

# Higher dietary nitrate intake is associated with better muscle function in older women

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## Abstract

**Background** In younger individuals, dietary nitrate supplementation has been shown to improve short-term vascular and muscle function. The role of higher habitual nitrate intake as part of a typical diet on muscle function in ageing has not been investigated. A cross-sectional study of relationships between dietary nitrate and measures of muscle function in older community-dwelling Australian women ( $n = 1420$ ,  $\geq 70$  years) was undertaken.

**Methods** Participants completed a semi-quantitative food frequency questionnaire assessing dietary intake over the previous year. Total nitrate from vegetables and non-vegetable sources was calculated from a validated instrument that quantified the nitrate content of food recorded within the food frequency questionnaire. Handgrip strength and timed-up-and-go (TUG) were assessed, representing muscle strength and physical function, respectively. Cut-points for weak grip strength ( $< 22$  kg) and slow TUG ( $> 10.2$  s) were selected due to their association with adverse outcomes. Linear and logistic regressions were used to examine the relationship between total nitrate intake and muscle function measures.

**Results** Mean  $\pm$  standard deviation (SD) total nitrate intake was  $79.5 \pm 31.2$  mg/day, of which 84.5% came from vegetables. Across the unadjusted tertiles of nitrate intake ( $< 64.2$  mg/day;  $64.2$  to  $< 89.0$  mg/day;  $\geq 89.0$  mg/day), women in the highest tertile had a 4% stronger grip strength and a 5% faster TUG performance compared with the lowest tertile. In multivariable-adjusted models, each SD higher nitrate intake (31.2 mg/day) was associated with stronger grip strength (per kilogram,  $\beta$  0.31,  $P = 0.027$ ) and faster TUG (per second,  $\beta$   $-0.27$ ,  $P = 0.001$ ). The proportion of women with weak grip strength ( $< 22$  kg) or slow TUG ( $> 10.2$  s) was 61.0% and 36.9%, respectively. Each SD higher nitrate intake (31.2 mg/day) was associated with lower odds for weak grip strength (OR 0.84, 95% CI 0.74–0.95,  $P = 0.005$ ) and slow TUG (OR 0.86, 95% CI 0.76–0.98,  $P = 0.021$ ). Compared with women in the lowest tertile of nitrate intake, women in the highest nitrate intake tertile had lower odds for weak grip strength (OR 0.65, 95% CI 0.49–0.87,  $P_{\text{trend}} = 0.004$ ) and slow TUG (OR 0.72, 95% CI 0.53–0.97,  $P_{\text{trend}} = 0.044$ ).

**Conclusions** This investigation highlights potential benefits of nitrate-rich diets on muscle strength and physical function in a large cohort of older women. Considering poor muscle strength and physical function is associated with a range of adverse health outcomes such as falling, fractures, cardiovascular disease, and mortality, increasing dietary nitrate, especially through vegetable consumption may be an effective way to limit age-related declines in muscle function.

**Keywords** Muscle strength; Physical function; Nutrition; Vegetables; Geriatrics

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## Introduction

Green leafy vegetables and beetroot contain high levels of inorganic nitrate.<sup>1</sup> Approximately 80% of total dietary nitrate intake is derived from vegetables, with the remaining majority being derived from fruit and meat products.<sup>2</sup> There is increasing evidence to indicate that some health benefits of vegetable-rich diets may be explained in part by higher nitrate intake.<sup>3,4</sup>

Ingesting dietary nitrate can increase nitric oxide bioavailability through the nitrate–nitrite–nitric oxide pathway.<sup>4</sup> In older populations, acute and short-term nitrate supplementation can lower blood pressure,<sup>5</sup> reverse vascular dysfunction,<sup>6</sup> and increase brain perfusion of the frontal lobe (involved in executive functioning).<sup>7</sup> Short-term physical performance benefits through improved efficiency of mitochondrial respiration and blood flow to active muscle have also been reported in athletic populations.<sup>8</sup> However, most studies reporting positive effects have used high doses of nitrate supplements (~400–700 mg) such as beetroot juice and nitrate salts. These supplements typically provide nitrate at levels that exceed those present in a diet rich in high-nitrate vegetables.

A healthy diet that includes vegetables is the cornerstone of many public health messages and are often promoted due to the relationship with better overall health, including functional measures. A clear link between poor function and adverse health outcomes, including disability, institutionalization, and mortality exists.<sup>9</sup> Therefore, strategies capable of limiting age-associated declines in muscle strength and/or physical function could have substantial impact on population health. Specifically, animal models suggest that nitrate supplementation improves force production in fast-twitch muscles.<sup>10</sup> Short-term high-dose nitrate supplementation (~600–700 mg) has also been reported to enhance skeletal muscle contractile properties in healthy individuals<sup>11,12</sup> and those with heart failure.<sup>13</sup> Furthermore, positive effects on physical capacity may also be maintained for up to ~2 weeks if supplementation is continued.<sup>14</sup> However, the longer term (habitual) impact of higher nitrate intakes on muscle function as part of a normal diet in the general population are uncertain. Therefore, our aim was to examine the cross-sectional relationship between total dietary nitrate and measures of muscle strength and physical function in a large cohort of older Australian women.

## Materials and methods

### Study population

The population included participants from the Perth Longitudinal Study of Ageing in Women (PLSAW; <http://www.lsaw.com.au>). Women with an expected survival beyond 5 years

were originally recruited to a 5-year, double-blind, randomized controlled trial, the Calcium Intake Fracture Outcome Study (CAIFOS), whereby daily calcium supplementation was provided to prevent fracture.<sup>15</sup> Women ( $n = 1500$ ) were recruited from the Western Australian general population aged  $\geq 70$  years by mail using the electoral roll; 1485 women completed a food frequency questionnaire (FFQ) at baseline (1998). Participants ( $n = 17/1485$ , 1.1%) with implausible energy intakes ( $< 2100$  kJ or  $> 14\,700$  kJ) or undertaking vitamin D supplementation (due to its link with muscle strength,<sup>16</sup>  $n = 39/1485$ , 2.6%) were not included in the analysis. An additional nine women were excluded due to missing measures of muscle strength and/or physical function measures. As a result, the current study included 1420 women. All participants provided written informed consent. Ethics approval was granted by the Human Ethics Committee of the University of Western Australia.

### Baseline characteristic assessment

Physical activity and smoking history questionnaires were completed at baseline to assess values for potential confounding variables. Participants were asked about participation in sport, recreation, and/or regular physical activities undertaken in 3 months prior to their baseline visit. This has previously been described in a greater detail within this cohort.<sup>17,18</sup> Briefly, the level of activity, expressed in expended kilojoules per day, was calculated from the questionnaire using a validated method applying the type of activity, time engaged in the activity, and the participant's body weight.<sup>19</sup> Smoking status was coded as non-smoker or ex-smoker/current smoker if they had consumed more than one cigarette per day for more than 3 months at any time in their life. Weight was assessed using digital scales with participants wearing light clothes and no shoes. Height was assessed using a stadiometer, and the body mass index (BMI) was calculated in kilogram per square metre. Current medication use at baseline was used to assess prevalent diabetes mellitus. Medications were verified by participants' general practitioner where possible and were coded (T89001–T90009) using the International Classification of Primary Care-Plus method that allows aggregation of different terms for similar pathologic entities as defined by the ICD-10 coding system.<sup>20</sup> Prevalent atherosclerotic vascular disease (ASVD) was determined from primary discharge diagnoses from hospital records for 18 years prior to baseline assessment. Socio-economic status (SES) was calculated using the Socio-economic Indexes for Areas developed by the Australian Bureau of Statistics that ranked residential postcodes according to relative socio-economic advantage and disadvantage.<sup>21</sup> Participants were then coded into six groups from the top 10% most highly disadvantaged to the top 10% least disadvantaged.

## Dietary assessment

A semi-quantitative FFQ developed by the Cancer Council of Victoria was used to assess dietary intake.<sup>22–24</sup> This validated FFQ (see Supporting Information, *Data S1* for a link to the FFQ) measures the usual frequency of food intake during a period of 12 months and comprises a list of 74 food items with 10 frequency response options ranging from ‘never’ to ‘3 or more times per day’; it is complemented by another 27 food and alcoholic beverage items that ask various questions such as ‘how many different vegetables do you usually eat per day?’ The FFQ calculates portion size by using three photographs of scaled portions for four different commonly consumed food types. Nutrient intake calculations were analysed by Cancer Council Victoria through the NUTTAB95 food nutrient database and were supplemented by other data where necessary. The process of collection was identical for each individual, whereby a research assistant supervised the completion of the questionnaire. Food models, cups, spoons, and charts were provided for accuracy. This enabled the calculation of daily total energy, protein, alcohol, and vegetable intake. Total dietary nitrate (mg/day) consisted of both vegetable and non-vegetable derived nitrate sources. Vegetable-derived nitrate was estimated from a recently developed comprehensive nitrate database for vegetables.<sup>1</sup> The median nitrate value (mg/g) for each vegetable in the FFQ was obtained from the database and multiplied with gram per day vegetable consumption to determine nitrate intake. Total nitrate intake from vegetables per day was calculated by totalling the nitrate intake from individual vegetables. An estimate of nitrate concentration (mg/g) in each of the non-vegetable items listed in the FFQ was derived using estimates from three published sources.<sup>25–27</sup> The nitrate database used to estimate nitrate intake from vegetables has been validated in a study of men and women by estimating nitrate intake using 24 hr dietary recalls and FFQs comparing these values with urinary nitrate excretion.<sup>1</sup> Nitrate concentration of 67 of the 77 non-vegetable items were obtained from Inoue-Choi *et al.*<sup>27</sup> Five values were obtained from the Food Standards Australia New Zealand survey of nitrates and nitrites in food and beverages in Australia<sup>25</sup> and three values from Griesenbeck *et al.*<sup>26</sup> Where no value was available (two foods: vegemite and jam), a value of 0 mg/g was used. Finally, as drinking water in Perth (Western Australia) contains very low levels of nitrate (~0.6 mg in 2 L of water including coffee and tea), these values were not included in the estimation of total nitrate intake.<sup>2</sup>

## Markers of muscle function

Grip strength and timed-up-and-go (TUG) were assessed at baseline as a surrogate for muscle strength and physical function, respectively. Grip strength was used to quantify upper-

limb muscular strength by measuring the amount of force (kg) the forearm flexors can produce using a dynamometer (Jamar Hand Dynamometer, Lafayette Instrument Company, USA). One practice and three test trials were performed using the participants’ dominant hand. The peak value was recorded. TUG measures the time it takes an individual to rise from a chair (46 cm seat height), walk 3 m, and turn around to return to sit on the chair. Participants performed one practice trial before undertaking a recorded trial. TUG is a commonly adopted method to assess functional mobility among older adults in geriatric clinics to evaluate physical performance. Cut-points for weak grip strength (<22 kg)<sup>28</sup> and slow TUG (>10.2 s)<sup>29</sup> were selected due to their potential association with adverse health outcomes (e.g. weakness, risk of falling, and fractures). The inter-observer coefficient of variation error was 7% for hand grip strength and 6% for TUG in our laboratory, as assessed on a random sample of 30 subjects.

## Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows, version 24.0 (IBM Corp., Armonk, NY, USA) and R (version 3.4.2, R Foundation for Statistical Computing, Vienna, Austria). Statistical significance was set at a two-sided type one error rate of  $P < 0.05$  for all tests. Descriptive statistics of normally distributed continuous variables were expressed as mean  $\pm$  standard deviation (SD). Non-normally distributed continuous variables were expressed as median and interquartile range. Categorical variables were expressed as number and proportion (%). Baseline characteristics were tested for differences across tertiles of total nitrate intake using one-way analysis of variance (ANOVA) for normally distributed continuous variables, the Kruskal–Wallis test for non-normally distributed variables, and the  $\chi^2$  test for categorical variables. The primary outcome variables of interest were muscle strength (weak grip strength; <22 kg)<sup>28</sup> and physical function (slow TUG; >10.2 s).<sup>29</sup> The relationship between total nitrate, grip strength, and TUG was investigated using Spearman rank order correlation ( $\rho$ ). Generalized additive modelling was used to examine the dose–response relationship between nitrate intake and measures of muscle function such as grip strength and TUG, separately. Linear regression was used to examine the association between grip strength and TUG with total nitrate intake (mg/day). Odds ratio (OR) for weak grip strength (<22 kg), slow TUG (>10.2 s), and total nitrate was investigated using logistic regression. Total nitrate intake was entered both as a continuous and categorical variables (tertiles). We also tested for evidence of linear trends across tertiles of total nitrate intake using the median value for each category as continuous variables in separate logistic regression models. Model calibration was tested using the Hosmer–Lemeshow goodness of fit test for logistic regression based on deciles of predicted risk vs.

actual risk (all  $P > 0.05$  indicating models were considered well calibrated). Two models of adjustment were adopted: minimally adjusted model (age and BMI) and multivariable-adjusted model (age, BMI, prevalent ASVD, prevalent diabetes mellitus, SES, physical activity, smoking history, total energy, protein, and alcohol intake).

### Additional analysis

In addition to the fully adjusted models, we also considered the potential confounding from other dietary components. Correlation analysis between total nitrate intake and various nutrients that have been reported to affect muscle function in previous work was performed. Given the potential for multicollinearity between many of these confounders, we considered their impact by entering them into the multivariable-adjusted models separately, each as a continuous variable, for grip strength and TUG. These included total daily intakes for polyunsaturated fat,<sup>30</sup> folate,<sup>31</sup> magnesium,<sup>32</sup> beta-carotene,<sup>33</sup> and calcium.<sup>34</sup> As some women were taking medications containing nitrate, we performed sensitivity analysis where we included these individuals as a covariate in the multivariable-adjusted linear regression models examining the relationship between nitrate intake, grip strength, and TUG. Because of the potential influence that exercise has on muscle function, we examined if the covariate physical activity was an independent model parameter in the aforementioned multivariable-adjusted analysis between total nitrate intake, weak grip strength, and slow TUG. Subsequently, we categorized women with lower (LN) or higher (HN) nitrate intake based on median nitrate intake (76.8 mg/day) and created physical activity categories for women who were sedentary (SED) vs. those who were physically active (PA). This enabled us to create four different groups: LN + SED; LN + PA; HN + SED; and HN + PA. We ran  $\chi^2$  test comparing the proportion of women with weak grip strength (<22 kg) and slow TUG (>10.2 s) in the aforementioned categories, separately. Considering that higher vitamin D levels (25OHD) have also been linked to better muscle function, we wanted to determine if 25OHD status influenced the relationship between nitrate and muscle function measures. As such, baseline 25OHD levels (plasma 25OHD<sub>2</sub> and 25OHD<sub>3</sub>; see Supporting Information, *Data S2*, for detailed information) and the corresponding season (summer/autumn vs. winter/spring) that the blood sample was obtained were added to the multivariable-adjusted model and analysed using linear regression. Finally, the relationship between nitrate intake and functional measures was also examined separately using linear regression in four subsets of individuals (i) women without ASVD, (ii) women with prevalent ASVD, (iii) women without diabetes, and (iv) women with diabetes.

## Results

Baseline characteristics according to tertiles of total nitrate per day are presented in *Table 1*. Mean  $\pm$  SD daily total nitrate was 79.5  $\pm$  31.2 mg/day, of which 84.5% and 15.5% came from vegetable and non-vegetable sources, respectively. Mean  $\pm$  SD of nitrate from vegetables and non-vegetable sources (predominantly meat and fruit) was 67.2  $\pm$  29.3 and 12.4  $\pm$  4.7 mg/day, respectively. Percentage contribution of vegetable nitrate from individual vegetables according to tertiles of total nitrate intake is displayed in Supporting Information, *Figure S1*. Women with higher total nitrate intake had greater total energy (kJ/day), protein (g/day), and vegetable intake (g/day). Compared with women in the lowest tertile of nitrate intake, those in the highest tertile had a 4% higher grip strength (kg); for TUG, tertiles 2 and 3 were 4% and 5% faster, respectively (*Table 1*).

### Nitrate intake and muscle function

Total nitrate intake was weakly correlated with grip strength ( $\rho$  0.09,  $P = 0.001$ ) and TUG ( $\rho$  -0.08,  $P = 0.002$ ). A graphic representation of the dose-response relationship between nitrate intake, grip strength, and TUG analyzed in separate generalized additive multivariable-adjusted models are presented in *Figure 1*. The unadjusted models are presented in Supporting Information, *Figure S2*. *Figure 1* suggest a different dose-response relationship for nitrate intake on grip strength and TUG. Specifically, the influence of nitrate on grip strength is linear across the range reported. However, for TUG, the influence of nitrate did not improve for intakes above ~80 mg/day, as also identified in the tertile analysis (*Table 1*). Using a linear regression multivariable-adjusted model with the backwards function, the most parsimonious model for grip strength included: age ( $P < 0.001$ ), physical activity ( $P = 0.007$ ), total nitrate intake ( $P = 0.014$ ), and BMI ( $P = 0.045$ ); while for TUG, this included age ( $P < 0.001$ ), BMI ( $P < 0.001$ ), physical activity ( $P = 0.001$ ), total nitrate ( $P = 0.001$ ), prevalent ASVD ( $P = 0.007$ ) and alcohol ( $P = 0.037$ ). In the multivariable-adjusted model, per 31.2 mg/day (1 SD) higher total nitrate intake was associated with stronger grip strength (per kilogram,  $\beta$  0.31,  $P = 0.027$ ) and faster TUG (per second,  $\beta$  -0.27,  $P = 0.001$ ) (*Table 2*). Overall, the proportion of women presenting with weak grip strength (<22 kg) and slow TUG (>10.2 s) was 61.0% and 36.9%, respectively (*Table 2*). After adjustment for covariates, each 31.2 mg/day (1 SD) higher dietary nitrate was associated with a 16% and 14% reduction in the odds for weak grip strength (<22 kg) and slow TUG (>10.2 s), respectively (*Table 2*). Compared with women with the lowest nitrate intake (<64.2 mg/day), women with moderate (64.2  $\leq$  89.0 mg/day) and higher nitrate intake ( $\geq$ 89.0 mg/day) had lower odds (24% and 35%,  $P_{\text{trend}} = 0.004$ ) for weak grip strength (<22 kg)

**Table 1.** Baseline characteristics in all women by tertiles of total dietary nitrate intake<sup>a</sup>

	All participants	Total nitrate intake tertiles		
		<64.2 mg/day	64.2 to <89.0 mg/day	≥89.0 mg/day
Number	1420	473	474	473
<b>Demographics</b>				
Age, years	75.2 ± 2.7	75.2 ± 2.8	75.1 ± 2.7	75.2 ± 2.7
Calcium treatment group	711 (50.1)	224 (47.4)	247 (52.1)	240 (50.8)
BMI, <sup>b</sup> kg/m <sup>2</sup>	27.2 ± 4.8	27.1 ± 4.8	27.2 ± 4.6	27.3 ± 4.8
Previous prevalent atherosclerotic vascular disease	169 (11.9)	57 (12.1)	52 (11.0)	60 (12.7)
Smoked ever <sup>c</sup>	525 (37.2)	172 (36.7)	171 (36.1)	182 (38.8)
Prevalent diabetes mellitus	87 (6.1)	27 (5.7)	30 (6.3)	30 (6.3)
Plasma 25OHD, <sup>d</sup> nmol/L	67.1 ± 28.7	64.5 ± 28.2	68.0 ± 29.8	68.9 ± 27.9
<b>Socio-economic status<sup>e</sup></b>				
Top 10% most highly disadvantaged	63 (4.5)	23 (4.9)	17 (3.6)	23 (4.9)
Highly disadvantaged	169 (12.0)	58 (12.3)	60 (12.8)	51 (10.9)
Moderate to highly disadvantaged	228 (16.2)	69 (14.7)	84 (17.9)	75 (16.0)
Low to moderately disadvantaged	214 (15.4)	72 (15.3)	62 (13.2)	80 (17.1)
Low disadvantaged	296 (21.0)	91 (19.4)	106 (22.6)	99 (21.2)
Top 10% least disadvantaged	438 (31.1)	157 (33.4)	141 (30.0)	140 (29.9)
<b>Nutrition</b>				
Total energy intake, kJ/day	7104 ± 2081	<b>6273 ± 1762</b>	<b>7148 ± 1923</b>	<b>7892 ± 2210</b>
Protein intake, g/day	79.5 ± 26.6	<b>67.5 ± 21.7</b>	<b>80.0 ± 23.3</b>	<b>91.1 ± 28.8</b>
Alcohol intake, g/day	6.7 ± 9.8	6.6 ± 9.9	6.4 ± 9.4	7.2 ± 10.1
Vegetable intake, g/day	196.9 ± 79.3	<b>131.8 ± 46.6</b>	<b>196.3 ± 53.3</b>	<b>262.6 ± 72.8</b>
<b>Functional measures</b>				
Physical activity, <sup>f</sup> kJ/day	471 (123–856)	452 (0–850)	464 (154–850)	489 (203–876)
Grip strength, kg	20.5 ± 4.8	<b>20.0 ± 4.6</b>	<b>20.6 ± 4.7</b>	<b>20.8 ± 5.0</b>
Timed-up-and-go, s	9.4 (8.2–11.1)	<b>9.7 (8.3–11.3)</b>	<b>9.3 (8.1–10.9)</b>	<b>9.2 (8.0–11.0)</b>

25OHD, 25-hydroxyvitamin D level; BMI, body mass index.

<sup>a</sup>Data presented as mean ± SD, median (interquartile range), or number *n* and (%). Bolded numbers indicate *P* < 0.05. *P* values are a comparison between groups using analysis of variance, Kruskal–Wallis test, and  $\chi^2$  test where appropriate.

<sup>b</sup>*n* = 1418.

<sup>c</sup>*n* = 1412.

<sup>d</sup>*n* = 1315.

<sup>e</sup>*n* = 1408.

<sup>f</sup>*n* = 1418.

**Figure 1** Graphic presentation of the multivariable-adjusted dose–response relationship between total nitrate intake, (A) grip strength, and (B) timed-up-and-go obtained by generalized additive regression models. Dotted lines represent 95% confidence intervals. The reference value for grip strength and timed-up-and-go is the value associated with the mean nitrate intake for all women. The rug plot along the bottom of each graph depicts each observation.

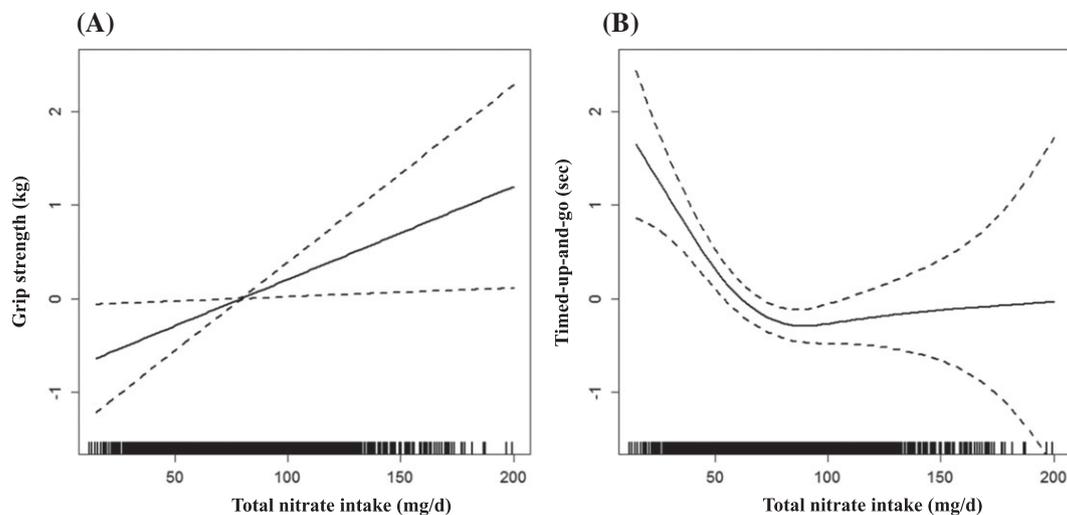


Table 2. Associations for functional measures grip strength and TUG with total nitrate intake

Functional measures	$\beta^a$ or OR <sup>b</sup> per SD, (31.2 mg/day) increase in nitrate intake	P value	Total nitrate intake tertiles			$P_{\text{trend}}^c$
			<64.2 mg/day	64.2 to <89.0 mg/day	≥89.0 mg/day	
Grip strength (kg)	0.35 ± 0.12	0.005	—	—	—	—
Multivariable adjusted	0.31 ± 0.14	0.027	—	—	—	—
Total number	1420	—	473	474	473	—
Weak grip strength (<22 kg)	866 (61.0)	—	317 (67.0)	284 (59.9)	265 (56.0)	—
Minimally adjusted	0.83 (0.74–0.92)	0.001	1.00	0.74 (0.56–0.96)	0.62 (0.48–0.81)	0.001
Multivariable adjusted	0.84 (0.74–0.95)	0.005	1.00	0.76 (0.57–1.00)	0.65 (0.49–0.87)	0.004
TUG (s)	–0.24 ± 0.08	0.002	—	—	—	—
Minimally adjusted	–0.27 ± 0.08	0.001	—	—	—	—
Multivariable adjusted	–0.27 ± 0.08	0.001	—	—	—	—
Total number	1420	—	473	474	473	—
Slow TUG (>10.2 s)	524 (36.9)	—	194 (41.0)	164 (34.6)	166 (35.1)	—
Minimally adjusted	0.88 (0.79–0.99)	0.030	1.00	0.73 (0.55–0.97)	0.73 (0.54–0.98)	0.056
Multivariable adjusted	0.86 (0.76–0.98)	0.021	1.00	0.73 (0.55–0.97)	0.72 (0.53–0.97)	0.044

Minimally adjusted model (age and BMI). Multivariable-adjusted model (age, BMI, prevalent atherosclerotic vascular disease, prevalent diabetes mellitus, socio-economic status, physical activity, smoking history, total energy intake, and protein intake). BMI, body mass index; CI, confidence interval; OR, odds ratio; TUG, timed-up-and-go.

<sup>a</sup>Unstandardized  $\beta$ -coefficient  $\pm$  standard error for total dietary nitrate intake in association with grip strength and TUG calculated using linear regression.

<sup>b</sup>Odds ratios (95% CI) for weak grip strength and slow TUG by total dietary nitrate intake (as a continuous or discrete variable) analysed using logistic regression.

<sup>c</sup>Test for trend conducted using median value for each tertile (tertile 1 = 50.0 mg/day; tertile 2 = 76.8 mg/day; tertile 3 = 108.4 mg/day).

(Table 2). For slow TUG, lower odds of 27% and 28% were also recorded for women with moderate (64.2 ≤ 89.0 mg/day) and higher nitrate intake (≥ 89.0 mg/day) compared with women with the lowest nitrate intake (< 64.2 mg/day) ( $P_{\text{trend}} = 0.044$ ), respectively (Table 2).

### Additional analysis

Correlational analysis between total nitrate intake and various nutrients previously linked with muscle function are presented in Supporting Information, Table S1. Dietary intake of these nutrients and foods according to tertiles of total nitrate intake are presented in Supporting Information, Table S2. In the separate linear regression analyses that additionally adjusted for individual dietary confounders, none of the nutrients changed the relationship between total nitrate intake and TUG. For grip strength, the inclusion of folate, magnesium, and beta-carotene slightly attenuated this relationship (Supporting Information, Table S3). Within our cohort, 4% (57/1420) of women were taking medication that contained some form of nitrate. When individuals taking nitrate supplements were included as an additional covariate in multivariable-regression models, the relationship between total nitrate intake, grip strength ( $\beta$  0.31,  $P = 0.039$ ), and TUG ( $\beta$  –0.27,  $P = 0.001$ ) was similar. The covariate physical activity was a significant model parameter in our multivariable-adjusted analysis that examined the relationship between nitrate intake and muscle function measures. Specifically, higher levels of physical activity (1 SD; 648 kJ/day) was associated with lower odds for weak grip strength (<22 kg; OR 0.85, 95% CI 0.76–0.95,  $P = 0.005$ ) and slow TUG (>10.2 s; OR 0.84, 95% CI 0.74–0.95,  $P = 0.007$ ). In a subsequent multivariable-adjusted analysis including an interaction term for total nitrate intake and physical activity, no relationship was observed for weak grip strength ( $P_{\text{interaction}} = 0.548$ ) and slow TUG ( $P_{\text{interaction}} = 0.775$ ). Compared with LN + SED, less women in HN + PA presented with weak grip strength (70.9% vs. 56.4%) and slow TUG (58.2% vs. 31.8%) (both  $P < 0.001$ ; Supporting Information, Figure S3). The proportion of women with weak grip strength (61.3% vs. 70.9%) and slow TUG (31.8% vs. 58.2%) were lower in LN + SED compared with HN + SED. Mean  $\pm$  SD 25OHD levels are displayed in Table 1. Including 25OHD to the multivariable-adjusted models did not influence the relationship between nitrate intake (per SD, 31.2 mg/day) and grip strength (per kilogram,  $\beta$  0.32,  $P = 0.027$ ) or TUG (per second,  $\beta$  –0.24,  $P = 0.005$ ). Similarly, excluding women with previous ASVD or prevalent diabetes did not influence the relationship between nitrate intake, grip strength, or TUG in the minimally adjusted models (Supporting Information, Table S4). In separate multivariable-adjusted models that included women with prevalent ASVD ( $n = 169$ ), higher dietary nitrate intake (per SD, 31.2 mg) was associated with faster TUG ( $\beta$  –0.55,

$P = 0.038$ ) but not stronger grip strength ( $\beta$  0.71,  $P = 0.092$ ) (Supporting Information, Table S4).

## Discussion

The main finding from this investigation was that higher dietary nitrate intake was associated with greater muscle strength and better physical function in older Australian women. Specifically, compared with women with lower nitrate intake, women with higher nitrate intake had reduced odds for weak grip strength and slow TUG. Considering that ~85% of total nitrate was derived from vegetables, these results are consistent with proposed benefits of vegetable-rich diets on muscle strength and physical function.<sup>35</sup> Our data suggest that the combination of higher physical activity and nitrate intake is most beneficial for muscle function. However, it appears that the beneficial relationship between dietary nitrate and physical function (TUG) may be most evident in women who are sedentary. This may be linked to the improved endothelial function reported after acute consumption of nitrate-containing vegetables (e.g. spinach),<sup>36</sup> which otherwise may only be achieved through exercise. However, as these analyses were hypothesis-generating, our findings should be interpreted with caution until tested in large randomized controlled trials.

Diets rich in vegetables are often promoted by public health organizations. Dietary patterns such as the Dietary Approaches to Stop Hypertension and the Mediterranean diet, which include high amounts of vegetables, have been associated with a range of health benefits. Specifically, higher vegetable (and fruit) intakes have been linked to greater physical function in older populations.<sup>35</sup> Although vegetables contain a range of bioactive compounds (vitamins, minerals, carotenoids, and flavanols) linked with better health,<sup>3</sup> our work supports the potential of additional benefits of vegetable-derived nitrate on muscle function. In our study, nitrate derived from spinach accounted for 7.4% vs. 15.8% of all vegetable nitrate in women with the lowest and highest nitrate intake, respectively (Supporting Information, Figure S1). Therefore, consuming spinach and similar green leafy (e.g. arugula and lettuce) vegetables present a simple way to increase daily nitrate intake.

In older populations, weak muscle strength is associated with a range of adverse outcomes such as falling, disability, and premature mortality.<sup>37,38</sup> Although muscle strength can be quantified in a number of ways (such as knee extensor strength), handgrip strength is often reported due to the ease of administration in large cohort studies, and because it is a low risk option for older populations. Similarities in the relationship between muscle strength quantified using the upper-limb and lower-limb with mortality have been reported in older American women ( $n = 1168$ , mean age ~73 years).

Specifically, lower quadriceps (per SD, 38 Nm) and grip strength (per SD, 10.7 kg) outcomes were both associated with greater relative hazards (65–67%) for mortality.<sup>38</sup> Therefore, preserving muscle strength in older populations is likely to have favourable consequences. In support of this concept, a small randomized controlled trial showed that acute dietary nitrate supplementation (beetroot juice containing ~700 mg of nitrate) increased peak knee extensor power (~10%) and velocity of knee extension (~12%) in heart failure patients ( $n = 9$ , mean age 57 years).<sup>13</sup> This finding is supported in the current study by faster TUG performance associated with higher nitrate intake for the subset of women with prevalent ASVD. When this experiment was repeated in healthy adults ( $n = 12$ , mean age 36 years), similar improvements were observed for knee extension power (6%) and velocity (11%).<sup>12</sup> Alternatively, in a small randomized controlled trial of older adults ( $n = 19$ ), large acute doses of beetroot concentrate supplemented over 7 days (~12 mmol/day of nitrate) only resulted in a trend for improved time-to-exhaustion ( $P = 0.10$ ) on an incremental cycle ergometer test.<sup>39</sup> No improvements in other functional tests (e.g. grip strength and TUG) were recorded. However, this is likely related to the small sample size with insufficient power to detect true effects for these tests. Intervention studies have yet to assess the long-term impact of lower nitrate intakes. In our study, higher habitual nitrate intake was associated with better muscle strength. Although the mechanisms are unclear, perhaps higher bioavailability of nitric oxide positively influences muscle contractile properties.<sup>40</sup> Such events may be associated with superior intracellular handling and/or cross-bridge sensitivity to calcium in fast-twitch muscle fibres.<sup>10</sup> This may lead to better force production, which can also contribute to superior physical function.

The TUG is commonly adopted to assess functional mobility among adults in geriatric clinics to evaluate physical performance. Compared with hand grip strength, TUG represents a more complex test incorporating manoeuvres typically used in daily life. Unsurprisingly, TUG correlates well with other physical measures such as balance, gait speed, and functional abilities.<sup>41</sup> Therefore, strategies (including nutrition) capable of improving any of the aforementioned physical qualities warrants investigation. Of interest, higher vegetable (and fruit) consumption has been linked with better functional health in activities of daily living (assessed via Short Form-36).<sup>35</sup> Considering that vegetables provided a large proportion of total nitrate in this cohort, the aforementioned result may be partly explained by the relationship between nitrate and muscle function. This relationship is supported by a range of potential mechanisms associated with nitrate supplementation. Mechanisms may include reduced oxygen costs due to better muscle and/or mitochondrial efficiency,<sup>8</sup> as well as improved endothelial function.<sup>36</sup> Specifically, as vascular disease has been associated with slower TUG,<sup>42</sup> better endothelial function within the

musculoskeletal system is likely to have favourable long-term effects on physical function.

Physical activity is an accepted determinant of muscle function. When comparing the magnitude of the relationship between dietary nitrate and muscle function, these were similar to physical activity. Considering one serve (equating to one cup) of raw spinach, arugula, or lettuce per day provides ~81, 196, and 85 mg of nitrate, respectively,<sup>1</sup> this study provides evidence that long-term, daily increases of this magnitude may translate to clinically meaningful improvements for grip strength (0.80–2.0 kg) and TUG (0.6–1.6 s). Comparable changes in grip strength and TUG have been associated with lower mortality risk in older populations. Specifically, a 33% increased risk of mortality was observed for every kilogram per year decline in grip strength, while a 1 s increase in TUG was associated with an 8% increase in mortality over 3 years.<sup>43,44</sup> Therefore, this association between vegetable-derived nitrate intake and muscle function could be a novel method to reduce the risk of adverse health outcomes. Future work should examine the effects of low-dose long-term (12 months) nitrate intake (though nitrate-rich vegetables or nitrate salts) on measures of muscle function and/or cardiorespiratory fitness in similar populations. Outcomes from such interventions could be especially useful in older people undergoing rehabilitation aimed at improving (or restoring) muscle function after an extended period of immobilization (e.g. bed rest) or general promotion of musculoskeletal health.

Strengths of the current study include the recruitment of a large cohort of community-dwelling women that were representative of the older Australian female population. Detailed information of potential confounders including plasma 25OHD, BMI, prevalent diabetes, ASVD history, SES, physical activity, as well as energy, protein, and alcohol intake were also considered. Dietary nitrate was calculated from a validated diet assessment tool in conjunction with comprehensive databases. Furthermore, hand grip strength and TUG, which are often used in geriatric clinics, were obtained using standardized assessments. However, several limitations need to be acknowledged.

Firstly, due to the cross-sectional nature of this investigation, causality cannot be established. This has the potential to increase the possibility of bias due to residual confounding. For example, higher nitrate intake may coincide with other diet and lifestyle patterns associated with better muscle function and general well-being (i.e. overall health interest and higher vegetable intake). Energy and protein intake were also not matched across nitrate intake tertiles, which has the potential to influence our results. To minimize this, total energy and protein intake were included in our multivariable-adjusted models as covariates. We have also performed sensitivity analysis that considered a range of other nutrients demonstrating the robust nature of the relationship between total dietary intake and muscle function. Secondly, the nitrate

content of food is influenced by cooking. As the FFQ does not detail method of food preparation, nitrate intake may have been overestimated. However, this will be consistent across tertiles of nitrate intake. Thirdly, there is no single gold standard biomarker of long-term nitrate exposure to validate nitrate intake calculated from our FFQ. Finally, current results might only be applicable to older women and may not be observed in men or younger populations.

In conclusion, this investigation highlights potential benefits of habitual nitrate-rich diets on muscle strength and physical function in a large cohort of older Australian women. Considering poor muscle strength and physical function are associated with a range of adverse health outcomes (e.g. falling, fractures, CVD, and mortality), increasing dietary nitrate especially although vegetables may be an effective way to limit any age-associated declines in muscle function.

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## Conflict of interest

The authors declare no conflicts of interest.

## Ethical standards

All participants provided written informed consent. Ethics approval was granted by the Human Ethics Committee of the University of Western Australia. CAIFOS and PLSAW studies were retrospectively registered on the Australian New Zealand Clinical Trials Registry (CAIFOS trial registration number #ACTRN12615000750583 and PLSAW trial registration number #ACTRN12617000640303) and complied with the Declaration of Helsinki. Human ethics approval for the use

of linked data was provided by the Human Research Ethics Committee of the Western Australian Department of Health (project number #2009/24).

## Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Data S1.** Cancer Council of Victoria Food Frequency Questionnaire

**Data S2.** Plasma 25OHD

**Table S1.** Correlation analysis between total nitrate intake and various nutrients associated with muscle function.

**Table S2:** Dietary intake of various nutrients associated with muscle function according to tertiles of total intake nitrate

**Table S3:** Multivariable-adjusted analyses for total nitrate intake with grip strength and timed-up-and-go that additionally adjusted for individual dietary confounders.

**Table S4:** Associations for functional measures including grip

strength and timed-up-and-go (TUG) with total nitrate intake for women (i) without atherosclerotic vascular disease (ii) women without diabetes at baseline and (iii) women with atherosclerotic vascular disease and/or diabetes.

**Figure S1:** Percentage contribution of the major contributors to vegetable nitrate presented by tertiles of total nitrate intake. Other vegetables consisted of tomatoes, capsicum, cucumber, cauliflower, peas, beans, bean sprouts, tofu, onion, garlic, mushrooms and zucchini.

**Figure S2.** Graphic presentation of the unadjusted dose-response relationship between total nitrate intake, (a) grip strength and (b) timed-up-and-go obtained by generalized additive regression models. Dotted lines represent 95% confidence intervals. The reference value for grip strength and timed-up-and-go is the value associated with the mean nitrate intake for all women. The rug plot along the bottom of each graph depicts each observation.

**Figure S3:** Proportion of women presenting with low grip strength (<22 kg) and slow timed-up-and-go based (>10.2 sec) based on nitrate intake and physical activity levels.

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