4.3 vs. beetroot juice; 11.7 ± 2.9 ml/s, P = 0.04, 95% CI -5.67, -0.13) but not in the young adults. See figure 3. Therefore, data were analysed using ANCOVA for the older adult cohort. There was no supplementation (absolute; young adults: $F_{(1, 15)}$ = 1.00, P = 0.33; older adults; $F_{(1, 12)}$ = 1.28, P = 0.28; or interaction effect (young adults: $F_{(3, 45)}$ = 0.34, P = 0.79; older adults: $F_{(2, 24)}$ = 0.68, P = 0.52) when comparing supplementation with inorganic dietary nitrate on portal vein flux compared to placebo.

289 *Portal Vein Velocity*:

290 There was a baseline difference for the older adults cohort (older adults: placebo; 13 ± 3.4 vs.
291 beetroot; 11.1 ± 3 cm/s, $P = 0.04$, 95% CI -3.7, -1.1) but not in the young adults. See figure 3.
292 Therefore, data were analysed using ANCOVA for the older adult cohort. Portal vein velocity
293 was decreased following beetroot juice, compared to placebo juice for the young adults: ($F_{(1,}$
294 $_{15)} = 2.9$, $P = 0.04$; however, no effect was seen in the older adults ($F_{(2, 24)} = 0.84$, $P = 0.44$).

295 *Plasma glucose concentration*:

296 There was a baseline difference for the older adults cohort (older adults: placebo; 5.3 ± 0.4 vs.
297 beetroot; 5.1 ± 0.4 cm/s, $P = 0.02$, 95% CI -3.6, -0.04) but not in the young adults. See figure
298 4. Therefore, data were analysed using ANCOVA for the older adult cohort. No effect of
299 supplementation (young adults: $F_{(1, 15)} = 0.96$, $P = 0.35$; older adults; $F_{(1, 12)} = 1.4$, $P = 0.25$) or
300 an interaction effect was present when comparing supplementation with inorganic dietary
301 nitrate on plasma glucose concentrations compared to placebo (young adults: $F_{(3, 45)} = 0.96$, P
302 $= 0.42$; older adults: $F_{(3, 42)} = 1.07$, $P = 0.36$). See figure 4.

303 *Effects on incretins and C-Peptide*:

304 There was no effect of supplementation (young adults: $F_{(1, 15)} = 0.48$, $P = 0.49$; older adults;
305 $F_{(1, 13)} = 0.26$, $P = 0.62$) or an interaction effect when comparing supplementation with
306 inorganic dietary nitrate on total GLP-1concentrations compared to placebo (young adults: $F_{(2,}$
307 $_{30)} = 0.81$, $P = 0.45$; older adults: $F_{(2, 26)} = 1.63$, $P = 0.22$), active GLP-1 concentrations (young
308 adults: $F_{(2, 30)} = 0.85$, $P = 0.43$; older adults: $F_{(2, 24)} = 0.67$, $P = 0.09$) or C-peptide (young adults:
309 $F_{(3, 45)} = 0.79$, $P = 0.50$; older adults: $F_{(3, 42)} = 0.39$, $P = 0.76$). See figure 5.

310 *Effects on resting blood pressure:*

There was no effect of supplementation (young adults: $F_{(1, 15)} = 1.2$, $P = 0.28$; older adults; $F_{(1, 14)} = 1.7$, $P = 0.20$) or an interaction effect when comparing supplementation with inorganic dietary nitrate on systolic blood pressure compared to placebo (young adults: $F_{(3, 45)} = 0.20$, $P = 0.89$; older adults: $F_{(3, 42)} = 1.7$, $P = 0.18$) or diastolic blood pressure for supplementation (young adults: $F_{(1, 15)} = 2.6$, $P = 0.13$; older adults: $F_{(1, 14)} = 4.0$, $P = 0.06$) or an interaction (young adults: $F_{(3, 45)} = 0.25$, $P = 0.86$; older adults: $F_{(3, 42)} = 0.45$, $P = 0.72$). See figure 6.

Discussion

This is the first study to investigate the effects of dietary nitrate supplementation on hepatic blood flow, incretin and C-peptide concentrations in young and older adults. The primary outcomes were to assess changes in microvascular diffusion (ADC), portal vein flux and velocity. Nitrate supplementation increased plasma nitrate and nitrite in both the young and older individuals but did not alter portal vein flux, or affect ADC. There was however, an interaction effect in the young adults, however, no effect was present in the older adults between visits for portal vein velocity. Secondary outcomes were to assess plasma glucose, incretin, C-peptide concentrations and blood pressure changes. Nitrate supplementation did not alter plasma glucose, incretin or C-peptide concentration and the response was not different in young compared to older individuals. Nitrate supplementation did not lower systolic or diastolic blood pressure in young or older individuals.

Nitrate supplementation and plasma nitrate *and plasma nitrite concentration.*

Plasma nitrate concentration peaked (~2000%) at 2 hours post nitrate rich beetroot juice compared with placebo for both the young and older adult groups. Similarly, plasma nitrite concentration rose following nitrate rich beetroot juice compared to the placebo and peaked at 2 hours for the young group and was highest at 3 hours for the older adult group representing a ~230 and 460% increase, respectively. The pharmacokinetic response for both plasma nitrate

and nitrite concentrations of both cohorts are similar to a previously reported dose response study in young healthy individuals (66). However, the pharmacokinetic response of plasma nitrate and nitrite concentrations have not previously been reported in a group of healthy older adults. Kelly et al. (35) recently reported a rise in plasma nitrite concentration of a similar magnitude of 418% in a group of healthy older adults.

Nitrate supplementation and hepatic blood flow

Despite a statistically significant and physiologically meaningful rise in plasma nitrite concentration, this did not lead to an increase in microvascular diffusion (ADC) or portal vein flux. There was however, an interaction effect for the young adults for a reduction in portal vein velocity. The portal vein supplies 75% of inflow, with the remainder supplied by the hepatic artery (56) and though the blood it supplies is partially deoxygenated, the portal vein supplies ~50% of the liver's O_2 delivery (See (29, 62) for reviews). A higher concentration of deoxyhaemoglobin may lead to a faster conversion of nitrite to nitric oxide (16) in the portal vein. It is therefore likely that the highest concentrations of nitric oxide and plasma glucose concentrations would be found in the liver. There was a statistical difference in the older adult cohort for baseline portal vein flux and velocity on the two visits. This study was powered to detect a 1 SD change in our primary outcome, ADC, and as portal vein flux and velocity had poorer reproducibility this may have contributed to the apparent baseline differences. The mean interaction change for portal vein velocity is -0.81 ± 3, in a similarly designed study we would need to recruit 291 individuals to see this effect.

Nitrate supplementation and plasma glucose C -peptide and Incretin concentrations

There was no significant difference between active or placebo juice for plasma glucose concentration. It should be noted that in the older adult cohort there was a statistically significant baseline difference in plasma glucose concentration (mean difference = -0.2 mmol/l

or 3.8%) on the placebo and nitrate rich supplementation day. Given the order of the two arms was randomised this was unlikely to be due to carry over effect from the previous supplementation period. Nitric oxide has been shown to mediate glucose uptake from the intestines and mediate glucose uptake into skeletal muscle (27, 38, 51), Potential mechanisms for this include an elevated nitric oxide bioavailability which in turn may stimulate insulin secretion (54) and increase GLUT4 translocation (46). Increased levels of GLP-1 have been shown to slow gastric emptying and reduce levels of satiety which may have significant health benefits by controlling food intake (68). Increasing these incretin concentrations may also lower plasma glucose concentrations via their insulinotropic effects. This would be particularly important in individuals with T2DM as they have an impaired incretin response (53). Although there was no statistical difference for the active or total GLP-1 concentration there was a trend for an increase in plasma concentration of active GLP-1's in the older adult cohort in the active arm (mean difference 0.55±0.86 pg/ml representing a 32.5% increase; see figure 5, D). If this represents a real difference we would require 41 participants in order to detect this change. Further analysis is warranted in an older adult population. Despite this, no such trend was evident between the active or placebo juice for C-peptide concentrations. Beetroot juice has been shown to lower the postprandial insulin response in healthy adults; however, it is unclear whether this is due to nitric oxide, polyphenols or betalains (65). Results from the present study with a true placebo would suggest that nitric oxide is not the active ingredient and further exploration of antioxidants and polyphenols in this area are warranted.

Nitrate supplementation and resting blood pressure.

There was no significant difference in systolic or diastolic BP following nitrate rich beetroot juice supplementation (at peak plasma nitrite concentration) compared to placebo in the healthy young adult cohort. This is similar to some acute nitrate supplementation studies (15, 57, 64). However, most studies have shown hypotensive effects with acute supplementation regimens

(40, 41, 60, 61, 63). There was no significant reduction in systolic BP following nitrate rich beetroot juice compared to placebo in the healthy older adult cohort. We report a non-significant 5 mm Hg drop in systolic BP. Kelly et al. (35) report a statistically significant 5 mmHg drop in systolic BP, whilst another groups reported larger reductions (30). Another study in healthy older adults also reported no effect of nitrate supplementation on systolic BP (8). A detailed pharmacokinetic study in healthy older adults over a prolonged period may identify at what time peak plasma nitrite concentrations occur. If the pharmacokinetic response of plasma nitrite concentrations in the healthy older adult group continued to rise past 3 hours, a greater hypotensive effect than measured here may have occurred. However, a dose response study of young adults showed the elevation in plasma nitrite concentration described above was sufficient to cause a drop in systolic and diastolic blood pressure (66).

Strengths and limitations

This is the first study to examine the effect of nitrate supplementation on hepatic blood flow. This study has a robust experimental design as a double-blind, placebo controlled, crossover trial. A limitation to this study is that we did not measure plasma nitrite concentration beyond 3 hours supplementation. Future research should aim to elucidate if plasma nitrite concentration rises beyond 3 hours in healthy older adults. The study was powered to detect a change of 1 SD in the outcome measures, changes smaller than this may have occurred but not been noted as significant. Finally, variability in our baseline measurement from the MRI analysis were more than anticipated, however, analysis was performed on absolute and change from baseline, therefore, this should not be a significant impediment.

Conclusion

This was the first study to examine the hepatic blood flow response to nitrate supplementation. Despite physiologically meaningful elevation in plasma nitrite concentration following an

acute dose of 11.91 mmol of nitrate, there was no effect on hepatic blood flow, plasma glucose, incretin, C-peptide concentrations or systolic and diastolic blood pressure for young or older adults.

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Figure Legends

Figure 1. Plasma nitrate and nitrite concentration. Figures depict changes across time for beetroot and placebo juice. The open circles are placebo and the closed circles beetroot. A and B: young adult cohort and C and D shows the older adult cohort. The * represents a significant difference when beetroot juice is compared with placebo < 0.001. The # shows a significant difference in the beetroot condition when hour 2 is compared to hour 3.

Figure 2. Microvascular diffusion. Figure shows ADC changes across time for beetroot and placebo juice. A: young adult cohort and B: older adult cohort. The open circles are placebo and the closed circles beetroot.

Figure 3. Portal vein flux and Portal vein velocity. Figures depict changes across time for beetroot and placebo. A and B: young adult cohort and C and D shows the older adult cohort. The open circles are placebo and the closed circles beetroot. The * represents a statistically different baseline portal vein flux between conditions.

Figure 4. Plasma glucose and C-peptide concentration. Figures depict changes across time for beetroot and placebo juice. A and B: young adult cohort and C and D shows the older adult cohort. The open circles are placebo and the closed circles beetroot. The * represents a statistically different baseline glucose concentration between conditions.

Figure 5. Total GLP-1 and active GLP-1 concentration. Figures depict changes across time for beetroot and placebo juice. A and B: young adult cohort and C and D shows the older adult cohort. The open circles are placebo and the closed circles beetroot.

Figure 6. Systolic blood pressure and diastolic blood pressure. Figures depict changes across time for beetroot and placebo juice. A and B: young adult cohort and C and D shows the older adult cohort. The open circles are placebo and the closed circles beetroot.

Table legends.

Table 1. Participant characteristics included in the final analysis.