Association of Vegetable Nitrate Intake With Carotid Atherosclerosis and Ischemic Cerebrovascular Disease in Older Women

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Background and Purpose—A short-term increase in dietary nitrate (NO_3^-) improves markers of vascular health via formation of nitric oxide and other bioactive nitrogen oxides. Whether this translates into long-term vascular disease risk reduction has yet to be examined. We investigated the association of vegetable-derived nitrate intake with common carotid artery intima-media thickness (CCA-IMT), plaque severity, and ischemic cerebrovascular disease events in elderly women (n=1226).

Methods—Vegetable nitrate intake, lifestyle factors, and cardiovascular disease risk factors were determined at baseline (1998). CCA-IMT and plaque severity were measured using B-mode carotid ultrasound (2001). Complete ischemic cerebrovascular disease hospitalizations or deaths (events) over 14.5 years (15 032 person-years of follow-up) were obtained from the West Australian Data Linkage System.

Results—Higher vegetable nitrate intake was associated with a lower maximum CCA-IMT (B=−0.015, P=0.002) and lower mean CCA-IMT (B=−0.012, P=0.006). This relationship remained significant after adjustment for lifestyle and cardiovascular risk factors ($P \leq 0.01$). Vegetable nitrate intake was not a predictor of plaque severity. In total 186 (15%) women experienced an ischemic cerebrovascular disease event. For every 1 SD (29 mg/d) higher intake of vegetable nitrate, there was an associated 17% lower risk of 14.5-year ischemic cerebrovascular disease events in both unadjusted and fully adjusted models (P=0.02).

Conclusions—Independent of other risk factors, higher vegetable nitrate was associated with a lower CCA-IMT and a lower risk of an ischemic cerebrovascular disease event. **(*Stroke*. 2017;48:1724-1729. DOI: 10.1161/STROKEAHA.117.016844.)**

Key Words: atherosclerosis ■ cardiovascular disease ■ cerebrovascular disease ■ nitrates ■ vegetables

Our understanding of the health impact of dietary nitrate has recently undergone a radical shift. Originally, nitrate was linked with detrimental health outcomes such as cancer, a theory unsupported despite extensive epidemiological research spanning more than 50 years.[1] Currently, the potential vascular benefits of dietary nitrate is a major research focus. Dietary nitrate, found predominantly in green leafy vegetables and beetroot, dose-dependently enhances the circulating nitric oxide (NO) pool by increasing the levels of circulating nitrite, NO, and related nitroso compounds.[2–5] Dietary nitrate, through the enterosalivary nitrate–nitrite–NO pathway, is now recognized as an important alternate source of NO. NO was originally assumed to be solely produced through the oxygen-dependent L-arginine–NO synthase pathway. NO is a key regulator of vascular homeostasis and integrity, with decreased production and bioavailability of NO implicated in several cardiovascular disorders.[6,7] More than 30 clinical trials have now demonstrated, via effects on NO, a reduction in blood pressure or improvement in measures of vascular function with a short-term increase in nitrate intake.[8–10] However,

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epidemiological studies exploring the relationship between nitrate intake and vascular disease risk are scarce.

Common carotid artery intima-media thickness (CCA-IMT) is a surrogate measure of atherosclerosis[11] and is associated with increased vascular risk in general and cerebrovascular disease in particular, independent of conventional risk factors.[12] Changes in CCA-IMT are used to assess the success of intervention studies in preventing or reducing the progress of atherosclerotic disease. Although specific dietary factors have been associated with CCA-IMT,[13] the association of nitrate intake with CCA-IMT has not been studied. Furthermore, the association of nitrate intake with risk of an ischemic cerebrovascular event has yet to be explored.

Thus, the primary aim of this study was to investigate the association between intake of nitrate from vegetables and CCA-IMT, plaque severity and risk of an ischemic cerebrovascular disease event in a population-based study of older women rather than total nitrate intake. Our focus on vegetable-derived nitrate is because most dietary nitrate derives from vegetables.[14] In addition, processed meat, another source of nitrate in the diet, is strongly linked to detrimental health effects.[15] We hypothesized an inverse association between vegetable-derived nitrate intake and CCA-IMT, plaque severity and risk of an ischemic cerebrovascular disease.

Subjects and Methods

Ethics Statement

The Human Ethics Committee of the University of Western Australia approved the study, and written informed consents were obtained from all participants. The study complies with the Declaration of Helsinki.

Participants

In 1998, 1500 Western Australian women over the age of 70 years were recruited to a 5-year randomized, controlled trial of oral calcium supplements to prevent osteoporotic fractures (ACTRN12615000750583).[16] Of these, 99% (n=1485) had complete food frequency questionnaires (Figure I in the online-only Data Supplement). After excluding implausible energy intakes <2100 kJ (500 kcal) or >14 700 kJ (3500 kcal) per day, 1468 (98%) were included. Participants with preexisting atherosclerotic vascular disease and diabetes mellitus were excluded (n=242), leaving n=1226 (82%) for the ischemic cerebrovascular disease analysis. Of these participants, 65% (n=968) had plaque data and 64% (n=954) had CCA-IMT data (Figure I in the online-only Data Supplement). A study of the demographic variables of the recruited women revealed disease burden, and pharmaceutical utilization was similar to whole populations of this age group.[17]

Baseline Assessment

A baseline assessment was performed in 1998. This included weight, height, medical history, and lifestyle factors (online-only Data Supplement) and completion of a validated Food Frequency Questionnaire (FFQ).

Assessment of Nitrate Consumption

An estimate of nitrate concentration (mg/g) in each of the vegetable items listed in the Cancer Council of Victoria FFQ was obtained using a recently developed comprehensive nitrate content of vegetables database. This database, compiled using a systematic approach, contains 4254 records sourced from 256 references and includes data on 178 vegetables as well as 22 herbs and spices from 56 countries. The median nitrate value (mg/g) for each vegetable in the FFQ was obtained from the database and multiplied with g/d 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 nonvegetable items listed in the Cancer Council of Victoria FFQ was derived using estimates from 3 published sources[18–20] (online-only Data Supplement).

Assessment of CCA-IMT and Plaque Severity

Carotid high-resolution B-mode ultrasonography was used to assess common carotid intima-media thickness and focal carotid plaques at year 3 (2001) of the study (online-only Data Supplement).

Assessment of Ischemic Cerebrovascular Disease Event

The first episode of ischemic cerebrovascular hospitalizations or death was retrieved from the Western Australian Data Linkage System for each study participant from their baseline clinic visit date in 1998 until 14.5 years after their baseline visit (online-only Data Supplement).

Statistics

A protocol for the statistical analysis of the data was established before analysis began. Data were analyzed using IBM SPSS Statistics (version 21; IBM Corp, Armonk, NY) and SAS (version 9.4; SAS Institute Inc, Cary, NC). The relationship between nitrate intake (vegetable total and nonvegetable nitrate) and CCA-IMT (maximum and mean) was examined using unadjusted, age- and energy-adjusted and baseline risk factor–adjusted linear regression. The baseline risk factor adjusted model included baseline age, body mass index, energy intake, alcohol intake, energy expended in physical activity, antihypertensive medication, statin medication, low-dose aspirin medication, organic nitrate medication, history of smoking, and supplementation group. Results are presented as unstandardized B±SE. Vegetable nitrate consumption was then categorized into tertiles for further analysis by ANCOVA with Bonferroni adjustment for multiple comparisons. The relationship between tertiles of nitrate intake and CCA-IMT (maximum and mean) was examined using unadjusted, age- and energy-adjusted and baseline risk factor–adjusted models as before. The relationship between atherosclerotic plaque severity and nitrate intake was examined using binary logistic regression using unadjusted, age- and energy-adjusted and baseline risk factor–adjusted models as before. Cox proportional hazards models were used for ischemic cerebrovascular disease events in unadjusted, age- and energy-adjusted and baseline risk factor–adjusted models as before. We tested for evidence of a linear trend for vegetable nitrate intakes as continuous variables by using the median value for each tertile of vegetable nitrate intake in separate Cox proportional hazards models. Cox proportional hazards assumptions were tested using log–log plots, which were shown to be parallel. Thus, the proportional hazards assumptions were not violated. P values of <0.05 in 2-tailed testing were considered statistically significant.

In a sensitivity analysis, we assessed the potential impact of other possible dietary confounders on CCA-IMT and ischemic cerebrovascular disease events. Daily intakes of total fat (g/d), protein (g/d), carbohydrate (g/d), and fiber (g/d) were investigated in multivariable adjusted Cox proportional hazards models on a variable-by-variable basis. We further explored the relationship of CCA-IMT and plaque severity with ischemic cerebrovascular disease events using Cox regression in unadjusted, age- and energy-adjusted and baseline risk factor–adjusted models as described above. As this current cohort excluded older women with prevalent atherosclerotic vascular disease and diabetes mellitus, we repeated the analysis including older women with prevalent atherosclerotic vascular disease and diabetes mellitus.

Results

Baseline characteristics of the study participants are presented in Table 1. Classification of vegetables in the Cancer Council

of Victoria FFQ according to nitrate content is presented in Table I in the online-only Data Supplement. Total nitrate intake from all vegetables was 67±29 mg/d (range: 6–224 mg/d). Total nitrate intake from all foods was 79±31 mg/d (range: 12–231 mg/d). Vegetables accounted for 84% of total nitrate intake (Figure II in the online-only Data Supplement). After vegetables, fruit, and meat were the greatest contributors to total nitrate intake contributing 6% and 3%, respectively. Participants with higher nitrate intake also had higher intakes of energy, total fat, protein, carbohydrate, and fiber (Table 1).

Nitrate Intake, CCA-IMT, and Plaque Severity

Vegetable Nitrate Intake

Vegetable nitrate consumption was linearly inversely associated with maximum CCA-IMT and mean CCA-IMT (Table 2). A 29-mg (≈1 SD) higher vegetable nitrate intake was associated with a 0.015-mm lower maximum CCA-IMT and a 0.012-mm lower mean CCA-IMT. These effects remained significant after adjustment for age and energy intake as well as after adjustment for baseline risk factors (Table 2).

The effect of vegetable nitrate intake was explored further by dividing the participants into tertiles: <53, 53–76, and >76 mg/d vegetable nitrate consumption (Table 2). Participants who consumed >76 mg/d nitrate (top tertile) had a significantly lower CCA-IMT than participants in the bottom tertile with consumption <53 mg/d. This relationship remained significant after adjustment for age and energy intake for maximum CCA-IMT but was slightly attenuated for mean CCA-IMT. A similar relationship was observed after adjustment for baseline risk factors.

Vegetable nitrate intake (odds ratio, 0.994; 95% confidence interval, 0.806–1.227; P=1.0) was not a predictor of plaque severity.

Table 1. Baseline Lifestyle, Cardiovascular Risk, and Dietary Factors for the Total Cohort* and by Tertiles of Vegetable Nitrate Intake (mg/d)

Characteristic	Total Cohort	Tertiles of Vegetable Nitrate Intake			
		<53 mg/d	53–76 mg/d	>76 mg/d	*P* Value†
N, %	1226	409 (33)	408 (33)	409 (33)	
Vegetable nitrate, mg/d‡	67±29	37±11	64±7	100±21	<0.001
Total nitrate, mg/d‡	79±31	48±12	76±8	114±22	<0.001
Non-vegetable nitrate, mg/d‡	12±5	11±4	12±4	14±5	<0.001
Age, y‡	75±3	75±3	75±3	75±3	0.3
Body mass index, kg/m^2‡	27±5	27±5	27±4	27±5	0.8
Energy intake, kJ/d†	7146±2092	6479±1893	7167±1930	7793±2230	<0.001
Physical activity, kJ/d‡	460 (102 – 861)	403 (0 – 873)	483 (178 – 854)	478 (193 – 880)	0.1
Hypertension medication, n (%)	493 (40)	164 (40)	166 (34)	163 (40)	1.0
Statin medication, n (%)	184 (15)	57 (14)	67 (16)	60 (15)	0.6
Low-dose aspirin medication, n (%)	193 (16)	71 (17)	67 (16)	55 (13)	0.3
Organic nitrate medication, n (%)	19 (2)	6 (2)	6 (2)	7 (2)	0.9
Smoked ever, n (%)	441 (36)	142 (35)	148(37)	151 (37)	0.8
Total fat, g/d‡	65±23	60±22	64±22	69±26	<0.001
Saturated fat, g/d‡	26±11	25±11	26±10	27±12	0.05
Protein, g/d‡	79±26	69±23	80±24	90±29	<0.001
Carbohydrate, g/d‡	191±58	172±51	193±55	208±62	<0.001
Fiber, g/d‡	23±8	19±6	23±7	27±8	<0.001
Alcohol, g/d§	2.1 (0.3–10.4)	1.6 (0.3–9.9)	1.9 (0.3–9.8)	2.9 (0.3–11.4)	0.3
Calcium treatment, n (%)	641 (52)	205 (50)	222 (54)	214(53)	0.5
Max CCA-IMT, mm‡	0.922±0.152	0.941±0.170	0.921±0.141	0.905±0.143	0.01
Mean CCA-IMT, mm‡	0.778±0.129	0.793±0.145	0.777±0.118	0.765±0.122	0.02
Plaque severity: moderate to high (≥25%),‖ n (%)	120 (12)	39 (13)	38 (12)	43 (13)	0.9
Ischemic cerebrovascular disease hospitalization or death, n (%)	186 (15)	78 (19)	51 (13)	57 (14)	0.02

CCA-IMT indicates common carotid artery intima-media thickness, n=954.
*n=1226.
†ANOVA or a χ^2 test was used as appropriate.
‡Mean±SD.
§Median (interquartile range).
‖n=968.

Table 2. Relationship of Baseline Vegetable Nitrate Intake (mg/d) With CCA-IMT*

	Vegetable Nitrate Intake, per SD (29 mg/d)†		Tertiles of Vegetable Nitrate Intake‡			
	Total Cohort	*P* Value	<53 mg/d	53–76 mg/d	>76 mg/d	*P* Value
n (%)			303 (32)	324 (34)	327 (34)	
Max CCA-IMT, mm						
Unadjusted	−0.015±0.005	0.002	0.941±0.009	0.921±0.008	0.905±0.008	0.010
Age and energy adjusted	−0.014±0.005	0.007	0.940±0.009	0.922±0.008	0.905±0.008	0.021
Baseline risk factor adjusted§	−0.015±0.005	0.004	0.939±0.009	0.921±0.008	0.904±0.008	0.020
Mean CCA-IMT, mm						
Unadjusted	−0.012±0.004	0.006	0.793±0.007	0.777±0.007	0.765±0.007	0.024
Age and energy adjusted	−0.010±0.004	0.021	0.791±0.007	0.778±0.007	0.766±0.007	0.053
Baseline risk factor adjusted§	−0.011±0.004	0.014	0.791±0.007	0.776±0.007	0.765±0.007	0.053

CCA-IMT indicates common carotid artery intima-media thickness.
*n=954.
†Linear regression, results are presented as unstandardized B±SE.
‡ANCOVA, results are presented as estimated mean±SEM.
§Baseline risk factor adjusted for age, body mass index, energy intake, alcohol intake, energy expended in physical activity, antihypertensive medication, statin medication, low-dose aspirin medication, organic nitrate medication, history of smoking, and treatment.

Total and Nonvegetable Nitrate Intake

Total nitrate consumption was linearly inversely associated with maximum CCA-IMT and mean CCA-IMT (Table II in the online-only Data Supplement). This relationship was not observed for nonvegetable nitrate intake (Table II in the online-only Data Supplement).

Neither total nitrate intake (odds ratio, 1.006; 95% confidence interval, 0.810–1.250; *P*=0.95) nor nonvegetable nitrate intake (odds ratio, 1.148; 95% confidence interval, 0.865; 1.524; *P*=0.34) were predictors of plaque severity.

Vegetable Nitrate Intake and Ischemic Cerebrovascular Disease Hospitalizations and Deaths

During 15032 person-years of follow-up, 186 of 1226 (15%) participants had an ischemic cerebrovascular disease event (hospitalization or death). For every 1 SD (29 mg/d) higher intake of vegetable nitrate, there was an associated 17% lower risk of 14.5-year ischemic cerebrovascular disease event in both unadjusted and baseline risk factor adjusted models (Table 3). A similar relationship was observed for total nitrate intake (*P*<0.05) but not for nonvegetable nitrate intake (*P*>0.05).

Across tertiles of vegetable nitrate intake, compared with the lowest tertile (<53 mg/d), intake of the highest tertile (>76 mg/d) nitrate from vegetables was associated with a lower risk of an ischemic cerebrovascular disease event (*P* for trend <0.05 for all models). The multivariable-adjusted cumulative event rate for ischemic cerebrovascular disease according to tertiles of vegetable nitrate intake is presented in the Figure.

Sensitivity Analysis

In separate multivariable-adjusted analyses that adjusted for individual dietary factors, total fat, protein, carbohydrates, and fiber did not change the interpretation of the association of vegetable nitrate intake with maximum and mean CCA-IMT as well as ischemic cerebrovascular disease events (*P*<0.05).

Discussion

In this study of older women, we report an inverse association of vegetable nitrate intake with CCA-IMT and risk of an ischemic cerebrovascular event over 15 years. The relationship remained consistent for total nitrate intake, but not nonvegetable nitrate intake, with CCA-IMT and risk of an ischemic cerebrovascular disease event. These results add weight to the accumulating body of evidence that the vascular benefits associated with a vegetable-rich diet are due, in part, to dietary nitrate.

Table 3. Relationship of Baseline Nitrate Intake (mg/d) With Ischemic Cerebrovascular Disease Events for the Total Cohort*

	HR (95% CI)	*P* Value†
Vegetable nitrate intake per SD (29 mg/d)		
Unadjusted	0.83 (0.71–0.97)	0.016
Age and energy adjusted	0.82 (0.70–0.96)	0.013
Baseline risk factor adjusted‡	0.83 (0.70–0.97)	0.021
Total nitrate intake per SD (31 mg/d)		
Unadjusted	0.83 (0.71–0.96)	0.015
Age and energy adjusted	0.81 (0.70–0.95)	0.011
Baseline risk factor adjusted‡	0.82 (0.70–0.97)	0.017

CI indicates confidence interval; and HR, hazard ratio.
*n=1226.
†Cox regression.
‡Baseline risk factor adjusted for age, body mass index, energy intake, alcohol intake, energy expended in physical activity, antihypertensive medication, statin medication, low-dose aspirin medication, organic nitrate medication, history of smoking, and treatment.

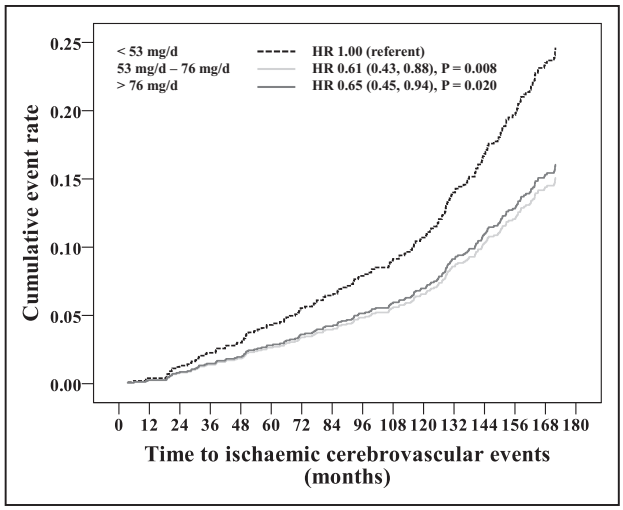

Figure. Cumulative event rate for ischemic cerebrovascular disease events. Baseline risk factor adjusted cumulative event rate for ischemic cerebrovascular disease events (n=186) by vegetable intake tertiles using Cox regression analysis. The dotted black line represents tertile 1 (vegetable nitrate intake <53 mg/d), the light grey line represents tertile 2 (vegetable nitrate intake 53–76 mg/d), and the dark grey line represents tertile 3 (vegetable nitrate intake >76 mg/d). The baseline risk factor–adjusted model included age, body mass index, energy intake, alcohol intake, energy expended in physical activity, antihypertensive medication, statin medication, low-dose aspirin medication, organic nitrate medication, history of smoking, and treatment.

The observed differences in CCA-IMT, although small, may be clinically important. An increase in CCA-IMT is indicative of arterial wall thickening, a form of atherosclerosis[11] and CCA-IMT is a predictor of future cardiovascular events across all age groups.[12] Vegetable nitrate intake was associated with a 17% lower risk of an ischemic cerebrovascular disease event per 29 mg/d (1 SD) higher vegetable nitrate intake, found in approximately half a serve (30 g) of leafy green vegetable intake. This association was significant before and after adjustment for lifestyle and other cardiovascular risk factors. Our data suggest a nonlinear relationship between nitrate intake and ischemic cerebrovascular events because no additional risk reduction was observed in those consuming >76 mg/d nitrate (median 100 mg/d) compared with 53 to 76 mg/d nitrate (median 64 mg/d). As little as one serve of nitrate-rich green leafy vegetable per day may provide adequate nitrate intake for ischemic cerebrovascular disease risk reduction.

A possible mechanism for the inverse association of vegetable nitrate intake with CCA-IMT and risk of an ischemic cerebrovascular event is the augmentation of NO status. Through the endogenous nitrate–nitrite–NO pathway, nitrate has the potential to be converted into NO and to form a large and abundant storage pool for this molecule in blood and tissues.[21] Recent studies demonstrating beneficial effects on blood pressure, endothelial function, platelet aggregation, ischemia reperfusion injury, and exercise performance after intake of dietary nitrate[8,22] are consistent with the proposal that nitrate intake contributes to cardiovascular health.

Approximately 80% of dietary nitrate intake is from vegetables.[14] In our study population, vegetables accounted for 84% of total dietary nitrate intake. Eighty five percent of vegetable nitrate intake was derived from 10 vegetables: lettuce, spinach, celery, beetroot, potatoes, cabbage, pumpkin, green beans, broccoli, and carrots. In contrast to other populations (Far Eastern, African, Latin American, and European) where potatoes often contribute well over 10% of total nitrate intake,[23] potatoes only provided 8% of total nitrate intake in our population. Nitrate intake varies greatly between individuals and populations with mean intakes for populations estimated to be between 0.4 to 2.6 mg/kg or 31 to 185 mg[24] and actual individual daily nitrate intakes ranging from <20 mg to >400 mg.[25,26] Our mean intake of nitrate from all food (79 mg/adult per day) fell into this range. It was slightly higher than the World Health organization (WHO) estimate of nitrate intake from food in Australia of 67 mg/adult per day[23].

Study Strengths and Limitations

Our study had several strengths. These included the use of a validated diet assessment tool, detailed information on lifestyle, and cardiovascular risk factors as well as the validated measure of CCA-IMT with repeated measurements leading to a high level of precision in measurement of the main outcome. Several potential limitations could be considered. First, a cross-sectional design for the relationship of dietary nitrate with CCA-IMT provides only weak evidence for causality. Second, although the possibility of potential reverse causation (changes in diet because of a disease diagnosis) exists, it is unlikely that individuals would knowingly alter their nitrate (or vegetable) intake on the basis of their IMT, which is an asymptomatic and preclinical marker of atherosclerosis, and its value would, therefore, most likely be unknown to them. Third, high nitrate intake may simply coincide with other lifestyle or dietary patterns that are associated with cardiovascular health. Although we adjusted for multiple lifestyle and cardiovascular risk factors as well as dietary factors in our analysis, residual or unmeasured confounders cannot be ruled out. A causal relationship of nitrate intake with CCA-IMT and ischemic cerebrovascular disease events cannot be established because of the observational nature of the study. Fourth, NO cannot be measured directly and endogenous levels have multifactorial influences.[27] There is also no reliable biomarker of nitrate intake. Nitrate levels in plasma, saliva, and urine are influenced by factors including dietary nitrate intake; metabolites of the L-arginine–NOS pathway; bacterial synthesis of nitrate within the gastrointestinal tract; denitrifying liver enzymes; and renal function. Fifth, in this study, carotid ultrasound measures were assessed in 2001. Since then, there have been numerous advances in the evaluation of sonographic characteristics of carotid plaques such as surface irregularity, ulceration and echogenicity that were not available in for this study. Although these newer measures may have provided further insight into how dietary nitrate may affect plaque stability and cerebrovascular events, this would not change our overall findings from the study that dietary nitrate is associated with both established measures of carotid atherosclerosis and long-term cerebrovascular events. Similarly, we did not assess resistive index and we only assessed the common CCA-IMT and not the bifurcation and internal CCA-IMT, which have been suggested as better predictors of future cardiovascular events.[28] Given this further studies investigating

the association of dietary nitrate on newer measures of carotid atherosclerosis with IMT of the 3 sites are warranted. Finally, the current data are limited to elderly women and needs to be confirmed in men and younger women.

Summary/Conclusions

Our study found an association of nitrate intake from vegetables with lower maximum and mean CCA-IMT as well as ischemic cerebrovascular disease events in a cohort of older women. The results are consistent with the proposal that increased nitrate consumption, primarily from vegetables, prevents thickening of the common carotid artery-intima-media, and may play a role in stroke and atherosclerosis prevention.

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Disclosures

Dr Lundberg is a named coinventor on patent applications relating to medical uses of nitrate- and nitrite salts. The other authors report no conflicts.

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Association of Vegetable Nitrate Intake With Carotid Atherosclerosis and Ischemic Cerebrovascular Disease in Older Women

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SUPPLEMENTARY MATERIAL

Supplementary Methods

Participants

A population based approach was used to recruit the women into the study. Letters of invitation were sent to a random selection (n=24800) of women over the age of 70 on the Western Australia electoral roll (a citizenship requirement). Of these women 22% (n=5586) responded to the letter of which 27% (n=1510) were eligible to participate. Exclusion criteria included use of bone active agents, including calcium supplements and hormone replacement therapy, current illness and any medical condition likely to influence 5-year survival.

Baseline assessment

A baseline assessment was performed in 1998. Weight and height was determined using digital scales and a stadiometer while the participant was wearing light clothes and no shoes. BMI was calculated in kg/m2. Vascular disease risk was calculated using previous medical history and current medications, as verified by the participant's general practitioner. These were coded using the International Classification of Primary Care-Plus (ICPC-Plus) method [1]. The occurrence of pre-existing atherosclerotic vascular disease (ASVD) were determined from the complete hospital discharge data from 1980-1998 and were defined using primary diagnosis codes from International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9-CM)[2]. These codes included: ischemic heart disease (ICD-9-CM codes 410-414); heart failure (ICD-9-CM code 428); cerebrovascular disease excluding hemorrhage (ICD-9-CM codes 433-438); and peripheral arterial disease (ICD-9-CM codes 440-444). The presence of baseline vascular disease risk factors included pre-existing diabetes (T89001-90009). Use of cardiovascular medications included β-blockers, angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers, 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors as well as antiplatelet agents. These medications were used to provide an estimate of prevalent hypertension and dyslipidaemia, risk factors classically used in calculating cardiovascular risk. Participants were coded as ex-smoker/current smoker (if smoked >1 cigarette/d for >3 months at any time) or non-smoker. The physical activity of participants in the 3 months prior to enrolment in the study was determined using a previously validated questionnaire which included questions relating to the hours spent per week in up to 4 sporting activities, recreational activities as well as other forms of regular physical activity such as walking. Energy costs of activity were used to determine the energy expenditure in kJ/d for these activities [3].

Baseline dietary intake was assessed using a validated semi-quantitative food frequency questionnaire (FFQ) developed by the Cancer Council of Victoria, Australia [4, 5]. The completion of the questionnaire was performed in small groups supervised by a research assistant with food models, cups, spoons and frequency charts. Frequency of consumption (servings per day) and an overall estimate of portion size were used to estimate energy and nutrient intakes [4, 5].

Assessment of nitrate consumption

Total nitrate intake

An estimate of nitrate concentration (mg/g) in each of the non-vegetable items listed in the Cancer Council of Victoria FFQ was obtained using estimates derived from three sources. Nitrate concentration of 67 of the 77 non-vegetable items were obtained from Inoue-Choi et al. [6], 5 values were obtained from the Food Standards Australia New Zealand (FSANZ) survey of nitrates and nitrites in food and beverages in Australia [7] and 3 values from Griesenbeck et al [8]. Where no value was available (two foods: vegemite and jam), a value of

0 mg/g was used. These values, together with the nitrate intake from vegetable values were used to determine total dietary nitrate intake as mg/d.

The concentration of nitrate in Perth drinking water was assessed using data from July 1997 to June 2001 for 24 regions around the Perth metropolitan area. The absolute maximum value at any time at any site was 0.0022 mg/g. The absolute 4-year mean (range of means) was 0.0003 mg/g (<0.00005; 0.0022) (the WA Water Corporation Water Quality Management System, personal communication). For a person drinking 2 L of water (including tea and coffee), this would equate to an estimated intake of 0.6 mg/d. These values were not included in the estimation of dietary nitrate intake as their contribution to nitrate intake in this population was negligible. The very low nitrate concentrations in Perth drinking water therefore allowed us to focus on nitrate intake from food.

Assessment of common carotid artery intima-media thickness (CCA-IMT) and presence of plaque

Carotid high-resolution B-mode ultrasonography was used to assess common carotid intima-media thickness and focal carotid plaques at year 3 (2001) of the study. The ultrasonic examinations were performed using an 8.0-MHz linear array transducer fitted to an Acuson Sequoia 512 ultrasound machine and a standard image-acquisition protocol. The far-wall carotid IMT at the distal 2 cm of both the left and right arteries were visualised from 3 different angles: anterolateral, lateral and posterolateral. The images were digitally captured at end-diastole for offline measurement by the same observer using semi-automated edge-detection software. The mean, maximum, minimum and SD was calculated for each of the 3 views from both sides. An overall mean CCA-IMT was determined from the average of the CCA-IMT of each of the 6 images. Plaque was defined as a distinct area of focal increased thickness (≥ 1 mm) of the intima-media layer. The severity of the carotid plaque was further categorised by the degree of carotid stenosis as either none to minimal (<25%) or moderate to high (≥25%) plaque. The precision of these measurements was determined in a previous study which reported a CV of 5.9% (root-mean-square error method) for repeat measures between 7 to 10 days apart [9].

Assessment of ischemic cerebrovascular disease event

The first episode of ischemic cerebrovascular hospitalizations or death was retrieved from the Western Australian Data Linkage System (WADLS) for each of the study participants from their baseline clinic visit date in 1998 until 14 ½ years after their baseline visit. Ischemic cerebrovascular disease hospitalizations were retrieved from the Hospital Morbidity Data Collection (HMDC) which provides a complete record of every participant's primary diagnosis at hospital discharge (hospital separation) using coded data from all hospitals in Western Australia. Ischemic cerebrovascular disease deaths were retrieved from the Death Registry by using the coded death certificate or Parts 1 and 2 of the death certificate where coded cause of death data was not yet available. Both HMDC and Death Registry data are linked through the WADLS. If the women remained in Western Australia we had complete follow-up for hospitalizations and deaths. Cerebrovascular hospitalization separations and deaths were defined from the International Classification of Diseases, 9th Revision, Clinical Modification (ICD-9-CM) [10] and the International Statistical Classification of Diseases and Related Health Problems, 10th Revision, Australian Modification (ICD-10- AM) [11]. These codes for cerebrovascular disease, excluding hemorrhage, included: ICD-9-CM codes 433-438 and ICD-10-AM, codes I63-I69, G45.9.

Supplementary Table I: Classification of vegetables in the Cancer Council of Victoria Food Frequency Questionnaire according to nitrate content (mg/g)

Very low **<0.2**	**Low** **0.2 to <0.5**	**Middle** **0.5 to <1.0**	**High** **≥1.0**
Bean sprouts	Baked beans	Zucchini	Beetroot
Capsicum	Broccoli		Celery
Carrot	Cabbage		Lettuce
Cauliflower	Green beans		Spinach
Cucumber	Other beans		
Garlic	Pumpkin		
Mushroom			
Onion			
Peas			
Potato			
Tofu			
Tomato			

Supplementary Table II: Relationship of total nitrate and non-vegetable nitrate intake to common carotid artery intima-media thickness (CCA-IMT) for the total cohort [1]

	B ± SE	P value[2]
Total nitrate per SD (31 mg/d)		
Max CCA-IMT (mm)[3]		
Unadjusted	-0.015 ± 0.005	0.002
Age and energy-adjusted	-0.014 ± 0.005	0.010
Baseline risk factor adjusted[4]	-0.015 ± 0.005	0.006
Mean CCA-IMT (mm)[3]		
Unadjusted	-0.011 ± 0.004	0.007
Age and energy-adjusted	-0.010 ± 0.004	0.032
Baseline risk factor adjusted[4]	-0.011 ± 0.005	0.020
Non-vegetable nitrate intake per SD (5 mg/d)		
Max CCA-IMT (mm)[3]		
Unadjusted	-0.005 ± 0.005	0.333
Age and energy-adjusted	0.004 ± 0.007	0.529
Baseline risk factor adjusted[4]	0.003 ± 0.007	0.661
Mean CCA-IMT (mm)[3]		
Unadjusted	-0.004 ± 0.004	0.371
Age and energy-adjusted	-0.005 ± 0.006	0.378
Baseline risk factor adjusted[4]	-0.004 ± 0.006	0.492

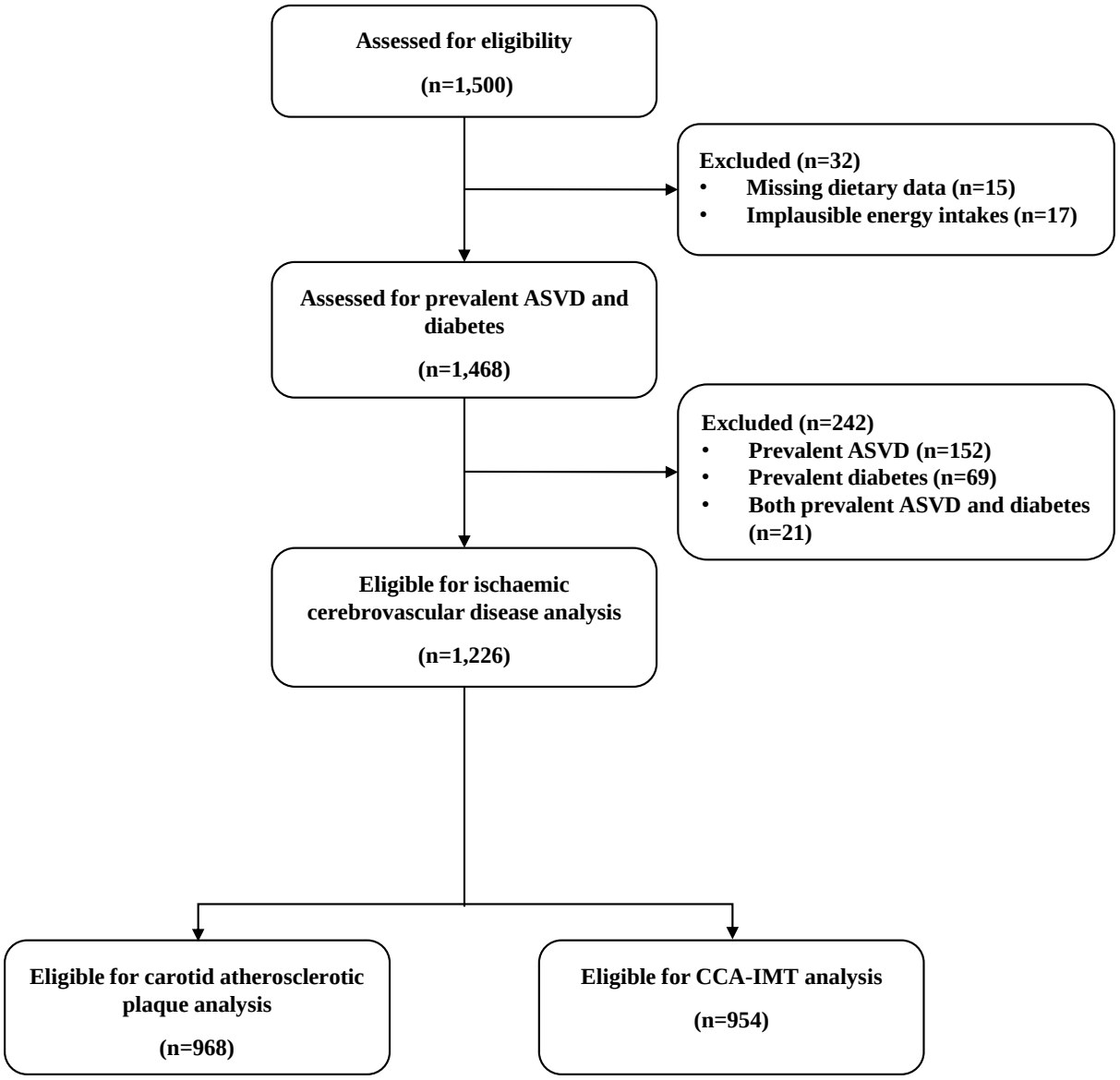
[1] n = 954

[2] Results are presented as unstandardized B ± SE using linear regression.

[3] CCA-IMT: Common carotid artery intima-media thickness.

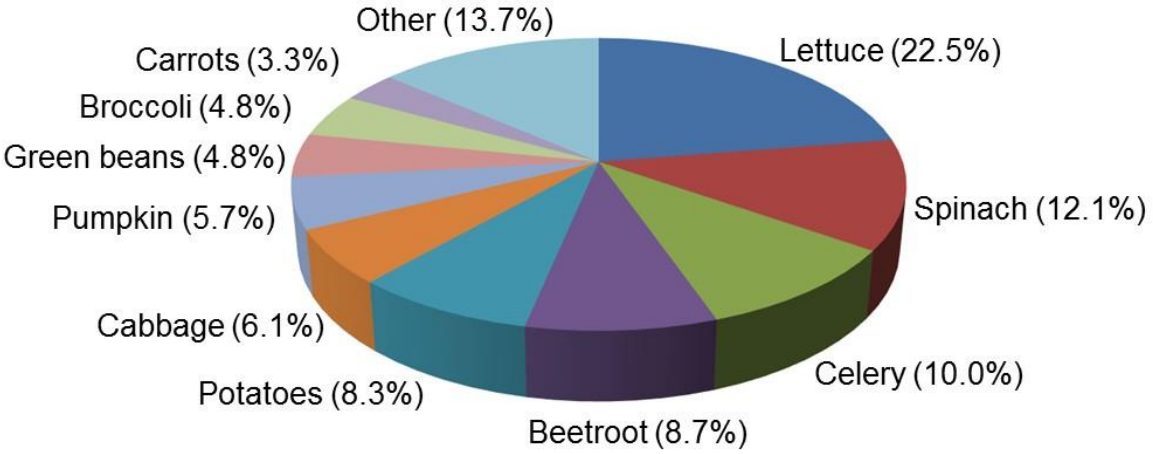
[4] Baseline risk factor-adjusted for age, body mass index, energy intake, alcohol intake, energy expended in physical activity, antihypertensive medication, statin medication, low-dose aspirin medication, organic nitrate medication, history of smoking and treatment.

Supplementary Figure I



Consort flow diagram. ASVD, atherosclerotic vascular disease; CCA-IMT, common carotid artery – intima media thickness; FFQ, food frequency questionnaire.

Supplementary Figure II



Major contributors to total vegetable nitrate intake. Proportional contribution to total vegetable nitrate intake of the greatest individual nitrate contributors.

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