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Relationship of dietary nitrate intake from vegetables with cardiovascular disease mortality: a prospective study in a cohort of older Australians

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Abstract

Purpose: Short-term trials indicate inorganic nitrate and nitrate-rich vegetables may have vascular health benefits. However, few observational studies have explored the relationship between nitrate intake and long-term cardiovascular disease (CVD) outcomes. The primary aim of this study was to investigate the association of nitrate intake from vegetables with CVD mortality in a sample of older Australians.

Methods: A subgroup of participants without diabetes or major CVD at baseline (1992-1994) were included from the Blue Mountains Eye Study, a population-based cohort study of men and women aged ≥49 years. Diets were evaluated using a validated food frequency questionnaire at baseline, 5 years and 10 years of follow-up. Vegetable nitrate intake was estimated using a comprehensive vegetable nitrate database. Cox proportional hazards regression was used to explore the association between vegetable nitrate intake and CVD mortality.

Results: During 14 years of follow-up, 188/2229 (8.4%) participants died from CVD. In multivariable-adjusted analysis, participants in quartile 2 [69.5-99.6 mg/d; HR: 0.53 (95%CI: 0.35, 0.82)], quartile 3 [99.7-137.8 mg/d; HR: 0.51 (95%CI: 0.32, 0.80)], and quartile 4 [>137.8 mg/d; HR: 0.63 (95%CI: 0.41, 0.95)] of vegetable nitrate intake had lower hazards for CVD mortality compared to participants in quartile 1 (<69.5 mg/d).

Conclusions: In older Australian men and women, vegetable nitrate intake was inversely associated with CVD mortality, independent of lifestyle and cardiovascular risk factors. These findings confirm a recent report that intake of vegetable nitrate lowers the risk of CVD mortality in older women and extend these findings to older men.

Key words: cardiovascular disease, mortality, nitrate, vegetables, coronary heart disease, stroke

Introduction

Cardiovascular disease (CVD) is the leading cause of death in both developed and developing countries, accounting for nearly one third of all deaths worldwide [1]. The majority of CVD deaths are caused by stroke or coronary heart disease (CHD) [2,3].

Nitric oxide (NO) plays a key role in cardiovascular health. It is continuously produced at low levels in the endothelium, controlling basal vascular tone and integrity, with effects on blood flow, blood pressure, endothelial function and platelet function [4]. Decreased levels are associated with dysfunction to the vasculature with increased arterial stiffness and higher blood pressure observed after inhibition of NO synthesis [5]. Originally thought to be derived solely through the L-arginine-NO synthase pathway, the discovery of an alternate nitrate-nitrite-NO pathway raised the possibility that dietary nitrate could be an important source of NO [6,7]. Vegetables contribute 80-90% to the total nitrate consumed in the diet [7,8], with the remainder coming from other food sources such as cured meats [9]. The addition of nitrate and nitrite to cured meats is controversial due to the potential of nitrate and nitrite to form N-nitrosoamines, which are potential carcinogens [10]. Compounds such as polyphenols, vitamins C and E, and other antioxidants found abundantly in vegetables inhibit the formation of N-nitrosoamines [10]. The evidence of nitrate from specific foods and the risk of certain cancers remains unclear [11]. It is now well-established that after ingestion of nitrate there is an active uptake of plasma nitrate by the salivary glands. In the oral cavity nitrate is converted to nitrite by enzymatic action of bacteria on the dorsal surface of the tongue. After swallowing, this nitrite is absorbed becoming a source of NO [12]. Indeed, data from acute and short-term clinical trials indicate that dietary nitrate may lower blood pressure and improve vascular function via effects on NO [13-20]. The potential impact of these functional changes on long-term CVD risk remains uncertain [9,21].

Data from epidemiological studies have indicated a link between diets rich in vegetables [22-24], particularly green leafy vegetables [25,26], and lower risk of CVD. Few epidemiological studies have investigated the associations between nitrate intake from vegetables and CVD mortality. We have recently observed a significantly lower risk of atherosclerotic vascular disease mortality and ischemic cerebrovascular disease events with higher vegetable nitrate intake in a cohort of elderly Australian women [27,28]. The aim of this study was to confirm these findings in another Australian cohort that included both men and women. Nitrate intake from vegetables was our primary focus due to the large proportion of nitrate in the diet coming from vegetables alone [7,8].

Methods

Study population

Data used in this prospective cohort study were obtained from the Blue Mountains Eye Study (BMES). The BMES was the first population-based study of common eye diseases and other health outcomes in a representative older Australian population. The population was predominantly Caucasian [29], aged 49 years and older, better educated than Australia overall, and fairly stable in terms of follow-up [30,31]. Participants were initially examined in 1992-1994 for baseline characteristics and were followed up at 5-, 10- and 15-year time points. Mortality data were obtained via data linkage with the Australian National Death Index (NDI) [30,31]. Further study details of the BMES have been previously reported [32,33].

The BMES was approved by the Sydney West Area Health Service and the University of Sydney Human Research Ethics Committees. The study was conducted according to guidelines from the Declaration of Helsinki. Written informed consents were obtained from all participants.

Of the 3654 participants that had completed the main study, 3267 responded to a food frequency questionnaire (FFQ) and 2900 gave FFQ responses of sufficient quality for inclusion. Previous data cleaning processes have been reported elsewhere [32,34]. The eligibility criteria for the present study included available data on all exposure and outcome variables. The exclusion criteria included a report of extreme energy intake (< 500 and < 800 kcal/d or > 3500 and > 4000 kcal/d in females and males, respectively), and baseline self-reported history of major CVD (myocardial infarction (MI) and stroke) and/or diabetes. Of the 2900 participants, 163 (5.6%) were excluded for missing data on mortality (n=17), energy intake (n=3), and baseline history of major CVD and diabetes (n=143), 40 (1.4%) were excluded for reporting extreme energy intakes, and data cleaning excluded an additional participant. Participants who reported a baseline history of major CVD (n=264) or diabetes (n=157), or both (n=46) were further excluded (n=467, 16.1%). In total, 2229 out of 2900 (76.9%) participants with FFQ data were eligible for the present study (**Figure 1**).

Dietary assessment

A 145-item self-administered semi-quantitative FFQ, modified from a previous version by Willett et al. [35], was used to assess dietary intake. Participants were required to estimate their usual frequency of consumed food items during the past year. A 9-category frequency scale was used, ranging from never to four or more times per day.

Portion sizes were given in household measures (e.g. cup, teaspoon). Twenty-five vegetable items were included in the questionnaire. Reproducibility and validity of the FFQ were verified in a subgroup of the study population (n=79) using three, four-day weighed food records completed four months apart [32]. Nutrient data were analysed using the Australian Tables of Food Composition (NUTTAB90 and NUTTAB95) databases [36,37].

Vegetable nitrate intake

Vegetable nitrate intake was calculated from consumed vegetables, which were assessed using the FFQ, and quantified in grams per day. A recently published comprehensive database, which has nitrate data for 178 vegetables from over 250 publications, was applied to assess nitrate intake for each vegetable item [38]. Nitrate intake (mg/d) was calculated by multiplying the amount of each vegetable (g/d) by the median nitrate content (mg/g) for that individual vegetable. The nitrate values from each individual vegetable were then summated to obtain the total daily nitrate intake derived from vegetables.

Total nitrate intake

Nitrate intakes from other food items in the FFQ were also calculated. Nitrate levels from food items were obtained from Inoue-Choi et al [39], the Food Standards Australia New Zealand survey of nitrate and nitrite in food and beverages in Australia [40], and Griesenbeck et al [41]. The value of nitrate was given zero if the food item was not listed in any of the databases aforementioned and if the food item was not similar to any other food item listed with a nitrate value, which was vegemite only. Nitrate intake (mg/d) was calculated by multiplying the daily consumption of that food item (g/d) by the assigned mean nitrate value (mg/g) for that food item. Total nitrate intake (mg/d) was calculated by summating daily nitrate intakes from all other food items together with vegetable items.

Baseline demographic assessments

Demographic information, socio-economic status and lifestyle factors were obtained from the participants through a detailed questionnaire administered by trained interviewers [30,31]. Smoking status was determined using categories of “never smoked”, “past smoker” and “current smoker”, with classification of current smoker including those who stopped smoking within the past 12 months. Weight (kg) and height (m) were assessed using digital scales and a stadiometer, respectively. Body mass index (BMI, kg/m^2) was calculated using weight and height measurements. Energy and alcohol intake were estimated using the FFQ described above [30,31]. Physical

activity was categorised into three groups according to activity level (not active, light exercise, and vigorous exercise). Assessment of physical activity has been described elsewhere [42].

Baseline questionnaires were used to determine values for other potential confounding variables including use of organic nitrate medication, use of antihypertensive medication, use of cholesterol-lowering medication (statins), and use of low-dose aspirin. Fasting blood samples were used to determine total cholesterol levels and glucose concentrations. Renal function was evaluated using Modification of Diet in Renal Disease (MDRD) study equation for estimated glomerular filtration rate (eGFR) [43]. Systolic and diastolic blood pressures were measured using a mercury sphygmomanometer after participants were seated and rested for at least 5 minutes [30,31].

The participants provided their previous medical history and current medications, which were verified by their general practitioner. These data were coded using the International Classification of Primary Care-Plus method. This coding methodology allows aggregation of different terms for similar pathologic entities as defined by the International Classification of Disease (ICD-10) coding system. These data were used to determine the presence of pre-existing diabetes mellitus and major CVD (MI and stroke).

Cardiovascular disease mortality assessment

The primary outcome of this study was CVD mortality during 14 years of follow-up, the period available for linkage with the mortality data. CVD mortality data were retrieved by matching the causes of death by CHD or stroke to the NDI. Causes of death were determined by the use of diagnosis codes from the International Classification of Diseases Code, 9th revision (ICD-9) and the International Statistical Classification of Diseases, 10th revision (ICD-10). The following codes were used for CHD: (ICD-9: 410.0 to 410.9, 411.0 to 411.8, 412.0, 414.0 to 414.9 and ICD-10: I21.0 to I21.9, I22.0 to I22.9, I23.0 to I23.8, I24.0 to I24.9 and I25.0 to I25.9) and stroke: (ICD-9: 430.0 to 438.9 and ICD-10:I60.0 to I69.9). The Australian NDI has been validated and proven to be highly sensitive and specific for CVD mortality (92.5% and 89.6%, respectively) [44].

Statistical analysis

An analytic protocol was developed before formal analyses were performed. Data were analysed using IBM SPSS Statistics for Windows, version 21.0 (IBM) and SAS software, version 9.4 (SAS Institute Inc). Statistical significance was set at a two-sided Type 1 error rate of $P<0.05$ for all tests. Normally distributed continuous

variables were reported as mean ± standard deviation (SD), non-normally distributed continuous variables were expressed as medians and interquartile ranges (IQRs), and categorical variables were expressed as numbers and proportions (%). Differences in baseline characteristics were tested using one-way ANOVA for normally distributed continuous variables, the Kruskal-Wallis test for non-normally distributed variables, and Chi-squared test for categorical variables.

The primary outcome of interest was CVD mortality. Follow-up was calculated in years from each participant's baseline visit until death or 14-year follow-up. Cox proportional hazards (PH) regression was used to investigate the association between exposure variables (vegetable nitrate, total nitrate and non-vegetable nitrate) and CVD mortality. Further analyses exploring the relationship between exposure variables and CHD, stroke, non-CVD, and all-cause mortality were also analysed. We used the mean vegetable nitrate, total nitrate, and non-vegetable nitrate intake, calculated throughout the duration of follow-up (baseline, 5-year and 10-year). We tested for associations between outcomes and exposure variables by comparing a linear relationship with a categorical relationship using quartiles. Because most outcome-exposure relationships had a better fitting model using the exposure variable entered as a categorical variable, we used this approach in all models. We also categorised vegetable nitrate intake using previously reported cut-offs (<52.7 mg/d; 52.7-76.4 mg/d; and >76.4 mg/d) [27,28]. These cut-offs were used to compare the results of the present study with reported results from another Australian population [27,28]. Three models of adjustment were used including: (1) unadjusted; (2) age-, gender- and energy-adjusted; and (3) multivariable-adjusted models. The covariates included in the multivariable-adjusted model were age (years), gender (female or male), BMI (kg/m^2), physical activity (not active, light exercise or vigorous exercise), alcohol intake (grams per day), smoking history (never smoked, previous smoker or current smoker), socio-economic status (home/unit owner), use of organic nitrate medications (yes or no), use of antihypertensive medications (yes or no), use of statin medications (yes or no), use of low-dose aspirin (yes or no), renal function (MDRD eGFR, expressed as $ml \cdot min^{-1} \cdot 1.73m^{-2}$) and energy intake (kcal per day). Age, alcohol intake, renal function and energy intake were entered into models as continuous covariates, with the rest entered as categorical covariates. Cox PH assumptions were tested using log-log plots, which were shown to be parallel indicating that PH assumptions were not violated.

Sensitivity analyses

In order to rule out potential bias from reverse causality, we excluded all deaths that occurred in the first 24 months of follow-up, and repeated the multivariable-adjusted Cox PH models for quartiles of vegetable nitrate intake and

CVD, CHD and stroke mortality. Pearson product-moment correlation coefficient (r) was used to investigate the relationship between vegetable nitrate intake and total vegetable intake. A forward stepwise Cox PH model for CVD, CHD and stroke mortality including all adjustment variables plus quartiles of vegetable nitrate intake (entered as a categorical variable) and total vegetable intake (entered as a categorical variable) was used to assess multicollinearity between vegetable nitrate intake and total vegetable intake. Total vegetable intake was categorised into servings of vegetables (<2; 2 to <3; 3 to <4; 4 to <5; and ≥5). As vegetable nitrate intake may also be a marker for a healthier diet, we further adjusted for diet quality using a modified version of the Healthy Eating Index for Australians. This diet quality index called the Total Diet Score (TDS) has been described elsewhere [31]. The TDS was entered in the multivariable-adjusted model with quartiles of vegetable nitrate intake (entered as a categorical variable) for CVD, CHD and stroke mortality. In addition, greater fish consumption has been shown to be inversely associated with cardiovascular health in the BMES cohort [30]. Therefore, we further adjusted for fish intake in the multivariable-adjusted model. In addition to fish intake, we also individually adjusted for fibre and total flavonoid intakes. Estimation of total flavonoid intake has been described elsewhere [45]. Interaction terms were used to investigate whether there was any evidence of different relationships according to gender and participants aged < and ≥70 years.

Results

Characteristics of the study population

Baseline characteristics and dietary intakes for all participants and by quartiles of vegetable nitrate intakes are shown in **Table 1** and **Table 2**, respectively. Differences were observed between quartiles of vegetable nitrate intake for gender, age, physical activity, and smoking history (Table 1). The mean (SD) vegetable nitrate intake was 109.7 (59.2) mg/d, and total nitrate from all foods was 128.8 (62.0) mg/d. Vegetables contributed 85.2%, fruit 5.5%, meat (processed and unprocessed) 3.7%, grains 1.7%, fish 0.2%, and dairy 0.1% to the total nitrate intake. The highest contributing vegetables towards nitrate intake were lettuce (32.2%), spinach and silverbeet (13.2%), and potato (10.7%). Differences in all nutrients were observed between quartiles of vegetable nitrate, except for saturated fat (g/d) (Table 2).

Cardiovascular disease mortality

Over 14 years of follow-up, 188 of 2229 participants (8.4%) died because of CVD. The relationship between quartiles of vegetable nitrate intake and CVD mortality are presented in **Table 3**. Compared with quartile 1, quartiles 2, 3 and 4 were associated with a lower relative hazard of CVD mortality in the multivariable-adjusted model. Multivariable-adjusted cumulative survival curves for CVD by quartiles of vegetable nitrate intake are shown in **Figure 2**. Associations by quartiles of non-vegetable nitrate intake and total nitrate intake with CVD mortality are presented in **Supplemental Table 1** and **Supplemental Table 2**, respectively. Associations for intakes of total nitrate were similar to vegetable nitrate, but non-vegetable nitrate was not associated with CVD mortality.

Coronary heart disease, stroke, non-cardiovascular disease and all-cause mortality

Over 14 years of follow-up, 125 of 2229 participants (5.6%) died of CHD and 63 participants (2.8%) died of stroke. Furthermore, 422 of 2229 participants (18.9%) died of non-CVD causes and 610 of 2229 participants (27.4%) died of any cause. The relationship between quartiles of vegetable nitrate intake and CHD, stroke, non-CVD, and all-cause mortality are presented in Table 3. For CHD, quartile 2 (69.5-99.6 mg/d), compared to the referent, was associated with a lower relative hazard of mortality in the multivariable-adjusted model (P=0.008). Compared with quartile 1, quartile 3 (99.7-137.8 mg/d) was associated with a lower hazard of stroke mortality in the multivariable-adjusted model (P=0.002) (Table 3). Compared with quartile 1, quartile 4 (>137.8 mg/d) was

associated with a lower relative hazard of non-CVD (P=0.009) and all-cause (P=0.001) mortality in multivariable-adjusted models (Table 3). For non-vegetable nitrate intake and total nitrate intake, quartile results are presented in Supplemental Table 1 and Supplemental Table 2, respectively.

Sensitivity analyses

Using previously reported cut-offs in older women [27,28], compared with low intakes of vegetable nitrate (<52.7 mg/d), moderate (52.7-76.4 mg/d) and high (>76.4 mg/d) intakes were associated with 50 to 55% lower relative hazard of CVD mortality in multivariable-adjusted models (P=0.001) (**Table 4**). Multivariable-adjusted cumulative survival curves for CVD by categories of vegetable nitrate intake using previously reported cut-offs are shown in **Supplemental Figure 1**.

Similarly, compared with low intakes of vegetable nitrate (<52.7 mg/d), moderate (52.7-76.4 mg/d) and high (>76.4 mg/d) intakes were associated with 51 to 53% lower relative hazard of CHD mortality (P=0.017), and 44 to 61% lower relative hazard of stroke mortality (P=0.036), in multivariable-adjusted models (Table 4). For non-CVD and all-cause mortality, moderate (52.7-76.4 mg/d) and high (>76.4 mg/d) intakes were associated with 25 to 27% (P=0.111) and 34 to 37% (P=0.001) lower relative hazard, respectively, in comparison with low intakes of vegetable nitrate (<52.7 mg/d) in multivariable-adjusted models (Table 4).

To assess the likelihood of reverse causality bias (sick people eat less food/vegetables), we excluded all deaths that occurred in the first 24 months. The observed association between quartiles of vegetable nitrate intake and CVD, CHD, and stroke mortality remained significant in multivariable-adjusted models (P<0.05 for all).

A strong positive correlation between vegetable nitrate intake and vegetable intake was observed (r=0.79, P<0.001). In a forward-stepwise Cox PH model for the prediction of CVD mortality, which included all adjustment variables as well as total vegetable intake and vegetable nitrate intake, age (P<0.001), gender (P=0.002), smoking (P<0.001), use of organic nitrate medication (P=0.001), and vegetable nitrate intake (P=0.008) remained in the final parsimonious model. For CHD mortality, age (P<0.001), gender (P=0.003), smoking (P<0.001), and use of organic nitrate medication (P=0.004) remained in the final parsimonious model. For stroke mortality, age (P<0.001), BMI (P=0.023), use of antihypertensive medication (P=0.051), and vegetable nitrate intake (P=0.009) remained in the final parsimonious model.

After additional adjustment for the TDS, in multivariable-adjusted Cox PH models, results for quartiles of vegetable nitrate intake remained similar for CVD (P=0.006), CHD (P=0.069) and stroke (P=0.013) mortality (**Supplemental Table 3**). After additional adjustment for fish intake, in multivariable-adjusted Cox PH models, results for quartiles of vegetable nitrate intake were similar for CVD (P=0.011) and CHD (P=0.060) mortality, but for stroke mortality, the relationship became borderline significant (P=0.052) (Supplemental Table 3). After individual adjustments for fibre and total flavonoid intakes, in multivariable-adjusted Cox PH models, results for quartiles of vegetable nitrate intake were similar for CVD ($P<0.01$ for both) and stroke ($P<0.05$ for both) mortality. For CHD mortality, the relationship was similar after adjusting for total flavonoid intake (P=0.064), and became statistically significant after further adjusting for fibre intake (P=0.035). For CVD, CHD and stroke mortality, there was no evidence that the relationships were different according to gender ($P_{interaction}>0.05$ for all) or for ages < 70 and ≥ 70 years ($P_{interaction}>0.05$ for all). Quartiles of vegetable nitrate and CVD, CHD and stroke mortality stratified by gender are presented in **Supplemental Table 4**.

Discussion

This 14-year prospective study demonstrated an inverse association between vegetable nitrate intake and CVD mortality in an older Australian population without diabetes and/or major CVD at baseline. The association remained significant after adjustment for lifestyle and CVD risk factors as well as diet quality and fish, fibre and total flavonoid intakes. To our knowledge, this study is one of the first prospective cohort studies to investigate the association of dietary nitrate intake with CVD in an older general population that includes both men and women.

Since Larsen et al. [13] reported a blood pressure lowering effect of dietary nitrate in 2006, numerous small-scale clinical trials have been conducted to investigate dietary nitrate intake with blood pressure, endothelial function and other CVD-related outcomes. Most studies investigating the acute effects of nitrate ingestion have demonstrated positive findings [15,16,46]. However, studies investigating chronic effects have shown contradictory results [14,18,47-50]. Our previous studies have failed to demonstrate blood pressure lowering effects after 7-day consumption of nitrate-rich green leafy vegetables or beetroot juice in pre-hypertensive and treated hypertensive participants, respectively [48,49]. A meta-analysis [51] including 16 acute and short-term studies indicated consumption of inorganic nitrate or nitrate-rich beetroot juice was associated with a reduction in systolic blood pressure. Another meta-analysis [52] including 13 short- and medium-term studies showed positive effects of nitrate ingestion on measures of clinic blood pressure, but not on more accurate measures of ambulatory or home blood pressure. Effects on clinic blood pressure were removed when the authors excluded all studies investigating exercise performance from the analysis. In addition to these meta-analyses on blood pressure, a meta-analysis [53] including 12 acute, short-, and medium-term studies demonstrated beneficial effects of nitrate ingestion on endothelial function. These effects were attenuated in older individuals and individuals with increased CVD risk. Due to these inconsistencies, there is a need to investigate the effects of nitrate ingestion in older and at risk individuals. There is also a need to explore the relationship of habitual intake of dietary nitrate with long-term risk of cardiovascular-related outcomes.

To our knowledge, few studies have investigated the long-term associations of nitrate intake with cardiovascular-related outcomes. Bahadoran et al. [54] reported habitual intake of nitrite, rather than nitrate, was inversely associated with the incidence of hypertension over 5.8 years of follow-up. In an Australian population of older women aged ≥70 years, we have previously shown an inverse association between vegetable nitrate intake and atherosclerotic vascular disease mortality, which was defined as any death attributed to CHD, ischaemic

cerebrovascular disease, peripheral arterial disease and heart failure [27]. We have also shown an inverse relationship between vegetable nitrate intake and ischaemic cerebrovascular disease hospitalisations and deaths [28]. In both prospective studies, the lowest relative hazards were observed for vegetable nitrate intakes between 52.7-76.4 mg/d, equivalent to ~1-2 cups of leafy green vegetables per day. Epidemiological studies have also been conducted using biological samples, such as plasma nitrate [55] and urinary nitrate excretion [56]. In a cross-sectional study, Smallwood et al [56] demonstrated urinary nitrate excretion over 24 hours was inversely associated with blood pressure. In a 17-year prospective cohort study, Maas et al [55] found no evidence for an association between plasma nitrate and incident CVD, and demonstrated higher plasma nitrate was associated with an increased risk of death from all causes. This latter relationship is suggested to be in part attributable to higher plasma nitrate being correlated with lower renal function [55]. Using biological samples, such as plasma nitrate and urinary nitrate excretion as markers of nitrate exposure are somewhat controversial. For example, plasma nitrate is likely a representative of both protective and detrimental processes within the body [55]. Therefore, caution needs to be taken when interpreting the findings from these studies.

In the present study, we demonstrated an inverse relationship between vegetable nitrate intake and CVD mortality. Even though the lowest relative hazard for CVD was observed in participants consuming vegetable nitrate intake ranging from 99.7-137.8 mg/d, the greatest benefit could not be determined as there was overlapping of the confidence intervals of quartiles 2, 3 and 4. However, in agreement with our recent study in older women, the findings suggest that older individuals consuming even relatively low levels of vegetable nitrate (>52.7 mg/day, equivalent to ~1 cup of leafy green vegetables per day), have half the risk of 14-year CVD mortality even after adjusting for cardiovascular risk factors. In sub-group analyses, an inverse relationship between vegetable nitrate intake and stroke was also demonstrated, which aligns with our previous study findings [28]. The lowest relative hazard for stroke mortality was observed in individuals consuming vegetable nitrate intake ranging from 99.7 mg/d to 137.8 mg/d, equivalent to ~2-3 cups of leafy green vegetables per day. However, it is important to note that, as mentioned above, the confidence intervals overlap for each quartile and therefore the greatest benefit cannot be determined. It is also important to note that the apparent J-shaped relationship may be an artefact of the cut-points used i.e. quartiles. There is a possibility that by chance there were a lower number of stroke events than expected in the 3rd quartile. The J-shaped relationship in Table 3 disappeared in Table 4 when we collapsed vegetable nitrate intake into three groups based on previously reported cut-points. When collapsing into three groups there is less chance of an artificially small number of events in a single category.

297 As discussed above, clinical trials have demonstrated reductions in blood pressure with nitrate supplementation.
298 In addition, blood pressure is strongly linked with stroke outcomes [57]. This could indicate that moderate habitual
299 intakes of vegetable nitrate could be beneficial for stroke outcomes through blood pressure lowering effects.
300 However, this needs to be explored further. In our study, no difference in blood pressure between quartiles was
301 observed. A single clinic measure may not be sufficient in capturing accurate blood pressure. Home and
302 ambulatory measures of blood pressure are widely accepted to be more accurate [58,59].

303 The intake of vegetable nitrate is likely to vary between populations [60-62]. In the current study, which included
304 both men and women, the mean vegetable nitrate intake was 110 mg/d. In our previously reported study of older
305 women, the mean vegetable nitrate intake was 67 mg/d (difference equivalent to ~0.5-1 cup of leafy green
306 vegetables) [27,28]. Differences between these two cohorts may be due to different ages and/or demographic areas.
307 In the present study, the participants were somewhat younger (mean age 65 versus 75 years) and lived in a semi-
308 urban area, while participants in the previous study were from a metropolitan area. The use of different FFQs in
309 the two studies may also have influenced the estimated nitrate intake. In addition, geographic variability in the
310 nitrate content of specific foods are likely to occur in Australia as it does in the United States and could affect
311 nitrate intake [21].

312 There are several strengths to this study. Firstly, we used a validated dietary assessment tool for dietary evaluation.
313 A comprehensive newly-developed vegetable nitrate database was used to calculate nitrate intake [38]. Secondly,
314 we removed the likelihood of reverse-causality by excluding all deaths in the first 24 months of follow-up. This
315 resulted in a stronger relationship between vegetable nitrate intake and CVD mortality. Thirdly, the long-term
316 nature of the BMES captures an important period in the life course of these older individuals when cardiovascular
317 disease deaths become more common, increasing the power to detect an association between the exposure variable
318 and outcome of interest. Fourthly, using pre-specified cut points, we were able to replicate our previous findings
319 in older women [27,28]. Lastly, the participants of this study were representative of an older Australian population
320 [30,34].

321 Limitations also need to be considered. Due to the observational nature of the study, we are not able to infer
322 causality or rule out residual confounding or the introduction of bias. Vegetable nitrate intake was inversely
323 associated with non-CVD mortality even though the major proposed mechanisms appear specific to CVD. This
324 may support the hypothesis that residual confounding by other dietary or lifestyle factors that may be affecting
325 both CVD and non-CVD mortality. However, evidence suggests nitrate intake may be associated with other

health-related outcomes such as increased mitochondrial efficiency [63], improvements in features of metabolic syndrome [64], reductions in pro-inflammatory cytokines [65], and improvements in cognitive function and increased cerebral blood flow [66], all factors that could possibly lead to a reduction in death from other non-CVD causes. In addition, NO has beneficial health effects that go beyond vascular health, including neurotransmission and non-specific host defence [67-69]. The use of vegetable nitrate intake averaged over the period of follow-up could also be considered as a limitation. Compared to baseline vegetable nitrate intake, even though it is more likely to better represent participants' eating habits and minimize the random bias from a single FFQ response, it may also attenuate the strength of the relationship. However, we observed similar results when assessing the relationship using only baseline vegetable nitrate intake in multivariable-adjusted models (data not shown). In addition, vegetable nitrate intake may be a surrogate marker for vegetable consumption and/or diet quality. To address this matter, we conducted a forward-stepwise Cox PH model including both vegetable nitrate intake and total vegetable consumption. In the final parsimonious model, vegetable nitrate intake remained together with age, gender, smoking status, and use of organic nitrate medication. We then conducted further analyses separately adjusting for diet quality (i.e. TDS) and fish [30], fibre and total flavonoid intakes. After the additional separate adjustments for TDS and fish, fibre and total flavonoid intakes, the relationship between vegetable nitrate intake and CVD mortality remained statistically significant in multivariable-adjusted models. However, we still cannot rule out the possibility that vegetable nitrate may be a marker for healthy eating and other beneficial foods/nutrients. In particular, the major source of nitrate intake from vegetables is leafy green vegetables. Leafy green vegetables are inversely associated with cardiovascular disease outcomes [70] and indeed have other beneficial nutrients and phytochemicals that may contribute to these potential benefits [71-73].

To conclude, our study demonstrated an inverse relationship between vegetable nitrate intake and CVD and stroke mortality, independent of lifestyle and CVD risk factors in an older Australian population of men and women. We also demonstrated an inverse relationship with non-CVD and all-cause mortality. Further studies are needed in other populations to confirm these results and to evaluate the optimum intake of nitrate from vegetables for the possible prevention of cardiovascular disease.

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Table 1. Baseline characteristics of study participants by categories of vegetable nitrate intake[1]

	All participants n=2229	Quartile 1 (<69.5 mg/d) n=557	Quartile 2 (69.5-99.6 mg/d) n=558	Quartile 3 (99.7-137.8 mg/d) n=557	Quartile 4 (>137.8 mg/d) n=557	P-value[2]
Participants demographics						
Gender, male	915 (41.0)	268 (48.1)	244 (43.7)	217 (39.0)	186 (33.4)	<0.001
Age, year	64.5 ± 9.1	65.1 ± 9.3	64.4 ± 8.7	63.5 ± 9.0	65.1 ± 9.4	0.010
BMI[3], kg/m^2	26.0 ± 4.4	26.1 ± 4.5	26.0 ± 4.4	26.4 ± 4.3	25.7 ± 4.2	0.122
SBP[4], mmHg	145.4 ± 20.8	145.6 ± 20.7	145.8 ± 20.3	145.4 ± 20.4	144.9 ± 21.7	0.890
DBP[4], mmHg	83.4 ± 9.8	84.0 ± 10.3	83.3 ± 9.4	83.5 ± 9.5	83.0 ± 9.9	0.319
Physical activity[5]						<0.001
Not active	476 (21.4)	149 (27.5)	137 (25.1)	104 (18.8)	86 (15.7)	
Light exercise	973 (43.7)	247 (45.6)	231 (42.3)	255 (46.2)	240 (43.8)	
Vigorous exercise	739 (33.2)	146 (26.9)	178 (32.6)	193 (35.0)	222 (40.5)	
Alcohol intake, g/d	2.8 (0.2-14.4)	2.5 (0.2-17.0)	4.5 (0.3-16.8)	3.6 (0.4-15.7)	1.9 (0.2-11.5)	0.058
Smoking history[6]						0.035
Never smoked	1079 (48.4)	243 (45.3)	262 (48.0)	282 (51.2)	292 (53.3)	
Previous smoker	790 (35.4)	201 (37.5)	196 (35.9)	197 (35.8)	196 (35.8)	
Current smoker	312 (14.0)	92 (17.2)	88 (16.1)	72 (13.1)	60 (10.9)	
Socio-economic status[7]						
Home/unit owner	1982 (88.9)	481 (88.4)	504 (91.5)	504 (91.5)	493 (90.0)	0.261
Medication use						
Organic nitrate	26 (1.2)	7 (1.3)	6 (1.1)	7 (1.3)	6 (1.1)	0.984

Antihypertensive	632 (28.4)	166 (29.8)	170 (30.5)	154 (27.6)	142 (25.5)	0.243
Statins	66 (3.0)	18 (3.2)	20 (3.6)	15 (2.7)	13 (2.3)	0.615
Low-dose aspirin[8]						0.766
Less than once per month	1532 (68.7)	374 (73.8)	376 (75.5)	389 (75.8)	392 (77.2)	
More than once per month but less than once per week	124 (5.6)	38 (7.5)	27 (5.4)	32 (6.2)	27 (5.3)	
Once per week or more	371 (16.6)	95 (18.7)	95 (19.1)	92 (17.9)	89 (17.5)	
Biochemical analyses						
MDRD eGFR [9], mL/min per 1.73 m^2	79.1 ± 18.7	78.2 ± 22.2	78.8 ± 17.7	79.3 ± 17.1	80.2 ± 17.1	0.336
Total cholesterol[10], mmol/L	6.1 ± 1.1	6.0 ± 1.1	6.0 ± 1.1	6.1 ± 1.0	6.1 ± 1.1	0.583
Fasting glucose[9], mmol/L	4.9 ± 0.6	5.0 ± 0.6	5.0 ± 0.6	5.0 ± 0.6	4.9 ± 0.5	0.135

[1] Vegetable nitrate intake was calculated using the mean intake over the 14 years of follow-up. Values are means ± SDs, medians (IQRs), or n (%). SBP, systolic blood pressure; DBP, diastolic blood pressure; MDRD, Modification of Diet in Renal Disease study equation; eGFR, estimated glomerular filtration rate.

[2] P-values are a comparison between categories by ANOVA, Kruskal-Wallis test, or chi-square test where appropriate.

[3] Mearued in 2209 participants.

[4] Measured in 2225 parcipants.

[5] Measured in 2188 participants.

[6] Measured in 2181 participants.

[7] Measured in 2194 participants.

[8] Measured in 2026 participants.

[9] Measured in 2155 participants.

[10] Measured in 2156 participants.

Table 2. Baseline dietary intakes of all study participants by categories of vegetable nitrate intake[1]

	All participants n=2229	Quartile 1 (<69.5 mg/d) n=557	Quartile 2 (69.5-99.6 mg/d) n=558	Quartile 3 (99.7-137.8 mg/d) n=557	Quartile 4 (>137.8 mg/d) n=557	P-value[2]
Total vegetable nitrate, mg/d	109.7 ± 59.2	48.3 ± 15.2	85.0 ± 8.8	117.3 ± 10.9	188.7 ± 53.8	<0.001
Total nitrate, mg/d	128.9 ± 62.0	63.9 ± 17.4	103.5 ± 11.1	137.0 ± 12.5	211.1 ± 55.8	<0.001
Baseline dietary intakes						
Energy intake, kcal	2063.9 ± 599.1	1860.7 ± 600.3	2043.8 ± 561.9	2118.5 ± 576.0	2232.8 ± 597.1	<0.001
Total sugar, g/d	124.1 ± 49.8	109.1 ± 48.3	121.0 ± 46.5	126.2 ± 47.6	140.1 ± 51.7	<0.001
Fibre, g/d	27.3 ± 10.4	20.8 ± 8.9	25.5 ± 8.5	28.6 ± 8.7	34.3 ± 10.6	<0.001
Protein, g/d	87.1 ± 27.0	75.1 ± 25.8	86.0 ± 26.0	90.4 ± 23.9	96.9 ± 27.6	<0.001
Fat, g/d	75.1 ± 27.7	69.5 ± 28.1	74.9 ± 26.8	77.0 ± 26.2	79.0 ± 28.8	<0.001
Carbohydrate, g/d	232.7 ± 75.1	206.5 ± 73.3	226.9 ± 68.7	237.6 ± 71.9	259.7 ± 76.5	<0.001
Sodium, mg/d	2034.8 ± 712.4	1847.0 ± 712.1	2005.5 ± 674.1	2090.9 ± 646.9	2196.0 ± 755.6	<0.001
Potassium, mg/d	3721.1 ± 1161.9	2960.5 ± 971.1	3561.5 ± 926.9	3868.8 ± 973.7	4493.9 ± 1197.0	<0.001
Calcium, mg/d	880.8 ± 405.9	767.6 ± 403.7	851.0 ± 394.2	900.9 ± 386.5	1003.5 ± 403.6	<0.001
Phosphorous, mg/d	1513.2 ± 503.8	1304.3 ± 497.5	1478.2 ± 473.3	1569.5 ± 460.8	1700.8 ± 499.6	<0.001
Magnesium, mg/d	336.2 ± 106.1	280.5 ± 97.7	324.9 ± 92.9	349.1 ± 96.8	390.4 ± 105.9	<0.001
Zinc, mg/d	11.5 ± 3.7	9.9 ± 3.6	11.4 ± 3.7	12.0 ± 3.2	12.9 ± 3.8	<0.001
Vitamin C, mg/d	182.4 ± 94.2	131.9 ± 81.3	169.6 ± 78.7	193.3 ± 81.7	234.8 ± 102.3	<0.001
Cholesterol, mg/d	294.0 ± 136.9	268.8 ± 134.1	294.1 ± 128.0	303.3 ± 141.3	309.8 ± 140.7	<0.001
Saturated fat, g/d	29.5 ± 13.0	28.2 ± 13.5	29.7 ± 12.7	30.1 ± 12.7	29.9 ± 13.0	0.073
Monounsaturated fat, g/d	26.6 ± 10.1	24.6 ± 10.1	26.5 ± 9.7	27.3 ± 9.4	28.0 ± 10.8	<0.001

Polyunsaturated fat, g/d	12.5 ± 5.4	11.0 ± 5.2	12.3 ± 5.3	12.9 ± 5.0	13.9 ± 5.7	<0.001

[1] Total and vegetable nitrate intakes are the mean calculated using baseline, 5-year, and 10-year dietary data. Values are means ± SDs.

[2] P-values are a comparison between categories by ANOVA.

Table 3. Cardiovascular disease, coronary heart disease, stroke, non-cardiovascular disease and all-cause mortality for all participants and by quartiles of vegetable nitrate intake[1]

	Quartile 1 (<69.5 mg/d) n=557	Quartile 2 (69.5-99.6 mg/d) n=558	Quartile 3 (99.7-137.8 mg/d) n=557	Quartile 4 (>137.8 mg/d) n=557	P-value[2]
Cardiovascular disease mortality					
Death, n (%)	61 (11.0)	39 (7.0)	35 (6.3)	53 (9.5)	
Unadjusted	1.00 (Referent)	0.59 (0.40, 0.88)	0.53 (0.35, 0.80)	0.79 (0.55, 1.15)	0.008
Age, gender and energy adjusted	1.00 (Referent)	0.60 (0.40, 0.90)	0.56 (0.37, 0.86)	0.71 (0.48, 1.05)	0.023
Multivariable adjusted[3]	1.00 (Referent)	0.53 (0.35, 0.82)	0.51 (0.32, 0.80)	0.63 (0.41, 0.95)	0.006
Coronary heart disease mortality					
Death, n (%)	37 (6.6)	22 (3.9)	30 (5.4)	36 (6.5)	
Unadjusted	1.00 (Referent)	0.55 (0.33, 0.94)	0.75 (0.46, 1.21)	0.89 (0.57, 1.42)	0.143
Age, gender and energy adjusted	1.00 (Referent)	0.56 (0.33, 0.95)	0.78 (0.48, 1.28)	0.80 (0.49, 1.29)	0.201
Multivariable adjusted	1.00 (Referent)	0.46 (0.26, 0.82)	0.73 (0.43, 1.23)	0.68 (0.40, 1.15)	0.066
Stroke mortality					
Death, n (%)	24 (4.3)	17 (3.0)	5 (0.9)	17 (3.1)	
Unadjusted	1.00 (Referent)	0.65 (0.35, 1.21)	0.19 (0.07, 0.50)	0.64 (0.34, 1.19)	0.008
Age, gender and energy adjusted	1.00 (Referent)	0.67 (0.36, 1.26)	0.21 (0.08, 0.56)	0.58 (0.30, 1.12)	0.016
Multivariable adjusted	1.00 (Referent)	0.66 (0.34, 1.26)	0.18 (0.06, 0.52)	0.56 (0.28, 1.11)	0.014
Non-cardiovascular disease mortality					
Death, n (%)	134 (24.1)	105 (18.8)	104 (18.7)	79 (14.2)	
Unadjusted	1.00 (Referent)	0.73 (0.56, 0.94)	0.71 (0.55, 0.92)	0.54 (0.41, 0.72)	<0.001

Age, gender and energy adjusted	1.00 (Referent)	0.77 (0.59, 0.99)	0.81 (0.62, 1.05)	0.56 (0.42, 0.74)	0.001
Multivariable adjusted	1.00 (Referent)	0.89 (0.67, 1.17)	1.00 (0.75, 1.33)	0.65 (0.47, 0.90)	0.034
All-cause mortality					
Death, n (%)	195 (35.0)	144 (25.8)	139 (25.0)	132 (23.7)	
Unadjusted	1.00 (Referent)	0.69 (0.55, 0.85)	0.66 (0.53, 0.81)	0.62 (0.50, 0.78)	<0.001
Age, gender and energy adjusted	1.00 (Referent)	0.72 (0.58, 0.89)	0.73 (0.59, 0.91)	0.61 (0.49, 0.77)	<0.001
Multivariable adjusted	1.00 (Referent)	0.76 (0.60, 0.96)	0.82 (0.65, 1.04)	0.65 (0.51, 0.84)	0.009

[1] Results are presented as HRs (95% CIs).

[2] P-values are for a comparison between quartiles using the Wald test with Cox PH regression.

[3] Multivariable-adjusted models included age, gender, BMI, physical activity, alcohol intake, smoking history, socio-economic status, use of organic nitrate medications, use of antihypertensive medications, use of statin medications, low-dose aspirin use, renal function (MDRD eGFR) and energy intake.

Table 4. Cardiovascular disease, coronary heart disease, stroke, non-cardiovascular disease and all-cause mortality for all participants and by categories of vegetable nitrate intake[1]

	Low nitrate (< 52.7 mg/d) n=298	Moderate nitrate (52.7-76.4 mg/d) n=378	High nitrate (> 76.4 mg/d) n=1553	P-value[2]
Cardiovascular disease mortality				
Death, n (%)	37 (12.4)	29 (7.7)	122 (7.9)	
Unadjusted	1.00 (referent)	0.58 (0.36, 0.94)	0.57 (0.39, 0.82)	0.009
Age, gender and energy adjusted	1.00 (referent)	0.60 (0.37, 0.99)	0.57 (0.39, 0.84)	0.015
Multivariable adjusted[3]	1.00 (referent)	0.50 (0.30, 0.84)	0.45 (0.30, 0.69)	0.001
Coronary heart disease mortality				
Death, n (%)	24 (8.1)	16 (4.2)	85 (5.5)	
Unadjusted	1.00 (referent)	0.50 (0.26, 0.94)	0.62 (0.39, 0.97)	0.053
Age, gender and energy adjusted	1.00 (referent)	0.52 (0.27, 0.98)	0.61 (0.38, 0.97)	0.068
Multivariable adjusted	1.00 (referent)	0.47 (0.24, 0.90)	0.49 (0.29, 0.82)	0.017
Stroke mortality				
Death, n (%)	13 (4.4)	13 (3.4)	37 (2.4)	
Unadjusted	1.00 (referent)	0.73 (0.34, 1.58)	0.48 (0.26, 0.91)	0.061
Age, gender and energy adjusted	1.00 (referent)	0.76 (0.35, 1.65)	0.50 (0.26, 0.97)	0.098
Multivariable adjusted	1.00 (referent)	0.56 (0.24, 1.30)	0.39 (0.19, 0.81)	0.036
Non-cardiovascular disease mortality				
Death, n (%)	79 (26.5)	79 (20.8)	264 (17.0)	
Unadjusted	1.00 (referent)	0.75 (0.55, 1.02)	0.58 (0.45, 0.75)	<0.001

Age, gender and energy adjusted	1.00 (referent)	0.81 (0.59, 1.11)	0.65 (0.50, 0.84)	0.004
Multivariable adjusted	1.00 (referent)	0.75 (0.53, 1.08)	0.73 (0.55, 0.98)	0.111
All-cause mortality				
Death, n (%)	116 (38.9)	108 (28.6)	386 (24.9)	
Unadjusted	1.00 (referent)	0.69 (0.53, 0.90)	0.58 (0.47, 0.71)	<0.001
Age, gender and energy adjusted	1.00 (referent)	0.74 (0.57, 0.97)	0.62 (0.50, 0.77)	<0.001
Multivariable adjusted	1.00 (referent)	0.66 (0.50, 0.89)	0.63 (0.50, 0.80)	0.001

[1] Cut-off values for vegetable nitrate intake categories were determined from previously published data [27,28]. Results are presented as HRs (95% CIs).

[2] P-values are for a comparison between categories using the Wald test with Cox PH regression.

[3] Multivariable-adjusted models included age, gender, BMI, physical activity, alcohol intake, smoking history, socio-economic status, use of organic nitrate medications, use of antihypertensive medications, use of statin medications, low-dose aspirin use, renal function (MDRD eGFR) and energy intake.

Figure legends

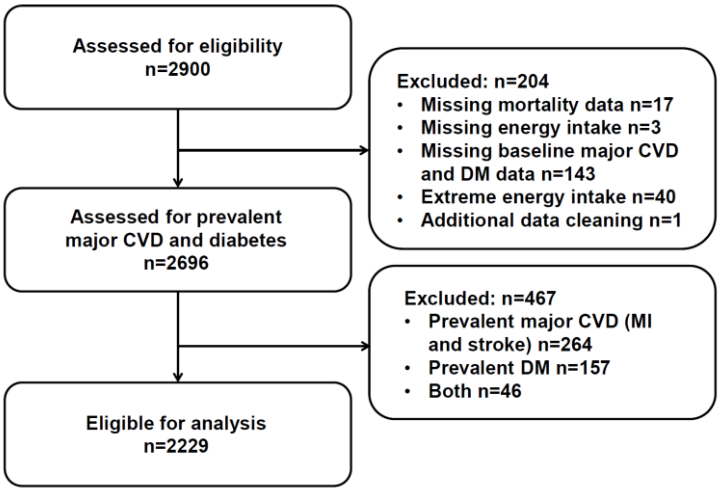

Figure 1. Participant flow chart. CVD, cardiovascular disease. DM, diabetes melletus. MI, myocardial infarction.

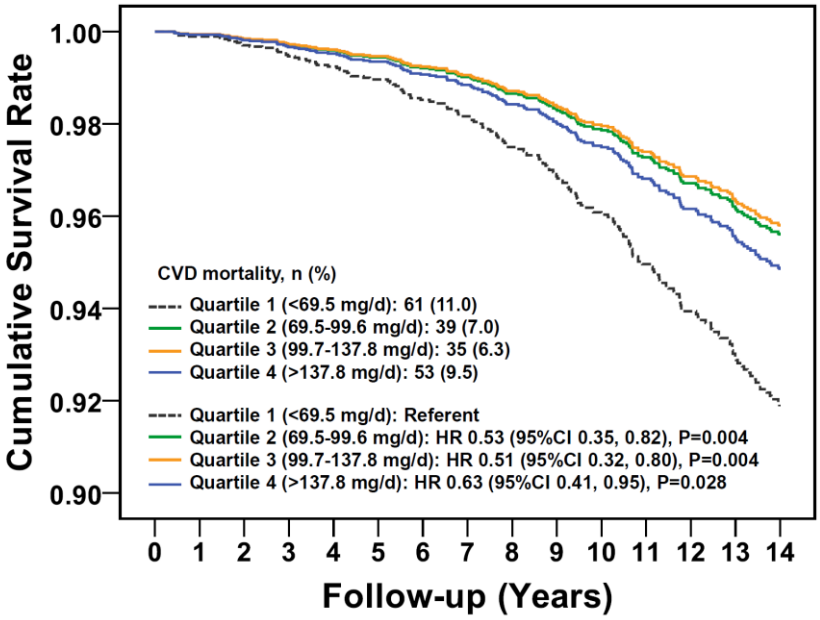

Figure 2. Multivariable-adjusted cumulative survival curves for cardiovascular disease mortality by quartiles of vegetable nitrate intake. Multivariable adjusted Cox PH model included age, gender, BMI, physical activity, alcohol intake, smoking history, socio-economic status, use of organic nitrate medications, use of antihypertensive medications, use of statin medications, low-dose aspirin use, renal function (MDRD eGFR) and energy intake. CVD, cardiovascular disease.