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Dietary flavonoids and risk of stroke in women

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Abstract

Background and Purpose—To date, few studies have examined associations between the wide range of flavonoid sub-classes and risk of ischemic, hemorrhagic and total stroke.

Methods—We conducted a prospective study among 69,622 women from the Nurses' Health Study. Total flavonoid and sub-class intakes were calculated from semi-quantitative food frequency questionnaires collected every 4 years using an updated and extended US Department of Agriculture (USDA) flavonoid database.

Results—During 14 years of follow-up, 1803 incident strokes were confirmed. After adjusting for potential confounders, women in the highest compared to lowest quintile of flavanone intake had a relative risk (RR) of ischemic stroke of 0.81 and 95% CI = 0.66-0.99, p= 0.04). Citrus fruits/ juices, the main dietary source of flavanones, tended to be associated with a reduced risk for ischemic stroke (RR= 0.90 and 95% CI = 0.77-1.05), comparing extreme quintiles.

Conclusions—Total flavonoid intake was not inversely associated with risk of stroke; however, increased intake of the flavanone sub-class was associated with a reduction in risk of ischemic stroke. Citrus fruit consumption may be associated with a reduction in stroke risk, and experimental data support these epidemiological associations that the flavanone content of citrus fruits may potentially be cardioprotective. Further prospective studies are needed to confirm these associations.

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Contributions: AC and KMR conducted the statistical analysis, interpreted the data and drafted the paper. AC, CK and EBR developed the flavonoid database. EBR, EO'R, CK and SEC provided critical review of the manuscript; all authors contributed to the manuscript and agreed the final version.

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Keywords

Flavonoids; flavanones; citrus fruits; stroke; ischemic; hemorrhagic; polyphenols

Introduction

A healthy lifestyle and adherence to a DASH-style or Mediterranean diet, high in fruits, vegetables and total flavonoids, are associated with reduced stroke risk[1-3]. Higher fruit and vegetable intake, per se, has also been associated with reduced risk of stroke, [4] and dietary flavonoids may explain some of this protective association. Experimental evidence suggests several protective mechanisms including improving vascular endothelial function, exerting neuroprotective and anti-inflammatory effects[5-7], enhancing nitric oxide (NO) production and binding to thromboxane A2 receptors [8-11].

However, the relative impact of different dietary flavonoid sub-classes on risk of stroke is unclear and small differences in chemical structure between sub-classes can alter bioactivity [10]. For several sub-classes, including the anthocyanins, flavanones and flavonols emerging *in vitro* and epidemiological data support a reduction in risk of ischemic stroke with increased intake [5,10]. A higher intake of anthocyanins was associated with a 12% reduction in risk of hypertension, a potent risk factor for stroke[12], and citrus fruits, a rich source of flavanones reduced ischemic stroke risk [13,14] although two studies that previously assessed flavanone intake did not observed protective effects in relation to risk of ischemic stroke[15,16]. Most previous prospective studies have predominantly focused on the flavonol sub-class with equivocal findings [15, 1716, 18, 19], However a recent meta-analysis showed that a high flavonol intake was associated with a reduced risk of both fatal and non-fatal stroke[20]. Potential reasons for the inconsistent findings may relate to measurement error in dietary assessment or the lack of availability of comprehensive food composition tables covering the range of flavonoid subclasses. Therefore no previous published prospective studies on stroke had the opportunity to use an updated food database that integrated levels of intake of the wide range of sub-classes consumed in the habitual diet [21,22].

Using the updated database to more accurately assess intake, we examined the relationship of the six main sub-classes of flavonoids commonly consumed in the US diet, flavanones, anthocyanins, flavan-3-ols, flavonoid polymers, flavonols, and flavones, with risk of ischemic, hemorrhagic and total stroke. We hypothesized, on the strength of the available mechanistic and human data that a high intake of anthocyanins, flavanones and flavonols would be associated with a reduced risk of ischemic stroke.

Methods

Study population

In 1976 the Nurses' Health Study (NHS) enrolled 121,700 female nurses aged 30-55 who returned a mailed questionnaire regarding lifestyle and medical history [23]. Participants received follow-up questionnaires biennially to record newly diagnosed illnesses and to update lifestyle factors, and every four years received semi-quantitative food frequency questionnaires (FFQ)[24,25]. The FFQ from 1990 was used as baseline because of the inclusion of a sufficient number of fruits and vegetables to more accurately estimate flavonoid intake. In 1990, a total of 80,332 women completed the FFQ. Those who had implausible values for total caloric intake (<500 or >3500 kcal/d) or too many missing items were excluded (1,448), resulting in 78,884 with FFQ data. We excluded those who reported a history of CVD or cancer prior to 1990 (9,262), leaving a total of 69,622 women for this analysis. We stopped updating dietary information if cancer or CVD was diagnosed during

follow-up, although these individuals continued to contribute follow-up time. The institutional review board at Brigham and Women's Hospital reviewed and approved this study and participants provided implied consent by virtue of returning their questionnaires.

Assessment of flavonoid intakes

Dietary intake data were collected from NHS participants in 1990 and subsequently every four years. FFQs administered prior to 1990 contained fewer questions on specific flavonoid-rich fruits and vegetables (for example, onions were absent from questionnaires before 1990). A database for assessment of intake of the different flavonoid sub-classes was constructed as previously described [12]. Intakes of individual compounds were calculated as the sum of the consumption frequency of each food multiplied by the content of the specific flavonoid for the specified portion size. We derived intakes of the six main sub-classes commonly consumed in the US diet; specifically, flavanones (eriodictyol, hesperetin, naringenin), anthocyanins (cyanidin, delphinidin, malvidin, pelargonidin, petunidin, peonidin), flavan-3-ols (catechins, epicatachins), flavonols (quercetin, kaempferol, myricetin, isohamnetin), flavones (luteolin, apigenin) and polymers (including proanthocyanidins, theaflavins and thearubigins). Total flavonoid intakes were derived by addition of the component sub-classes (flavanones, anthocyanins, flavan-3-ols, polymers, flavonols, flavones). Cumulative intakes (energy adjusted) were calculated for a given questionnaire cycle by averaging the intake for the current and preceding FFQs [26]. The validity and reproducibility of the FFQs have been reported previously; for example correlations between several major dietary sources of flavonoids including fruits, vegetables, tea and wine measured by diet-records and FFQ were 0.70, 0.50, 0.77 and 0.83 respectively [27,28].

Outcome assessment

We requested permission to review medical records of all participants who reported a physician diagnosis of a stroke during follow-up. Physicians blinded to risk factor status reviewed the medical records. Cerebrovascular pathology due to infection, trauma or malignancy was excluded and "silent" strokes discovered only by radiologic imaging were also excluded. Strokes were confirmed using the National Survey of Stroke criteria, [29] requiring neurological deficit of rapid or sudden onset lasting ≥24 hours or until death. Fatal strokes were identified by next of kin, postal authorities, or the National Death Index and confirmed by medical records, autopsy reports, and death certificates with stroke listed as the underlying cause. We categorized types of stroke as ischemic (embolic or thrombotic), hemorrhagic (subarachnoid or intraparenchymal), and unknown. Strokes that required hospitalization and for which confirmatory information was obtained but medical records were unavailable were designated as probable (25% of total strokes). Because the exclusion of probable strokes did not alter the results, we included both confirmed and probable strokes in this analysis.

Statistical methods

Participants contributed person-time of follow-up from the date of return of the 1990 questionnaire to the date of stroke diagnosis, death, or end of follow-up (June 2006). Mantel-Haenzel age-adjusted incidence rate ratios (RR) and 95% confidence intervals (CI) were obtained relative to the incidence rates among those in the lowest quintile of nutrient of interest. We used Cox proportional-hazard modeling to assess the associations between different flavonoid sub-classes and risk of stroke. We used separate models for ischemic and hemorrhagic stroke, given that the underlying biological mechanisms for the subtypes may differ. In multivariate models, we adjusted for age (continuous), body mass index (BMI; <25, 25-29.9, or ≥30 kg/m^2), physical activity (metabolic equivalents/week, in quintiles), alcohol consumption (0, 0.1-4.9, 5-14.9, 15-29.9, ≥30 g/day), energy intake (kcal/day, in

quintiles), use of multivitamin supplements (yes, no), use of aspirin (non-user, <6/week, 6+ per week), menopausal status (premenopausal, dubious menopause, postmenopausal), postmenopausal hormone use (never, past, or current hormone use), smoking (never, past and current), and history of type 2 diabetes, CHD, hypercholesterolemia, hypertension. We stopped updating dietary information if cancer (all except non-melanoma skin cancer) or CHD was diagnosed. All analyses were conducted with the SAS software, version 9 (SAS Institute, Inc., Cary, North Carolina). All *p* values are two sided.

In secondary analyses, we stratified by hypertension and aspirin use, which were updated through follow-up, to evaluate potential interactions.

Results

During 14 years of follow-up among the 69,622 participants included in the analyses, we documented 1803 cases of stroke (943 ischemic, 253 hemorrhagic and 607 of unknown type). Baseline characteristics of the study population according to quintiles of total flavonoid intake are presented in Table 1. Women with higher total flavonoid intake tended to smoke less, exercise more, have greater intakes of fiber, folate, fruits and vegetables, but lower intakes of caffeine and alcohol.

The median intake of total flavonoids was 232 mg/d and the median intakes of the flavonoid sub-classes across quintiles are shown in Table 1. The polymer subclass contributed most to total flavonoid intake while the contribution to total intake from flavones was negligible. Tea was the main food contributor to total flavonoid intake, with apples and oranges/orange juice as other significant contributors. Flavan-3-ols were predominantly consumed from tea, while blueberries were the main source of anthocyanins, and oranges/orange juice were the main contributors to flavanone and flavone intake (Supplement Figure 1).

Flavanone intake was inversely related to risk of ischemic stroke; women in the top quintile of flavanone intake had a relative risk (RR) of 0.81 and 95% CI = 0.66-0.99, p for trend =0.1, Quintile 5 vs. Quintile 1 (Q5 v Q1), p=0.038) compared with those in the lowest quintile (Table 2). When we further adjusted for calcium or magnesium the results were not materially altered (data not shown). Given that 95% of the flavanones were derived from citrus fruit and juice consumption (Supplement Figure 1), a high intake of citrus fruits/juice tended to be associated with a reduced risk of ischemic stroke (RR 0.90 and 95% CI= 0.77-1.05, Q5 vs. Q1). Citrus fruits and juices contain other constituents that may reduce the risk of stroke, including Vitamin C and potassium. The addition of vitamin C to the flavanone multivariate model had little effect on the RR (0.80 and 95% CI = 0.64-0.99), while potassium slightly attenuated the association (RR 0.83 and 95% CI = 0.67-1.03).

In models adjusting for age and smoking, flavone intake was inversely associated with risk of total and ischemic stroke; however the relative risk was attenuated following further adjustment for stroke risk factors (Table 2 and Supplement Table A).

There were no significant associations between any sub-class and hemorrhagic stroke (Supplement Table B). We stratified by hypertension and aspirin use to investigate if these factors modified the relationship between flavonoid sub-class intake and risk of stoke but there were no significant effect modifications (p for interaction >0.05).

Discussion

To our knowledge, this is the first prospective study to examine the main dietary flavonoid sub-classes on risk of stroke using an updated, comprehensive food database [12]. Over 14 years of follow-up, high flavanone intake was associated with a 19% lower risk of ischemic

stroke, a finding that fits with existing data on the protective effect of citrus fruit consumption [4,13,14]. The main dietary sources of flavanones were orange and grapefruit juices (63%), oranges (34%), AND grapefruits (4.8%) (Supplement Figure 1) and in food-based analyses we similarly observed a trend towards an inverse association for citrus fruit intake and total and ischemic stroke (RR 0.90 and 95% CI = 0.77-1.05, Q5 vs. Q1). On a mg/100g basis, oranges are a richer source of flavanones, however given the frequency of consumption of fruit juices, juices are the main source in the US diet [22]. Given the higher flavanone content of citrus fruits and the sugar content of commercial fruit juices, public health recommendations should focus on increasing citrus fruit intake.

In two previous prospective studies, citrus fruit (including juice) intake but not intake of other fruits protected against risk of ischemic stroke and intracerebral hemorrhage [13,14]. Vitamin C has often been cited as the potentially protective constituent and plasma vitamin C levels are inversely associated with stroke risk[3]. In our analyses, vitamin C intake was not associated with a reduction in total or ischemic stroke risk (data not shown). The addition of vitamin C to our model did not substantially attenuate the relationship between flavanones and ischemic stroke risk, suggesting that flavanones may be another important cardioprotective constituent of citrus fruits. However in a population-based study like ours it is impossible to disentangle the relative influence of all the constituents of citrus fruits. The risk reductions we observe with increased flavanone intake are supported by experimental data as naringenin and hesperetin exert a diverse array of neuroprotective effects *in vitro* by interacting with MAPK, P13 kinase/Akt and PKC signaling pathways [30-32]. Recently naringinen was the most potent anti-inflammatory flavonoid tested; it inhibited iNOS expression and NO release due to its ability to inhibit p38 MAPK and STAT-1 phosphorylation/activation [32]. The flavanone hesperetin has also been shown to increase NO release from endothelial cells and up-regulate eNOS expression [33].

We observed a modest inverse association between a higher intake of flavones and anthocyanins and risk of total and ischemic stroke, although these data did not reach statistical significance. Growing *in vitro* mechanistic evidence suggest potential beneficial effects of specific flavonoids and their biologically active metabolites in reducing ischemic stroke development including inhibitory effects on platelet function, thrombosis, inflammation, and protection against ischemia-reperfusion injury and arrhythmia [10,34]. Specifically, for several sub-classes, including flavones and anthocyanins evidence is emerging for beneficial effects by suppressing neuro-inflammation and improving cerebral blood flow [5]. Flavonoid metabolites may inhibit platelet function through multiple mechanisms (included blocking Fyn kinase activity and the tyrosine phosphorylation of Syk and PLCg2 following internalisation) which are differentially influenced by specific structural characteristics[10]; key structural attributes for the inhibition of platelet function include a B-ring catechol moiety, presence of a planar C-4 substitution and C-3 hydroxylated C ring [10] at concentrations known to be achieved in plasma following ingestion of flavone- and flavonol-rich foods [8,9].

We found no evidence for an inverse association for flavonols and risk of total, ischemic or hemorrhagic stroke. This contrasts with important data from a recent meta-analysis [35] which showed that a higher intake of flavonols was associated with a lower risk of stroke. Habitual intakes of flavonols in our study were low (median intake 14.5mg/d) while median intake of flavanones were much higher (30.4 mg/d). Further studies are therefore required to examine the effects of flavonols on stroke risk, particularly focusing on stroke type as we had few hemorrhagic stroke cases (n=253).

To date, most previous cohort studies have focused specifically on the flavonol quercetin [17, 35], or specific flavonoid-rich foods like tea [36]. In the Iowa women's study, no

association between intake of any flavonoid sub-class and stroke mortality was observed however data on non-fatal stroke were not available [16]. In the Finnish study, men in the highest, compared to lowest quartile of flavonol intake had a lower risk of ischemic stroke [15]

Limitations of our study warrant discussion. Although we adjusted for possible confounders, there is still the possibility of residual or unmeasured confounding. However given our detailed and updated adjustment for potential confounders it is unlikely that these would account for the observed results. Although our follow-up rates exceed 85%, follow-up is not perfect and could theoretically induce some dilution bias of effect estimates. We used repeated measurements of diet to obtain a more accurate assessment of long-term flavonoid intake and to reduce measurement error. Mean cumulative dietary flavonoid intakes were calculated from a database developed using the most recent USDA databases [21, 22], with additional input from other sources. These datasets allowed us to quantify a broad range of flavonoid sub-class intakes more robustly than previous analyses. However there is wide variability in flavonoid content of foods depending on geographical origin, growing season, different cultivars, agricultural methods and processing and a lack of biomarkers to integrate intake with the extensive metabolism these compounds undergo in vivo. Finally, it is possible that our findings for flavonoid subclasses might be due to other nutrients found in the foods that contribute most to these subclasses.

Increased consumption of fruits and vegetables has been associated with a reduced risk of stroke. In a meta-analysis of existing cohort studies [4] those consuming 3-5 servings/day and >5 servings/day had an 11% and 26% reduction in risk of stroke, compared to those consuming <3 servings/day. However, these data could not determine which specific fruits/vegetables or their constituents exerted these protective effects. Our findings suggest that bioactive compounds present in citrus may potentially be associated with a reduced risk of stroke. Further prospective studies are needed to confirm these associations together with further molecular mechanistic data on flavanones to inform and optimize the design of randomized trials of flavanone and citrus-based foods to potentially reduce ischemic stroke risk.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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TABLE 1

Age-standardized baseline characteristics of 69622 women in the Nurses' Health Study, according to quintiles of total flavonoid intake in 1990

	Quintiles (Q) of total flavonoid intake				
	Q1	**Q2**	**Q3**	**Q4**	**Q5**
Total Flavonoids (mg/d)	96.8	161.7	232.0	356.3	761.2
Postmenopausal %	70	69	69	70	69
History of high cholesterol %	36	38	37	38	37
History of diabetes %	4	4	4	4	5
History of hypertension %	30	29	29	30	30
Current smoking, %	27	17	13	12	14
Current postmenopausal hormone use %	26	29	30	30	28
Alcohol g/d	6.5	5.7	5.0	4.5	3.8
Regular aspirin use (6+ tablets per week)	10	10	10	10	10
BMI kg/m^2	26.0	25.7	25.6	25.5	25.6
Multivitamin users %	34	38	40	39	38
Physical activity mets/wk	13.1	15.8	16.7	17.4	15.2
Energy intake (Kcal/d)	1786	1812	1798	1747	1595
Caffeine mg/d	296	261	251	245	270
Fiber g/d	15.2	17.6	19.0	19.6	18.9
Fruits and vegetables (portions/d)	4.7	6.0	6.7	6.9	6.0
Wholegrains g/d	16.0	19.2	20.2	20.5	19.9
Folate ug/d	370	421	445	455	462
Calcium mg/d	941	990	1005	1000	978
Sodium mg/d	1896	1843	1821	1807	1837
Potassium mg/d	2612	2821	2934	2982	2990
Magnesium mg/d	281	302	310	312	309
Omega 3 g/d	1.2	1.2	1.2	1.2	1.2
Flavonols mg/d	9.6	12.5	15.3	19.6	34.3
Flavones mg/d	1.0	1.7	2.0	2.1	2.0
Flavonones mg/d	18.9	35.5	44.3	48.4	44.9

	Quintiles (Q) of total flavonoid intake				
	Q1	Q2	Q3	Q4	Q5
Flavan-3-ols mg/d	8.6	15.1	25.1	51.9	175.0
Anthocyanins mg/d	5.8	10.3	13.6	16.6	16.5
Polymers mg/d	48.6	86.7	134	227	669

All variables are age-standardized; all nutrients are energy adjusted

Table 2

Flavonoid sub-class intake and risk of ischemic stroke among 69,622 women in the Nurses' Health Study.

	Quintiles (Q) of flavonoid intake subclasses					
	Q1	**Q2**	**Q3**	**Q4**	**Q5**	**P for trend**
Flavonols (mg/d)	<10.15	10.16-13.62	13.63-17.86	17.87-25.14	>25.14	
No. of ISCHEMIC STROKE cases	191	181	169	203	199	
No. of person years	106193	106545	106375	106331	106354	
Age and smoking RR (95% CI)	1.0	0.91(0.74-1.12)	0.84(0.68-1.03)	0.96(0.78-1.17)	0.94(0.77-1.14)	0.94
Multivariate model *RR (95% CI)	1.0	0.94(0.76-1.15)	0.87(0.70-1.07)	1.00(0.82-1.22)	0.96(0.79-1.17)	0.94
Flavones (mg/d)	<0.94	0.94-1.45	1.46-2.00	2.01-2.69	>2.69	
No. of cases	189	175	187	194	198	
No. of person years	1062330	105439	106800	106820	106409	
Age and smoking RR (95% CI)	1.0	0.90(0.73-1.11)	0.89(0.73-1.09)	0.86(0.70-1.05)	0.81(0.66-0.99)	0.048
Multivariate model *RR (95% CI)	1.0	0.93(0.75-1.14)	0.92(0.75-1.13)	0.91(0.74-1.12)	0.88(0.72-1.08)	0.26
Flavanones (mg/d)	<13.72	13.72-27.50	27.51-42.71	42.72-62.95	>62.95	
No. of cases	199	164	184	199	197	
No. of person years	106354	106376	106379	106322	106367	
Age and smoking RR (95% CI)	1.0	0.82(0.67-1.01)	0.84(0.69-1.03)	0.84(0.69-1.03)	0.75(0.62-0.92)	0.02
Multivariate model *RR (95% CI)	1.0	0.84(0.69-1.04)	0.87(0.71-1.07)	0.89(0.73-1.09)	0.81(0.66-0.99)	0.10
Flavan-3-ols (mg/d)	<12.26	12.26-19.85	19.86-35.83	35.84-75.83	>75.83	
No. of cases	217	185	174	181	186	
No. of person years	106318	106381	106361	106375	106363	
Age and smoking RR (95% CI)	1.0	0.83(0.68-1.01)	0.79(0.64-0.96)	0.82(0.67-1.00)	0.85(0.70-1.04)	0.59
Multivariate model *RR (95% CI)	1.0	0.86(0.71-1.05)	0.82(0.67-1.00)	0.85(0.70-1.04)	0.87(0.72-1.06)	0.59
Anthocyanins (mg/d)	<5.40	5.40-9.19	9.20-13.56	13.57-20.21	>20.21	
No. of cases	195	185	186	196	181	
No. of person years	106315	106395	106387	106331	106370	
Age and smoking RR (95% CI)	1.0	0.87(0.71-1.07)	0.86 (0.70-1.06)	0.91(0.75-1.12)	0.82(0.67-1.01)	0.17
Multivariate model *RR (95% CI)	1.0	0.89(0.72-1.09)	0.89(0.73-1.10)	0.96(0.78-1.18)	0.89(0.72-1.11)	0.59

	Quintiles (Q) of flavonoid intake subclasses					
	Q1	**Q2**	**Q3**	**Q4**	**Q5**	**P for trend**
Polymers (mg/d)	<77.10	77.10-118.54	118.55-176.23	176.24-306.04	>306.04	
No. of cases	209	203	176	167	188	
No. of person years	106337	106346	106369	106381	106365	
Age and smoking RR (95% CI)	1.0	0.96(0.79-1.17)	0.85(0.69-1.04)	0.79(0.65-0.97)	0.89(0.73-1.09)	0.33
Multivariate model *RR (95% CI)	1.0	0.99(0.82-1.21)	0.90(0.73-1.10)	0.83(0.68-1.02)	0.93(0.76-1.14)	0.47
Total Flavonoids (mg/d)	<150.69	150.69-213.37	213.38-296.60	296.61-470.81	>470.81	
No. of cases	203	205	175	171	189	
Person years	106343	106345	106373	106377	106360	
Age and smoking RR (95% CI)	1.0	0.94(0.77-1.14)	0.80(0.65-0.98)	0.77(0.62-0.94)	0.86(0.70-1.05)	0.22
Multivariate model *RR (95% CI)	1.0	0.98(0.80-1.19)	0.84(0.68-1.03)	0.81(0.66-1.00)	0.90(0.73-1.11)	0.36

* **Multivariate model** – adjusted for age, physical activity, smoking, HRT, BMI, aspirin use, type 2 diabetes, hypercholesterolemia, history of CHD, alcohol, menopausal status, energy, use of multivitamins, history of hypertension