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Association of flavonoids and flavonoid-rich foods with all-cause mortality: The Blue Mountains Eye Study

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Short running head: Flavonoid intake and mortality

Abbreviations: BMES, Blue Mountains Eye Study; CHD, coronary heart disease; CVD, cardiovascular disease; FFQ, food frequency questionnaire; HR, hazard ratio; ICD, International Classification of Diseases; NCD, non-communicable disease; NDI, national death index; SES, socio-economic status; TDS, total diet score; USDA, United States Department of Agriculture.

ABSTRACT

Background: Higher intakes of flavonoids provide health benefits, however, the importance of each flavonoid class and which population groups may receive the greatest protection from higher flavonoid intake warrants further investigation.

Objective: To explore the associations of flavonoid and flavonoid-rich wholefood intakes with all-cause mortality and the moderating effects of early mortality risk factors.

Design: The study included 2 349 participants of The Blue Mountains Eye Study, with a mean±SD age at baseline of 64.7±9.2 years. We calculated flavonoid intake from baseline food frequency questionnaires using US Department of Agriculture food composition databases. Associations were examined using adjusted Cox proportional hazards models.

Results: After 14 years of follow-up, 677 participants died. There was a flavonoid threshold effect with the greatest risk reduction seen between low and moderate intakes of total flavonoids, flavonoid classes and flavonoid-rich foods. Amongst the whole cohort, participants in the highest tertile of anthocyanidin intake had a significantly lower risk of all-cause mortality [multivariable adjusted HR (95%CI): 0.76 (0.61, 0.94)] when compared to those in the lowest tertile. Amongst participants with at least one early mortality risk factor (smoking, high alcohol consumption, no regular exercise or obesity), risk of all-cause mortality was lower in those in the highest intake tertile for total flavonoids [adjusted HR: 0.77 (0.59, 1.00)], flavan-3-ols [0.75 (0.58, 0.98)], anthocyanidins [0.70 (0.54, 0.92)], and proanthocyanidins [0.69 (0.52, 0.92)], compared to those in the lowest tertile. No similar associations were observed among those without any risk factors. Similarly, consumption of apples, tea and the individual flavonoid compounds, quercetin and epicatechin, were

associated with a lower risk of all-cause mortality among participants with at least one risk factor, but not amongst other participants.

Conclusion: Moderate to high intakes of flavonoids and certain flavonoid subclasses may provide health benefits, particularly for individuals with at least one early mortality risk factor.

Keywords: Flavonoids, flavonoid-rich foods, all-cause mortality, prospective cohort study

INTRODUCTION:

Despite improvements seen in the past 25 years, dietary risk factors are still a major contributor to the burden of non-communicable diseases (NCDs) in Australia, with an estimated 19.7% of all deaths in 2015 attributable to dietary habits [1]. In particular an estimated 8.4% of NCD deaths were attributable to either a diet low in fruit or a diet low in vegetables. Of all NCD deaths attributable to dietary risks, 80.5% were related to cardiovascular disease (CVD).

Beneficial effects of a diet rich in fruits and vegetables have partly been attributed to flavonoids, a class of polyphenolic compounds found in plant-based foods and beverages [2]. Six main flavonoid subclasses have been defined: flavonols, flavan-3ols (including proanthocyanidins), flavones, flavanones, anthocyanidins and isoflavones. There are at least 300 different flavonoid compounds commonly consumed in the human diet. However, most of our total flavonoid intake is derived from fewer than 30 flavonoid compounds. The bioactivity of these compounds and their circulating metabolites differ [3], meaning it is likely that not all flavonoids will have the same impact on health outcomes [4, 5]. Several cohort studies have reported an inverse association between high flavonoid intakes and all-cause mortality [6], CVD-related mortality, and coronary heart disease (CHD)-related mortality [7]. These results are supported by short-term randomised controlled trials demonstrating that flavonoids and flavonoid-rich foods and beverages positively influence measures related to the development and progression of CVD [8-10]. Whilst such studies support a protective effect of a flavonoid rich diet, none have yet examined whether or not these effects are consistent amongst individuals with and without unhealthy lifestyle behaviours, particularly smoking and excessive alcohol intake, both of which increase the

risk of mortality [1]. Thus, the potential for a moderating impact of such behaviours on the beneficial effects of a flavonoid-rich diet is unknown.

Therefore, the primary aim of this study was to investigate the associations between intakes of total flavonoids, flavonoid subclasses and major individual flavonoid compounds, as well as flavonoid-rich wholefoods and beverages and all-cause mortality in a cohort of older Australians. Secondary aims were to investigate associations with CVD- and CHD-related mortality and to explore whether these associations are modified in the presence of risk factors for early mortality.

SUBJECTS AND METHODS:

Study Population

This was a prospective cohort study, conducted using data from the Blue Mountains Eye Study (BMES). Details of the BMES methods have been previously reported [11, 12]. Briefly, the BMES was the first large population-based assessment of visual impairment and common eye diseases, conducted in a representative older Australian community. The study recruited participants within a geographically defined area, in the Blue Mountains region of New South Wales (postcodes 2780 and 2782). The study population was representative of the Australian population demographically and for socio-economic status (SES), although they were slightly older on average [12]. All permanent, non-institutionalised residents, aged 49-97 years, identified in a door-to-door census, were invited to participate. Of the 4433 eligible residents, 3654 (82.4%) participated in baseline examinations during 1992–1994. Starting in 1992, the population has been followed for up to 15 years. The study was approved by the

Western Sydney Area Human Research Ethics Committee and was conducted in adherence to the tenets of the Declaration of Helsinki.

Participants who did not complete a food frequency questionnaire (FFQ) at baseline (n=756) and those with implausible energy intakes [<2,092 kJ/day (<500kcal/day) and >14,644 kJ/day (>3,500kcal/day)] (n=38) were excluded from the analysis. All participants with a history of diabetes (n=160) or major CVD (acute myocardial infarction or stroke, n=351) at baseline were excluded from the analysis (**Figure 1**). The participants provided their previous medical history and current medications 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, major CVD and/or stroke.

Dietary Assessment

Dietary data were collected using a 145-item self-administered semi-quantitative FFQ, modified for the Australian diet and vernacular from an early FFQ by Willett *et al.* [13]. Respondents were asked to indicate their usual frequency of consuming food items during the past year, using a nine-category frequency scale that ranged from never to four or more times per day. Each food was presented on the FFQ with a standard portion size. An allowance for seasonal variation of fruit and vegetables was made by weighting seasonal fruits and vegetables. The FFQ has been tested for reproducibility and validity in a subsample of the study population against weighed food records for nutrients and individual food items [14, 15].

Exposures

Estimates of the flavonoid content of foods in the FFQ and beverage questionnaire were derived from the US Department of Agriculture (USDA) Database for the Flavonoid Content of Selected Foods [2], the USDA Database for the Isoflavone Content of Selected Foods [16] and the USDA Database for the Proanthocyanidin Content of Selected Foods [17]. The method of estimating the flavonoid content of foods was similar to that outlined by Mink *et al.* [18]. For each food we estimated the intake of each individual flavonoid compound present. The total intake of each class of flavonoids was then calculated by summing each individual flavonoid compound within that flavonoid class. Total flavonoid intake was calculated by summing each of the flavonoid classes. The flavan-3-ol content of foods was considered to represent the average of total flavan-3-ol and proanthocyanidin monomer contents. For foods where only the flavan-3-ol or proanthocyanidin monomer content was available, the single value provided was used to represent the flavan-3-ol content. The proanthocyanidin content of foods was calculated by summing the proanthocyanidin dimers, trimers, 4–6mers, 7–10mers and polymers. Where multiple varieties of a food listed in the FFQ were reported in the databases, the average flavonoid content of all similar varieties was computed, consistent with the descriptors used in the FFQ output. Foods in the FFQ that were not in the flavonoid databases were assumed to contain no flavonoids. Intakes of flavonoid classes (in mg/d) were calculated by multiplying the estimated intake (g edible portion/d) from the FFQ and beverage questionnaire, with the flavonoid class content (mg/g edible portion) of each food item on the questionnaire. Estimations for some of the food items were made using generic recipes found online.

Study outcomes

The primary outcome of this study was death from any cause. Cause of death data were obtained from the Australian National Death Index (NDI). CVD mortality data were obtained

by matching cause of death codes for CHD and stroke (see below) to the codes recorded in the NDI up until 31 December 2007 (i.e., 14 years of follow up). Those who were unable to be matched to the NDI (5%) were excluded from the analysis. Causes of death in the NDI were defined using the 9th revision of International Classification of Diseases Code (ICD-9) and International Statistical Classification of Diseases, 10th revision (ICD-10), with the following codes 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) or stroke: (ICD-9:430.0 to 438.9 and ICD-10:I60.0 to I69.9). The data from the Australian NDI have been validated, and reported to be highly sensitive and specific for CVD mortality (92.5% and 89.6%, respectively) [38]. Over 14 years of follow-up there were 677 deaths with 548 recorded cases for CVD mortality in this cohort: 432 for CHD; 176 for stroke; and 60 for both CHD and stroke as the co-primary causes of death.

Covariates

A physical examination and detailed questionnaires administered by trained interviewers [19, 20], were used to determine values for potential confounding variables including age, gender, BMI, energy intake, physical activity, hypertension, hypercholesterolemia, smoking status and SES. Weight was assessed using digital scales with participants wearing light clothes and no shoes. Height was assessed using a stadiometer and BMI was calculated in kg/m^2 at baseline. Smoking status was determined using categories of never smoked, past smoker and current smoker; participants were classified as a current smoker if they had stopped smoking within the past 12 months [20]. Systolic and diastolic blood pressures were measured with the participants seated for at least 5 minutes using a mercury sphygmomanometer. Participants were deemed hypertensive if they were currently taking blood pressure-lowering medication or had a systolic blood pressure ≥140 mmHg. If participants were taking cholesterol-lowering

medication (statins) or had a total cholesterol ≥ 5.5 mmol/L, they were considered hypercholesteremic. Total cholesterol levels were measured using fasting blood samples [21]. Use of blood pressure-lowering medication and cholesterol-lowering medication (statins) were obtained from self-report and were verified by participants' General Practitioners. Participants were asked questions regarding walking exercise and the performance of moderate or vigorous activities, as detailed elsewhere [22]. These data were categorized as follows: 1) No physical activity; 2) No vigorous physical activity; 3) Vigorous physical activity. We used home/unit owner (yes/no) as a proxy for SES. Potential dietary confounding variables were calculated from the FFQ described above.

Statistical Analysis

An analytical protocol was developed prior to the commencement of analysis. Descriptive data are presented as mean ± SD for normally distributed continuous variables, median (IQR) for non-normally distributed continuous variables and as number (n) and percentage (%) for categorical variables. The exposure variables were categorized by tertiles of intake [T1: 0-33.3%; T2: 33.4-66.6%; T3: 66.7-100%]. Cox proportional hazard ratios (HR) and 95% confidence intervals (CIs) for all-cause, CVD- and CHD-related mortality were computed using tertiles of the exposure variables, where the lowest tertile (reflecting the lowest intakes) was the referent category. Schoenfeld residuals were used to check the Cox proportional hazards assumptions, with no evidence of violation for all outcomes. For all analyses, two models were fit: 1) minimally-adjusted (age and gender) and 2) multivariable-adjusted (age, gender, BMI, physical activity, alcohol intake, smoking status, SES, hypercholesterolemia and hypertension). Given the aetiological focus of our research hypotheses, deaths from non-CVD causes were censored rather than being treated as competing risks [23]. We tested for nonlinear relationships using restricted cubic splines, with the exposure variables treated as

continuous, excluding individuals with intakes more than 4 SD's above the mean for each exposure. The test of nonlinearity used analysis of variance to compare the model with only the linear term to the model that included both the linear and the cubic spline terms. To account for the possibility of reverse causality bias, we repeated all analyses after excluding all events that occurred within the first 2 years. We tested for effect modification by gender and age; interaction terms between flavonoid intakes and each of these factors were added to the models and likelihood ratio chi-square tests were used to formally test for statistical interaction. To test for potential modification by lifestyle behaviour pattern, we performed a stratified analysis according to an *a priori*–defined risk of early mortality characterized by at least one of the following risk factors: current smoking, alcohol intake >14 standard drinks/week, BMI ≥ 30 kg/m^2 or no physical activity. Hazard ratios were obtained from models including the two main effects and the interaction term. As flavonoid intake is not highly correlated with total energy intake, and we believe crude values to be more relevant than energy-adjusted values, we did not include total energy intake as a covariate in model 1 or 2. Energy intake was however added to model 2 in a sensitivity analysis to assess its effect on the primary outcome. In two additional sensitivity analyses, we separately added potential dietary confounders (fibre, saturated fat, polyunsaturated fat, dietary cholesterol and vitamin C) and a diet quality index to model 2. This diet quality index has been described elsewhere [24]. Analyses were undertaken using IBM SPSS® Statistics version 21 (2012, Armonk, NY: IBM Corp), STATA/IC 14.2 (StataCorp LLC) and R statistics (R Core Team (2016). URL http://www.R-project.org/.). Statistical significance was set at $p \leq 0.05$ (two-tailed) for all tests.

RESULTS:

Baseline characteristics

In this older Australian population (n=2 349), total flavonoid intake was normally distributed with a mean of 861.9 mg/d and a SD of 467.5 mg/d. The mean age ± SD was 64.7 ± 9.2 years. The baseline characteristics of the study population overall, and stratified by total flavonoid intake tertiles [T1 (8.8 - 599.9 mg/d); T2 (600 - 1105.9 mg/d); T3 (1106 - 1969 mg/d)], are shown in **Table 1**. Participants in the highest tertile of flavonoid intake were less likely to be current or past smokers, were more likely to be hypertensive and had the lowest intake of alcohol. Intakes of all dietary characteristics, including total energy, increased across flavonoid tertiles.

Flavonoid intake

Mean intakes of each flavonoid subclass, the primary flavonoid compounds within each subclass, and the top three dietary contributors to each subclass are presented in **Table 2**. Pearson correlations between flavonoid subclasses varied from weak (r = -0.03 for isoflavones and flavonols) to high (r = 0.97 for flavonols and total flavonoids). In this population, flavan-3-ols were the greatest contributors to total flavonoid intake, with 96.9% of flavan-3-ols in the diet coming from tea. Of those who drank at least one cup of tea per week (82.0 %), flavan-3-ols accounted for approximately 77.2 % of total flavonoid intake. The second highest contributors to total flavonoid intake were the proanthocyanidins, coming primarily from apples and pears, followed by the flavanones, coming mainly from oranges, orange juice and grapefruit and the flavonols, from tea. The flavonoid subclasses with the lowest mean intakes were the anthocyanidins, coming predominantly from red wine and port, the isoflavones, of which soybean intake contributed the most and lastly the flavones, found mainly in oranges.

Associations between flavonoid intake and mortality

During 28 608 person-years of follow-up, 677 out of 2 349 persons (28.8%) died from any cause. Minimal- and multivariable-adjusted associations of total flavonoid and flavonoid subclass intakes with all-cause mortality are shown in **Table 3.** There was a significant linear trend for a lower risk of all-cause mortality across intake tertiles for the flavone, flavanone, anthocyanidin and proanthocyanidins subclasses (p for trend <0.05), but only that for the anthocyanidin subclass remained significant after multivariable adjustment (p for trend = 0.003). For total flavonoid, flavonol and flavan-3-ol intakes, those in the second tertile were at the lowest risk of death from any cause. In the multivariable-adjusted models, the highest tertile of anthocyanidin intake was associated with a significantly lower risk of all-cause mortality (HR: 0.76; 95% CI: 0.61, 0.94), compared to the lowest tertile. The associations between flavonoid intakes and death due to CVD and CHD are provided in **Supplemental Tables 1 and 2** under "Supplemental data" in the online issue. No significant associations with any flavonoid subclasses were seen after adjustments were made for potential confounders.

There was evidence that the relationship between flavonoid intake and all-cause mortality was non-linear (Table 3) with the greatest hazard reductions seen in the second tertile for many of the flavonoid subclasses. Therefore, restricted cubic spline curves for the multivariable adjusted models were generated (**Figure 2**). The relationships between flavonoid intake and all-cause mortality seen in Figure 2, support the interpretation of Table 3, which suggests possible threshold associations. For total flavonoid intake, and intakes of flavonols, flavan-3-ols, flavanones and isoflavones, participants consuming less than the median intake in tertile 1, had a trend of increased hazard of death from any cause.

<u>Associations between flavonoid-rich food intake and all-cause mortality</u>

Associations between individual foods, contributing to greater than 20% of the intake of any flavonoid subclass (Table 2), and all-cause mortality are presented in **Table 4.** In the minimally-adjusted models, participants in the highest intake tertile had a significantly lower hazard of mortality for tea, apple and pear, orange and red wine intakes when compared to those in the lowest intake tertile. These relationships were attenuated in the fully-adjusted models. For orange juice and grapefruit intakes, those in the second tertile appeared to be at the lowest risk of dying from any cause.

In accordance with the flavonoid subclass results, multivariable-adjusted cubic splines (**Supplemental Figure 1**) illustrate a threshold rather than a dose-response effect for flavonoid-rich wholefoods.

Associations between individual flavonoid compounds and all-cause mortality

We only examined the relationship of individual flavonoid compounds with mean intakes >10 mg/day from sources other than tea with all-cause mortality (**Table 5**). Several individual flavonoid compounds are found almost exclusively in tea, and thus their relationship with all-cause mortality will be a proxy for tea intake. In the minimally-adjusted models, participants in the third tertile for epicatechin, catechin, malvidin and naringenin had a significantly lower risk of all-cause mortality. For quercetin and hesperetin intakes, the greatest risk reductions were seen for participants in the second tertile. These relationships were weakened in the fully-adjusted models. Fully-adjusted spline curves for the relationship between all-cause mortality and major flavonoid compounds are presented in **Supplemental Figure 2.**

Effect modification

No effect modification by age or gender was observed for total flavonoids or any flavonoid subclass (p-interaction >0.1). The associations of total flavonoid and flavonoid subclass intakes with all-cause mortality, stratified by risk of early mortality, are shown in **Table 6**. The protective effect of total flavonoids, flavonols, flavan-3-ols and proanthocyanidins was limited to those participants with at least one risk factor (p for interaction <0.05). The risk of all-cause mortality for flavone, flavanone and anthocyanidin intakes did not differ significantly between the two groups. The associations of flavonoid-rich foods and intakes of individual flavonoid compounds with all-cause mortality, stratified by risk of early mortality, are shown in **Supplementary Tables 3 and 4**. The reduced risk associated with higher intakes of apples, tea, quercetin and epicatechin was also limited to those participants in the 'at risk' group (p for interaction <0.05).

Sensitivity analyses

Including energy intake in model 2 did not change the estimates. Adjusting for other dietary confounders such as fibre, saturated fat, polyunsaturated fat, dietary cholesterol and vitamin C or a diet quality index did not change the outcomes materially. Excluding participants who died within the first two years (n=59), to account for reverse causality bias, slightly strengthened the relationship between total and individual flavonoid subclass intake and all-cause mortality.

DISCUSSION

With almost 20% of NCD-related deaths and 42.3% of CVD deaths in Australia attributable to dietary risk factors [1], identifying optimal dietary patterns for disease prevention is crucial. In this prospective cohort study of 2 349 elderly Australians, we provide evidence of

329 a reduced risk of all-cause mortality with moderate to high intakes of flavonoids and
330 flavonoid-rich foods. Our results indicate that these effects plateau and that the greatest risk
331 reduction is seen when moving from a low to a moderate intake. We also demonstrate for the
332 first time that the inverse association between total flavonoid intake and all-cause mortality
333 was mostly apparent in participants with at least one risk factor for early mortality.

334 In this cohort, approximately 74% of total flavonoid intake was attributed to the flavan-3-ol
335 subclass and 15% came from the proanthocyanidin subclass, where tea and apples were the
336 major dietary contributors, respectively. Intakes of total flavonoids and of each flavonoid
337 subclass were comparable to those reported in another Australian prospective cohort study
338 examining the relationship between flavonoid intake and mortality [25]. In contrast, a
339 considerably lower total flavonoid intake has been reported in several other studies [6, 26, 27]
340 most likely explained by the lower intake of tea in these cohorts.

341 The findings in our study examining the relationship between total flavonoid intake and all-
342 cause mortality are consistent with other studies [18, 26-31]. Although there were apparent
343 risk reductions, they generally no longer remained significant after adjusting for potential
344 confounders. In a meta-analysis of eight studies, participants in the highest versus the lowest
345 category of total flavonoid intake had an 18% lower risk of all-cause mortality (RR: 0.82;
346 95% CI: 0.72–0.92) [7]. However, we and others have shown that the greatest risk reduction
347 is often seen in the moderate flavonoid intake groups [18, 27-29, 31]. Of these eight studies,
348 only one study demonstrated a significantly lower risk of all-cause mortality with high
349 compared to low flavonoid intake (HR: 0.38; 95% CI: 0.22–0.64) [6]. While there are several
350 key differences in study design, dietary assessment, and participant characteristics between
351 these prospective cohort studies, the age of the participants at baseline (80 ± 3 years) and the
352 inclusion of those with prevalent CVD in the study by Ivey *et al.*, could explain why such a

large risk reduction was observed as the population would have been at a very high risk of mortality. Similar non-significant reductions in CVD-related mortality risk with moderate to high flavonoid consumption have been demonstrated in the present study and others [18, 26].

Several studies have examined the relationship between individual flavonoid subclass intakes and all-cause mortality [18, 26-29]. However, results are too inconsistent to suggest that one flavonoid subclass may be more beneficial than another. In the present study, after adjusting for potential confounders, the anthocyanidin subclass was associated with the lowest risk of all-cause mortality while Ivey et al. showed that a high intake of the flavan-3-ol subclass was associated with the lowest risk [28]. For individual flavonoid compounds we show that after adjusting for potential confounders only high intakes of malvidin and moderate intakes of epicatechin were significantly associated with a lower risk of mortality. However, there was a trend for a lower risk with moderate intakes of quercetin and hesperetin and moderate to high intakes of naringenin. To date, only one other study [30] has examined the relationship between individual flavonoid compounds and all-cause mortality; Knekt *et al.,* demonstrated non-significant lower risks in all-cause mortality for quercetin, kaempferol, hesperetin and naringenin.

To our knowledge, this is the first observational study to provide evidence that the protective effects of flavonoids may be confined to populations with unhealthy lifestyle habits placing them at an increased risk of early mortality. Cigarette smoking [32], obesity [33], high alcohol consumption (>2 standard drinks per day) [34] and physical inactivity [35] have been shown to have harmful effects on nitric oxide bioavailability, endothelial function, blood pressure, inflammation, blood lipids, platelet function and thrombosis, while strong evidence suggests that flavonoids can ameliorate these intermediate risk factors for CVD [36-40]. We demonstrated that moderate total flavonoid, flavonol and flavan-3-ol intakes were associated

with a significantly lower risk of all-cause mortality in participants with at least one risk factor while little or no benefit was seen in participants with none of these risk factors. Consistent findings were observed with flavonoid-rich foods (apples and tea) and individual flavonoid compounds (quercetin and epicatechin). This finding may partly explain why we and others have shown only non-significant reductions in risk of all-cause mortality with moderate to high flavonoid consumption. Analyzing this relationship in the entire study population may have diluted the protective effect afforded by flavonoids to those at a higher risk. That anthocyanidin intake was associated with a reduced risk in both the 'at risk' and 'not at risk' subgroups explains why this was the only flavonoid subclass associated with a significantly lower risk of all-cause mortality in the whole study population. This finding warrants further investigation in both observational studies and clinical trials. Additionally, future clinical trials investigating the mechanisms behind the beneficial effects of flavonoids should be conducted in cohorts at risk for CVD, where the potential for improvement might be greater.

In the present study, our analyses suggest possible threshold levels for intakes of total flavonoid, flavonoid subclass, individual flavonoid compounds and flavonoid-rich wholefoods, rather than a dose response effect. Due to the observational nature of the study, we are not able to infer causality or rule out residual or unmeasured confounding. Although adjusting for dietary confounders such as fibre, saturated fat, polyunsaturated fat, dietary cholesterol and vitamin C did not change the outcomes materially, the possibility of flavonoids being a marker of other potentially protective unobserved dietary factors cannot be discounted. However, benefits of flavonoids and flavonoid-rich foods were independent of overall diet quality as associations were unchanged after adjustments for a healthy eating index. Additionally, grouping all risk factors for early mortality together does not allow for the examination of

potentially different modifying effects. For this study we only used baseline dietary intake and covariate data; it is possible that intakes may have changed over the 14 years of follow-up, resulting in some non-differential misclassification of the exposures attenuating the power to detect an association. A second FFQ was completed after 5 years of follow-up; out of 1480 participants who completed both FFQ's, 64% stayed in the same tertile for total flavonoid intake, 15.5% increased their total flavonoid intake and total 19.7% decreased their flavonoid intake. It should also be noted that the estimation of flavonoid intake was based on a US database, meaning that regional variation in the flavonoid content of foods could not be accounted for in this study.

In this prospective cohort study, we demonstrate that a moderate to high intake of flavonoids is protective against mortality in elderly Australians at an increased risk of early mortality. This protective effect was not observed in participants without these risk factors. Results also indicate threshold levels for which benefits are seen.

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Conflicts of Interest:

The authors declare no conflict of interest.

Authors' Contributions:

NPB, JRL and JMH contributed to the study concept and design; VF and PM collected the data; HS calculated the flavonoid intake from FFQ data; NPB conducted the data analysis; NPB drafted the manuscript; all authors critically reviewed the final draft of the manuscript.

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Table 1. Baseline characteristics of study population

	Total population n = 2349	Total flavonoid intake tertiles		
		1 n = 783	2 n = 783	3 n = 783
Total flavonoid intake (mg/day), median [IQR]	861.9 [421.4 – 1291.4]	286 [157.6 – 421.4]	915 [851.6 – 981.6]	1372.1 [1291.4 – 1462.9]
Sociodemographic characteristics				
Age (years)	64.7 ± 9.2	63.2 ± 9.0	65.7 ± 9.6	65.1 ± 8.9
Gender (male), n (%)	971 (41.3)	339 (43.3)	327 (41.8)	305 (39.0)
Body mass index (kg/m^2)	26.0 ± 4.4	26.4 ± 4.6	25.9 ± 4.4	25.8 ± 4.1
Physical activity, n (%)				
None	505 (22.0)	176 (23.2)	177 (23.0)	152 (19.9)
Not vigorous	1022 (44.6)	339 (44.7)	345 (44.8)	338 (44.3)
Vigorous	764 (33.4)	243 (32.1)	248 (32.2)	273 (35.8)
Smoking status, n (%)				
Never	1129 (49.4)	336 (44.6)	396 (41.5)	397 (52.1)
Quit	826 (36.2)	269 (35.7)	289 (37.6)	268 (35.2)
Current	329 (14.4)	148 (19.7)	84 (10.9)	97 (12.7)
Hypertensive, n (%)	1632 (69.7)	520 (66.7)	542 (69.3)	570 (73.1)
High cholesterol, n (%)	1578 (72.3)	509 (70.3)	541 (74.6)	528 (71.93)
SES (home/unit owner), n (%)	2079 (90.3)	664 (86.9)	720 (93.6)	695 (90.3)
Dietary characteristics				
Energy intake (kcals/day, ×1000)	2.1 ± 0.6	1.9 ± 0.6	2.1 ± 0.6	2.2 ± 0.6
Alcohol (drinks/week)	1.8 [0 – 10.5]	2.3 [0 – 10.5]	2.3 [0 – 10.5]	0.8 [0 – 8.3]

Dietary fibre (g/d)	27.3 ± 10.5	25.0 ± 9.7	28.0 ± 10.4	28.9 ± 11.0
Protein (g/d)	87.1 ± 27.1	81.7 ± 28.3	87.6 ± 25.9	91.8 ± 26.2
Carbohydrate (g/d)	233.1 ± 75.5	212.1 ± 74.6	235.9 ± 71.1	251.4 ± 75.7
Saturated fat (g/d)	29.6 ± 13.0	27.7 ± 13.1	29.5 ± 12.1	31.5 ± 13.5
Polyunsaturated fat (g/d)	12.5 ± 5.4	11.7 ± 5.4	12.6 ± 5.4	13.2 ± 5.2
Vitamin C (mg/d)*	182.5 ± 95.0	172.2 ± 87.7	186.1 ± 96.8	189.3 ± 99.4
Cholesterol (mg/d)	294.5 ± 136.4	283.5 ± 139.4	295.2 ± 130.4	304.8 ± 138.6

Data expressed as mean ± SD unless otherwise stated.

*Vitamin C without dietary supplementation

SES, social economic status.

Table 2. Flavonoid intake in study population

Flavonoid class	Respective compounds*	Top three dietary contributors (percentage contribution to class intake)	Intake (mg/d) #
Flavan-3-ols	Thearubigin; Epigallocatechin 3-gallate; Epigallocatechin; Epicatechin 3-gallate; Epicatechin; Catechin; Theaflavin3-apgallate; Theaflavin; Theaflavin3apdigallate; Gallocatechin	Tea (96.9%); apples and pears (1.0%); bananas (0.6%)	641.0 ± 426.1
Proanthocyanidins	Monomers; dimers; trimers; 4–6mers; 7–10mers; polymers	Apples and pears (42.8%); tea (15.0%); plums (6.0%)	134.2 ± 90.8
Flavanones	Hesperetin; Naringenin; Eriodictyol	Orange (46.8%); orange juice (26.1%); grapefruit (21.0%)	34.5 ± 37.6
Flavonols	Quercetin; Kaempferol; Myricetin; Isorhamnetin:	Tea (68.8%); apples and pears (6.8%); beer (2.9%)	32.6 ± 15.7
Anthocyanidins	Malvidin; Cyanidin Delphinidin; Petunidin; Peonidin; Pelargonidin	Red wine (56.8%); port (18.7%); apples and pears (8.9%)	17.1 ± 37.2
Isoflavones	Genistein; Daidzein; Glycitein	Soybeans (24.8%); white bread (14.9%); coffee (12.9%)	1.3 ± 1.6

Flavones	Luteolin; Apigenin	Orange (42.2%); meat and vegetable soup (12.2%); green peas (10.3%)	1.2 ± 0.8

*In descending order of mean intake

#Intakes are presented as mean ± SD.

Table 3. Hazard ratios of all-cause mortality by tertiles of flavonoid intake

	Flavonoid intake tertiles			Test for non-linearity[#]
	1	2	3	
Total Flavonoids				
No. deaths (%)	220 (28.1)	233 (29.8)	224 (31.2)	
Intake (mg/d)*	286.1 (8.8 – 599.9)	915.2 (600 – 1105.9)	1372.1 (1106 – 1969)	
HR (95% CI)				
Model 1	1.00	**0.81 (0.67, 0.97)**	0.90 (0.75, 1.09)	**0.0059**
Model 2	1.00	0.88 (0.72, 1.08)	0.98 (0.80, 1.20)	**0.0343**
Flavonols				
No. deaths	217 (27.7)	229 (29.2)	231 (29.5)	
Intake (mg/d)*	13.4 (1.7 – 25.0)	34.4 (25.0 – 42.3)	49.4 (42.3 – 84.8)	
HR (95% CI)				
Model 1	1.00	**0.81 (0.67, 0.97)**	0.93 (0.77, 1.12)	0.0896
Model 2	1.00	0.85 (0.69, 1.04)	0.98 (0.80, 1.20)	0.2418
Flavones				
No. deaths (%)	247 (31.5)	209 (26.7)	221 (28.2)	
Intake (mg/d)*	0.44 (0.02 – 0.72)	1.02 (0.72 – 1.32)	1.77 (1.32 – 7.70)	
HR (95% CI)				
Model 1	1.00	0.86 (0.71, 1.03)	**0.75 (0.62, 0.90)**	0.1199
Model 2	1.00	0.90 (0.74, 1.10)	0.83 (0.68, 1.02)	0.4691
Flavan-3-ols				
No. deaths (%)	224 (28.6)	231 (29.5)	222 (28.4)	
Intake (mg/d)*	49.1 (1.3 – 310.5)	729.7 (310.6 – 747.6)	1158.0 (747.9 – 1224.0)	
HR (95% CI)				
Model 1	1.00	**0.76 (0.63, 0.91)**	0.86 (0.71, 1.03)	**0.0270**
Model 2	1.00	0.82 (0.67, 1.01)	0.92 (0.75, 1.13)	0.0725
Flavanones				

No. deaths (%)	243 (31.0)	216 (27.6)	218 (27.8)	
Intake (mg/d)*	4.3 (0.05 – 10.3)	24.8 (10.3 – 38.9)	64.3 (38.9 – 548.9)	
HR (95% CI)				
Model 1	1.00	0.86 (0.72, 1.04)	**0.79 (0.66, 0.94)**	**0.0192**
Model 2	1.00	0.91 (0.75, 1.11)	0.91 (0.75, 1.16)	0.1243
Isoflavones				
No. deaths (%)	239 (30.7)	224 (28.6)	214 (27.3)	
Intake (mg/d)*	0.60 (0.01 – 0.81)	0.99 (0.82 – 1.22)	1.61 (1.23 – 29.89)	
HR (95% CI)				
Model 1	1.00	0.96 (0.80, 1.15)	0.98 (0.81, 1.18)	0.0630
Model 2	1.00	0.97 (0.79, 1.18)	0.99 (0.81, 1.21)	0.1482
Anthocyanidins				
No. deaths (%)	258 (33.0)	234 (29.9)	185 (23.6)	
Intake (mg/d)*	2.1 (0.0 – 3.8)	5.6 (3.9 – 9.2)	21.3 (9.2 – 333.7)	
HR (95% CI)				
Model 1	1.00	0.89 (0.74, 1.06)	**0.66 (0.55, 0.80)**	**<0.0001**
Model 2	1.00	1.04 (0.85, 1.26)	**0.76 (0.61, 0.94)**	**0.0015**
Proanthocyanidins				
No. deaths (%)	250 (31.9)	227 (29.0)	200 (25.5)	
Intake (mg/d)*	53.8 (0.2 – 85.9)	116.7 (86.0 – 155.4)	200.4 (155.5 – 747.8)	
HR (95% CI)				
Model 1	1.00	0.88 (0.74, 1.05)	**0.71 (0.59, 0.85)**	0.0999
Model 2	1.00	1.01 (0.83, 1.23)	0.86 (0.70, 1.05)	0.4308

Hazard ratios (95% CI) for 14-year all-cause mortality analysed using multivariate Cox proportional hazard models. Model 1 was adjusted for age and gender and model 2 was adjusted for age, gender, BMI, smoking status, physical activity, alcohol intake, hypertension, hypercholesterolemia and social economic status.

*Median; range in parentheses (all such values). [#]Tests for nonlinearity used analysis of variance to compare the model with only the linear term to the model that includes both the linear and the cubic spline terms.

Table 4. Hazard ratios of all-cause mortality by tertiles of flavonoid-rich foods

	Food intake tertiles			Test for non-linearity[#]
	1	2	3	
Tea				
No. deaths (%)	129 (28.0)	110 (27.4)	438 (29.5)	
Intake (ml/d)*	0 (0-35)	250 (108-250)	625 (625-1000)	
HR (95% CI)				
Model 1	1.00	0.80 (0.62, 1.03)	**0.77 (0.63, 0.94)**	0.0276
Model 2	1.00	0.84 (0.63, 1.10)	0.84 (0.68, 1.05)	0.0770
Apples and pears				
No. deaths (%)	279 (32.4)	222 (28.0)	176 (25.3)	
Intake (mg/d)*	10.5 (1 – 21.1)	64.7 (64.7 – 118.9)	150.5 (150.5 – 602)	
HR (95% CI)				
Model 1	1.00	0.89 (0.74, 1.06)	**0.70 (0.58, 0.85)**	0.1800
Model 2	1.00	1.02 (0.84, 1.23)	0.83 (0.68, 1.03)	0.4240
Oranges				
No. deaths (%)	301 (50.8)	186 (26.2)	190 (28.0)	
Intake (mg/d)*	2.5 (0 – 8.8)	53.8 (17.5 – 53.8)	125.0 (98.8 – 500)	
HR (95% CI)				
Model 1	1.00	**0.83 (0.69, 0.99)**	**0.75 (0.62, 0.90)**	0.0416
Model 2	1.00	0.87 (0.72, 1.06)	0.83 (0.68, 1.02)	0.1188
Orange juice				
No. deaths (%)	312 (31.9)	183 (22.3)	182 (33.1)	
Intake (ml/d)*	2.5 (0 – 2.5)	17.5 (8.8 – 53.8)	125 (98.8 – 500)	
HR (95% CI)				
Model 1	1.00	**0.79 (0.66, 0.95)**	1.00 (0.84, 1.21)	0.0198
Model 2	1.00	**0.80 (0.65, 0.97)**	1.05 (0.87, 1.28)	0.0251
Red wine				

No. deaths (%)	445 (31.5)	116 (27.0)	116 (22.8)	
Intake (ml/d)*	0 (0 – 0)	2.4 (2.4 – 2.4)	16.8 (8.4 – 480)	
HR (95% CI)				
Model 1	1.00	0.90 (0.73, 1.10)	**0.77 (0.63, 0.95)**	<0.0001
Model 2	1.00	0.91 (0.73, 1.14)	0.79 (0.62, 1.00)	0.0027
Grapefruit				
No. deaths (%)	434 (33.0)	114 (22.4)	129 (24.7)	
Intake (mg/d)*	0 (0 – 0)	2.7 (2.7 – 2.7)	18.6 (9.3 – 532)	
HR (95% CI)				
Model 1	1.00	**0.69 (0.56, 0.85)**	**0.74 (0.60, 0.90)**	0.0221
Model 2	1.00	**0.79 (0.63, 0.99)**	0.81 (0.66, 1.00)	0.1668

Hazard ratios (95% CI) for 14-year all-cause mortality analysed using multivariate Cox proportional hazard models. Model 1 was adjusted for age and gender and model 2 was adjusted for age, gender, BMI, smoking status, physical activity, alcohol intake, hypertension, hypercholesterolemia and social economic status.

*Median; range in parentheses (all such values). #Tests for nonlinearity used analysis of variance to compare the model with only the linear term to the model that includes both the linear and the cubic spline terms.

Table 5. Hazard ratios of all-cause mortality by tertiles of individual flavonoid compound intake

	Flavonoid compound intake tertiles			Test for non-linearity[#]
	1	2	3	
Quercetin				
No. deaths (%)	224 (28.6)	227 (29.0)	226 (28.9)	
Intake (mg/d)*	9.00 (1.33 – 15.83)	20.76 (15.84 – 25.16)	29.14 (25.17 – 54.81)	
HR (95% CI)				
Model 1	1.00	**0.82 (0.68, 0.98)**	0.86 (0.72, 1.04)	0.1191
Model 2	1.00	0.85 (0.69, 1.04)	0.96 (0.78, 1.17)	0.2001
Epicatechin				
No. deaths (%)	239 (30.6)	228 (29.1)	210 (26.9)	
Intake (mg/d)*	9.60 (0.59 – 16.10)	21.40 (16.11 – 25.21)	30.83 (25.22 – 66.42)	
HR (95% CI)				
Model 1	1.00	**0.80 (0.66, 0.96)**	**0.75 (0.62, 0.90)**	0.0148
Model 2	1.00	**0.80 (0.66, 0.98)**	0.86 (0.70, 1.05)	0.0340
Catechin				
No. deaths (%)	221 (28.3)	245 (31.3)	211 (27.0)	
Intake (mg/d)*	9.06 (0.20 – 13.46)	17.06 (13.47 – 20.22)	24.33 (20.23 – 57.22)	
HR (95% CI)				
Model 1	1.00	0.96 (0.80, 1.15)	**0.80 (0.67, 0.97)**	0.0048
Model 2	1.00	1.00 (0.82, 1.23)	0.90 (0.73, 1.10)	0.0527
Malvidin				
No. deaths (%)	254 (32.4)	231 (29.6)	192 (24.6)	
Intake (mg/d)*	0.08 (0 – 0.44)	1.34 (0.45 – 2.49)	12.73 (251.04)	
HR (95% CI)				
Model 1	1.00	0.96 (0.80, 1.14)	**0.73 (0.60, 0.88)**	0.0015
Model 2	1.00	0.98 (0.81, 1.19)	**0.75 (0.60, 0.94)**	0.0549
Hesperitin				

No. deaths (%)	249 (31.9)	207 (26.5)	221 (28.2)	
Intake (mg/d)*	2.28 (0 – 5.66)	14.39 (5.67 – 25.60)	37.79 (25.63 – 214.91)	
HR (95% CI)				
Model 1	1.00	**0.79 (0.65, 0.95)**	**0.79 (0.66, 0.95)**	0.0243
Model 2	1.00	0.84 (0.69, 1.03)	0.91 (0.75, 1.11)	0.1295
Naringenin				
No. deaths (%)	247 (31.6)	217 (27.7)	213 (27.2)	
Intake (mg/d)*	1.55 (0 – 3.37)	6.20 (3.38 – 10.39)	17.24 (10.40 – 333.11)	
HR (95% CI)				
Model 1	1.00	**0.82 (0.68, 0.98)**	**0.74 (0.62, 0.89)**	0.0036
Model 2	1.00	0.89 (0.73, 1.08)	0.87 (0.71, 1.07)	0.1301

Hazard ratios (95% CI) for 14-year all-cause mortality analysed using multivariate Cox proportional hazard models. Model 1 was adjusted for age and gender and model 2 was adjusted for age, gender, BMI, smoking status, physical activity, alcohol intake, hypertension, hypercholesterolemia and social economic status.

*Median; range in parentheses (all such values). #Tests for nonlinearity used analysis of variance to compare the model with only the linear term to the model that includes both the linear and the cubic spline terms.

Table 6. Hazard ratios of total mortality by tertiles of flavonoid intake stratified by risk of early mortality

	Not 'at risk' group (n=1 209)			'At risk' group (n=1 140)			P-value for interaction*
	Flavonoid intake tertile			Flavonoid intake tertile			
	1	2	3	1	2	3	
Total Flavonoids							
Model 1	1.00	1.02 (0.77, 1.36)	1.15 (0.87, 1.53)	1.00	**0.69 (0.54, 0.88)**	0.79 (0.61, 1.02)	**0.013**
Model 2	1.00	1.03 (0.76, 1.41)	1.20 (0.89, 1.63)	1.00	**0.69 (0.53, 0.89)**	0.77 (0.59, 1.00)	**0.006**
Flavonols							
Model 1	1.00	1.07 (0.80, 1.43)	1.18 (0.89, 1.57)	1.00	**0.64 (0.50, 0.82)**	0.80 (0.63, 1.03)	**0.015**
Model 2	1.00	1.03 (0.76, 1.40)	1.20 (0.89, 1.62)	1.00	**0.64 (0.49, 0.83)**	0.77 (0.59, 1.01)	**0.007**
Flavones							
Model 1	1.00	0.86 (0.65, 1.14)	0.86 (0.66, 1.12)	1.00	0.91 (0.72, 1.16)	**0.75 (0.58, 0.97)**	0.238
Model 2	1.00	0.90 (0.67, 1.21)	0.82 (0.62, 1.08)	1.00	0.89 (0.69, 1.15)	0.79 (0.61, 1.03)	0.620
Flavan-3-ols							
Model 1	1.00	1.00 (0.75, 1.33)	1.08 (0.81, 1.44)	1.00	**0.62 (0.49, 0.80)**	0.78 (0.61, 1.00)	**0.028**
Model 2	1.00	1.00 (0.74, 1.36)	1.10 (0.81, 1.49)	1.00	**0.61 (0.47, 0.80)**	**0.75 (0.58, 0.98)**	**0.012**
Flavanones							
Model 1	1.00	0.86 (0.64, 1.14)	0.87 (0.67, 1.13)	1.00	0.87 (0.68, 1.10)	0.78 (0.60, 1.00)	**0.063**

Model 2	1.00	0.90 (0.67, 1.22)	0.88 (0.66, 1.17)	1.00	0.88 (0.68, 1.14)	0.81 (0.62, 1.07)	0.160
Isoflavones							
Model 1	1.00	0.91 (0.50, 1.65)	1.31 (0.75, 2.31)	1.00	0.97 (0.55, 1.70)	1.13 (0.65, 1.96)	0.247
Model 2	1.00	1.02 (0.54, 1.94)	1.31 (0.70, 2.45)	1.00	1.05 (0.56, 1.97)	1.17 (0.64, 2.17)	0.440
Anthocyanidins							
Model 1	1.00	0.96 (0.73, 1.25)	**0.69 (0.52, 0.92)**	1.00	0.91 (0.72, 1.16)	**0.69 (0.53, 0.89)**	0.191
Model 2	1.00	1.03 (0.79, 1.37)	**0.71 (0.52, 0.96)**	1.00	0.95 (0.73, 1.22)	**0.70 (0.54, 0.92)**	0.130
Proanthocyanidins							
Model 1	1.00	0.95 (0.72, 1.27)	**0.89 (0.67, 0.17)**	1.00	0.90 (0.71, 1.14)	**0.65 (0.49, 0.85)**	**0.021**
Model 2	1.00	0.99 (0.73, 1.34)	0.91 (0.68, 1.22)	1.00	0.95 (0.74, 1.22)	**0.69 (0.52, 0.92)**	0.037

Hazard ratios (95% CI) for 14-year all-cause mortality analysed using multivariate Cox proportional hazard models.

Model 1 was adjusted for age and gender and model 2 was adjusted for age, gender, hypertension, hypercholesterolemia and social economic status.

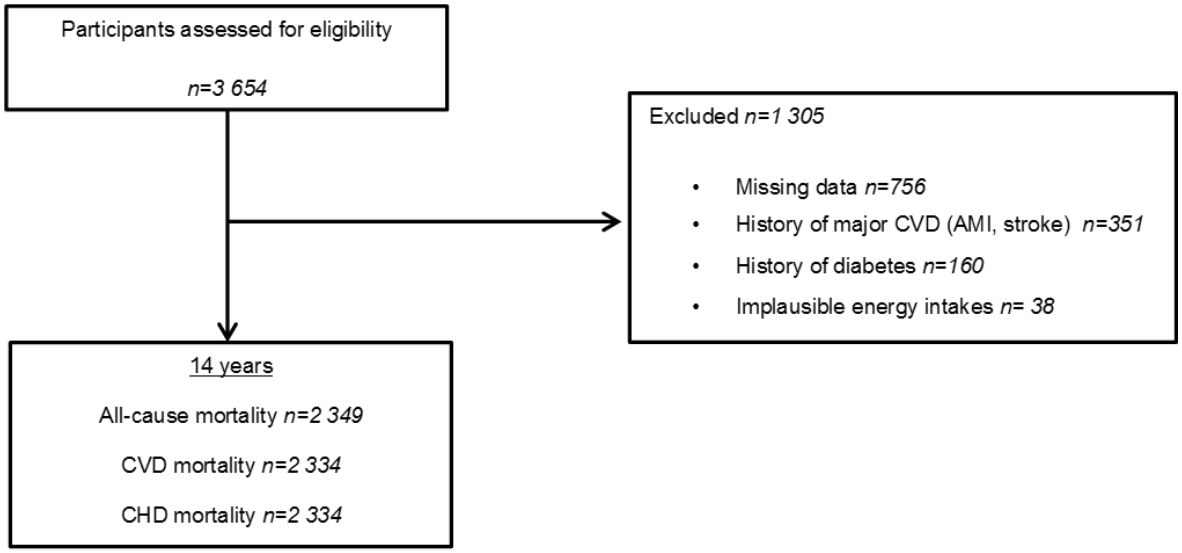
*Likelihood ratio test was used to calculate the P-value for interaction by comparing the model with the product term between flavonoid intake (continuous) and risk of early mortality (no/yes).

FIGURE LEGENDS

Figure 1. Consort flow diagram. AMI, acute myocardial infarction; CHD, coronary heart disease; CVD, cardiovascular disease.

Figure 2. Cubic spline curves for the association between flavonoid intakes (mg/day) and all-cause mortality among participants of the Blue Mountains Eye Study, adjusted for age, gender, BMI, smoking status, physical activity, alcohol intake, hypertension, hypercholesterolemia and social economic status. Individuals with intakes > mean+4 SD's were excluded for each subclass ($n \leq 46$).

Figure 1.


Participants assessed for eligibility
n=3 654
Excluded n=1 305
Missing data n=756
History of major CVD (AMI, stroke) n=351
History of diabetes n=160
Implausible energy intakes n= 38
14 years
All-cause mortality n=2 349
CVD mortality n=2 334
CHD mortality n=2 334