Plasma phospholipid and dietary α-linolenic acid, mortality, CHD and stroke: the Cardiovascular Health Study

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Abstract

Previous studies have suggested that long-chain n-3 fatty acids derived from seafood are associated with a lower risk of mortality, CHD and stroke. Whether α-linolenic acid (ALA, 18 : 3n-3), a plant-derived long-chain essential n-3 fatty acid, is associated with a lower risk of these outcomes is unclear. The aim of the present study was to examine the associations of plasma phospholipid and dietary ALA with the risk of mortality, CHD and stroke among older adults who participated in the Cardiovascular Health Study, a cohort study of adults aged ≥65 years. A total of 2709 participants were included in the plasma phospholipid ALA analysis and 2583 participants were included in the dietary ALA analysis. Cox regression was used to assess the associations of plasma phospholipid and dietary ALA with the risk of mortality, incident CHD and stroke. After adjustment for age, sex, race, enrolment site, education, smoking status, diabetes, BMI, alcohol consumption, treated hypertension and total energy intake, higher dietary ALA intake was found to be associated with a lower risk of total and non-cardiovascular mortality; on comparing the highest quintiles of dietary ALA with the lowest quintiles, the HR for total mortality and non-cardiovascular mortality were found to be 0·73 (95 % CI 0·61, 0·88) and 0·64 (95 % CI 0·52, 0·80), respectively. Dietary ALA was found to be not associated with the risk of cardiovascular mortality, incident CHD or stroke. In conclusion, the results of the present study suggest that dietary ALA, but not plasma phospholipid ALA, is associated with a lower risk of total and non-cardiovascular mortality in older adults.

Key words: Fatty acids: α-Linolenic acid: Mortality: CVD

Previous studies(1–7) have suggested that long-chain n-3 fatty acids derived from seafood are associated with a lower risk of mortality, CHD and stroke. Whether α-linolenic acid (ALA, 18 : 3n-3), a plant-derived long-chain essential n-3 fatty acid, is associated with a lower risk of these outcomes is less clear. Given concerns about the sustainability of fish populations and potential harm from fish contaminants(8,9), a cheaper, plant-derived alternative source of n-3 fatty acids might be important to public health. Therefore, it is essential to understand whether plant-derived ALA exhibits similar associations with the risk of cardiovascular-related morbidity and mortality as seafood-derived n-3 fatty acids.

ALA is found in selected seed and vegetable oils, such as soyabean and rapeseed oils. These oils are used alone or in combination with other vegetable and seed oils in varying concentrations in many foods. Consequently, estimation of dietary ALA using FFQ is prone to measurement error. Plasma phospholipid ALA is an objective biomarker of circulating levels of ALA over the past 1–2 months that reflects diet together with the metabolism of dietary ALA. Thus, dietary and biomarker measures provide complementary information on ALA exposure. In the present study, we investigated the associations of both plasma phospholipid and dietary ALA with the risk of mortality, CHD and stroke among adults aged ≥65 years who participated in the

Abbreviations: ALA, α-linolenic acid; CHS, Cardiovascular Health Study; FADS2, Δ-6-desaturase; LA, linoleic acid.

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Cardiovascular Health Study (CHS), a large community-based prospective cohort study.

Experimental methods

Design and population

The CHS is a community-based prospective cohort study of cardiovascular disease (CVD) and its risk factors among older adults from four geographical areas in the USA (Forsyth County, NC; Sacramento County, CA; Washington County, MD, and Allegheny County, PA). Previous publications have described the study rationale, study design and data collection methodology in detail(10). Briefly, non-institutionalised adults aged ≥65 years were randomly selected and enrolled in the study using Medicare eligibility lists. In total, 5201 participants were enrolled in 1989–1990 and 687 participants (predominantly African Americans) were enrolled 3–4 years later. The study included annual clinical visits with interim phone calls from 1989 to 1999 and phone contact two times per year thereafter. The study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the institutional review board of each centre. Written informed consent was obtained from all subjects/patients.

Among the 5565 study participants alive in 1992–3, fatty acids were quantified in 3941. After the exclusion of 1232 participants with prevalent CVD (myocardial infarction, angina, coronary revascularisation, stroke, transient ischaemic attack or heart failure) at the time of the 1992–3 blood draw, 2709 persons were included in the plasma phospholipid ALA analysis. Among the 3764 participants who completed a semi-quantitative FFQ in 1996, 1181 had prevalent CVD at the time of questionnaire administration and were excluded from the analysis. The remaining 2583 participants comprised the analytical cohort for the dietary ALA analysis. In total, 80·1% of the study participants included in the plasma phospholipid ALA analysis were also included in the dietary ALA analysis.

Data collection

Standardised interviews, physical examinations, medical history review, laboratory evaluations and diagnostic testing were performed at the annual clinic examinations. Fasting blood samples collected from all study participants were stored at −70°C.

Plasma phospholipid α-linolenic acid measurement

Plasma phospholipid ALA was measured using blood samples collected from 1992 to 1993. Plasma lipids were extracted using the methods of Folch et al.(11), as described previously. A one-dimensional TLC was used to separate phospholipids from neutral lipids. Phospholipid fractions were directly transesterified using the LePage & Roy(12) method to prepare fatty acid methyl esters. Individual fatty acid methyl esters were separated using GC, as described previously (Agilent 5890 Gas Chromatograph flame ionisation detector; Agilent Technologies; fused silica capillary column SP-2560 (100 m × 0.25 mm, 0.2 µm), Supelco Bellefonte; initial 160°C for 16 min, ramp 3°C/min to 240°C, hold at 160°C for 15 min)(13). Laboratory inter-assay CV was 3·1% for ALA(13). All fatty acids were processed at the Biomarker Laboratory of the Fred Hutchinson Cancer Research Center (Seattle, WA USA). ALA is expressed as a percentage of total plasma phospholipid fatty acids analysed.

Dietary α-linolenic acid measurement

Previous year’s dietary intake was measured using a Willett 131-item semi-quantitative FFQ administered in 1996. This questionnaire has known reliability and validity(14). To obtain a measure of dietary ALA, the dietary ALA content of each food item was multiplied with the participant’s frequency response and then summed for all foods. For consistency with the measurement of plasma phospholipid ALA, which is assessed as a percentage of total fatty acids, dietary ALA was evaluated as a percentage of total fat, which is correlated better than absolute intake (g/d) or percentage of total energy with plasma phospholipid ALA ($r 0·18)(15,16).

Mortality and CVD assessment

Cause of death and cardiovascular events were adjudicated by a centralised CHS events committee based on information from medical records, laboratory/diagnostic reports, death certificates and/or interviews with next of kin. Details of CHS methods for surveillance and disease classification have been reported in detail previously(10,17). For the purposes of this analysis, the association of plasma phospholipid or dietary ALA with the risk of total mortality, as well as CVD mortality, CHD mortality, and non-CVD mortality, and incident CHD and incident stroke was evaluated. Total CHD mortality was further subclassified as arrhythmic or non-arrhythmic deaths and strokes were subclassified as ischaemic, haemorrhagic or unknown type. The maximum duration of follow-up was 16 years for the plasma phospholipid ALA analysis and 12 years for the dietary ALA analysis.

Statistical analyses

Cox regression was used to examine the associations of plasma phospholipid and dietary ALA with the risk of total and cause-specific mortality, incident CHD and incident stroke (total, ischaemic and haemorrhagic). Circulating ALA and dietary ALA were assessed both categorically (indicator quintiles) and semi-parametrically (cubic splines). Loss to follow-up was considered a censoring event. Schoenfeld’s residuals were used to evaluate the proportional hazards assumption for both plasma phospholipid and dietary ALA. For consistency with the measurement of plasma phospholipid ALA (assessed as a percentage of total fatty acids), dietary ALA was adjusted for total energy to reduce measurement error and confounding by total reported energy(18). Covariates of interest included age, sex, race (European American or African American), enrolment site (Bowman Grey, Davis, Hopkins or Pittsburgh), education...
DHA may influence the associations of ALA with the outcomes of FADS2 genotype and ALA was also examined. As dietary EPA and \( \Delta^-6 \)-desaturase (FADS2) genotype on the risk of death or incident CHD or stroke was assessed. The statistical significance of each multiplicative interaction term was tested using likelihood ratio tests. Potential interactions of ALA and LA were examined as LA competes with ALA for elongation into longer-chain \( \Delta^-3 \) and \( \Delta^-6 \) fatty acids. Sensitivity analyses were also carried out by terminating follow-up at 8 years after the collection of plasma phospholipid or dietary ALA measures to minimise exposure misclassification, which may be higher in later years of follow-up.

The effects of potential interactions of ALA (modelled continuously) with sex, age, LA and \( \Delta^-6 \)-desaturase (FADS2) genotype on the risk of death or incident CHD or stroke was assessed. The statistical significance of each multiplicative interaction term was tested using likelihood ratio tests. Potential interactions of ALA and LA were examined as LA competes with ALA for elongation and desaturation into very-long-chain \( \Delta^-3 \) fatty acids\(^{(20)} \). Similarly, genetic variability in FADS2 may affect the conversion of ALA to EPA and DHA\(^{(20)} \), so the interaction between FADS2 genotype and ALA was also examined. As dietary EPA and DHA may influence the associations of ALA with the outcomes of interest\(^{(21)} \), stratification was done at the 25th percentile of fish intake (0.6 servings/d) in sensitivity analyses.

Single imputation was used to impute missing values for covariates (<2% missing values for all covariates) using data on age, sex, smoking status, education, race, BMI, physical activity, self-reported health status and diabetes at the time of ALA measurement. All statistical analyses were conducted using Stata version 10.0 (Stata Corporation).

## Results

### Association between plasma phospholipid \( \alpha \)-linolenic acid and risk of mortality, CHD and stroke

There were 2709 CHS participants free of CVD and with available plasma phospholipid ALA measures in 1992–3. Of these participants, 36-1% were male, 90-0% were Caucasian, and the median age was 73-0 years (interquartile range: 71-0–98-0 years). The baseline characteristics of the study participants according to quintile of plasma phospholipid ALA are given in online supplementary Table S1. There were 1757 deaths during 32 111 person-years of follow-up. In both age- and sex- and fully adjusted analyses, plasma phospholipid ALA was not associated with the risk of total or cause-specific mortality (Table 1). Similarly, plasma phospholipid ALA was not associated with the risk of incident CHD, stroke or

### Table 1. Hazard ratios (HR) for the association of plasma phospholipid \( \alpha \)-linolenic acid with the risk of total and cause-specific mortality among 2709 US adults

<table>
<thead>
<tr>
<th>Quintiles</th>
<th>I HR 95% CI</th>
<th>II HR 95% CI</th>
<th>III HR 95% CI</th>
<th>IV HR 95% CI</th>
<th>V HR 95% CI</th>
<th>P for trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total mortality</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Person-years</td>
<td>6483</td>
<td>6025</td>
<td>6315</td>
<td>6352</td>
<td>6936</td>
<td></td>
</tr>
<tr>
<td>No. of deaths</td>
<td>360</td>
<td>354</td>
<td>359</td>
<td>331</td>
<td>353</td>
<td></td>
</tr>
<tr>
<td>Age- and sex-adjusted</td>
<td>1.0 (ref.)</td>
<td>1.08</td>
<td>0.93–1.25</td>
<td>1.07</td>
<td>0.92–1.23</td>
<td>0.94</td>
</tr>
<tr>
<td>Non-CVD mortality</td>
<td>235</td>
<td>229</td>
<td>236</td>
<td>209</td>
<td>229</td>
<td></td>
</tr>
<tr>
<td>Age- and sex-adjusted</td>
<td>1.0 (ref.)</td>
<td>1.07</td>
<td>0.89–1.28</td>
<td>1.08</td>
<td>0.90–1.29</td>
<td>0.92</td>
</tr>
<tr>
<td>Total CVD mortality</td>
<td>101</td>
<td>108</td>
<td>102</td>
<td>102</td>
<td>106</td>
<td></td>
</tr>
<tr>
<td>Age- and sex-adjusted</td>
<td>1.0 (ref.)</td>
<td>1.16</td>
<td>0.88–1.52</td>
<td>1.07</td>
<td>0.81–1.41</td>
<td>1.02</td>
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<td>Total CHD mortality</td>
<td>68</td>
<td>64</td>
<td>69</td>
<td>64</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>Age- and sex-adjusted</td>
<td>1.0 (ref.)</td>
<td>1.03</td>
<td>0.74–1.46</td>
<td>1.11</td>
<td>0.79–1.56</td>
<td>0.99</td>
</tr>
<tr>
<td>Arrhythmic death</td>
<td>35</td>
<td>30</td>
<td>38</td>
<td>34</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Age- and sex-adjusted</td>
<td>1.0 (ref.)</td>
<td>0.93</td>
<td>0.57–1.52</td>
<td>1.16</td>
<td>0.73–1.83</td>
<td>1.00</td>
</tr>
<tr>
<td>Non-arrhythmic death</td>
<td>33</td>
<td>34</td>
<td>31</td>
<td>30</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Age- and sex-adjusted</td>
<td>1.0 (ref.)</td>
<td>1.14</td>
<td>0.71–1.85</td>
<td>1.06</td>
<td>0.65–1.74</td>
<td>0.98</td>
</tr>
</tbody>
</table>
| ref., Reference. *Additionally adjusted for race, enrolment site, education, smoking status, diabetes, BMI, waist circumference, physical activity, alcohol consumption and treated hypertension.
stroke subtypes (Table 2). Using restricted cubic splines to model plasma phospholipid ALA, additionally adjusting for plasma phospholipid LA or fish intake, terminating follow-up at 8 years, or performing subgroup analyses by sex or fish intake had no meaningful effect on reported hazard ratios (data not shown). There was no evidence of interactions of ALA with age, sex, FADS2 genotype or plasma phospholipid LA on the risk of death, CHD or stroke (data not shown).

**Association between dietary α-linolenic acid and risk of mortality, CHD and stroke**

In general, the characteristics of the study participants according to quintile of dietary ALA were similar to those according to quintile of plasma phospholipid ALA, except for the absence of observed differences in lipid-lowering medication use or BMI according to quintile of dietary ALA (online supplementary Table S2). In total, 1517 deaths occurred during 25849 person-years of follow-up among participants with dietary intake data. In both minimally and fully adjusted analyses, higher dietary ALA intake was found to be associated with a lower risk of total mortality and non-CVD mortality (Table 3). Hazard ratios did not change materially after additionally adjusting for other dietary factors, such as dietary LA or fish intake. There were no statistically significant associations of dietary ALA with the risk of total CVD mortality, total CHD mortality or CHD-related non-arrhythmic or arrhythmic deaths after adjustment for age, sex, energy intake, race, enrolment site, education, smoking status, alcohol consumption, BMI, diabetes and treated hypertension (Table 3). There was no significant association of dietary ALA with the risk of incident CHD or stroke (Table 4). Similar to the results of the plasma phospholipid analysis, it was found that additionally adjusting for dietary LA or fish intake, terminating follow-up at 8 years, or performing subgroup analyses by fish intake did not affect the results (data not shown). There was also no evidence of interactions between dietary ALA and age, sex, FADS2 genotype or LA on the risk of death, CHD or stroke (data not shown).

In exploratory secondary analyses, the association of dietary ALA with the risk of non-CVD mortality subtypes (i.e. deaths from dementia, cancer, infection, trauma/fracture and respiratory diseases) was further examined. Approximately 60% of the participants who died from non-CVD causes had cancer or dementia listed as causes of death in death records. Higher dietary ALA intake was associated with a lower risk of death from dementia and cancer. There were no statistically significant associations of dietary ALA with the risk of deaths from respiratory diseases, infection or trauma/fracture, although power was limited due to a small number of deaths from these causes (online supplementary Table S3).

**Discussion**

In this large prospective cohort study of older adults, we found no significant associations of plasma phospholipid ALA with the risk of total or cause-specific mortality, CHD or stroke. Dietary ALA was associated with a lower risk of total mortality, which appeared to be related to a significantly lower risk of non-CVD deaths. Dietary ALA was not associated with the risk of CVD mortality, CHD or stroke.

It is interesting that higher dietary ALA intake was associated with a lower risk of total mortality in the study cohort. When specific types of deaths were evaluated, this inverse association was only statistically significant for non-CVD...
deaths, in particular, deaths due to cancer and dementia. Biological mechanisms by which dietary ALA may reduce the risk of non-CVD mortality are not well established. In vitro and rodent studies of cancer suggest that ALA may suppress cancer cell proliferation, inhibit tumour growth and increase apoptosis (22–24). Additionally, animal models indicate that ALA deficiency may promote abnormalities in cerebral structures, and it is hypothesised that low levels of ALA may be associated with poor cognitive function in humans (25). However, the results of epidemiological studies that have examined the relationships of ALA with cancer or dementia in human populations are conflicting (25–29). More research is needed to better understand the mechanisms by which ALA may be associated with a lower risk of non-CVD mortality.

On the other hand, the inverse associations of dietary ALA with the risk of non-fatal CHD or stroke were found (36). On the other hand, the results of a recent meta-analysis of previous observational studies (45) and a small meta-analysis (44) that indicate no association of ALA with the risk of non-fatal or fatal heart disease and with those of two prospective studies (46,47) that found no statistically significant association of circulating ALA with the risk of fatal and non-fatal CHD or stroke (48).

Relatively few studies have assessed the relationship of dietary ALA with the risk of mortality, stroke and CHD, and the findings are inconsistent. The results of the present study support the findings of the Nurses' Health Study that found dietary ALA to be associated with a lower risk of all-cause mortality (51). The findings are consistent with the results of a recent meta-analysis that found no statistically significant association of circulating ALA with the risk of fatal and non-fatal CHD or stroke (52).

In four previous prospective studies that have examined the association of circulating ALA with the risk of incident CHD, stroke or mortality, circulating ALA was found to be not significantly associated with the development of CHD (52,53), stroke (44) or total/CVD mortality (55). Our findings are also consistent with the results of a recent meta-analysis that found no statistically significant association of circulating ALA with the risk of fatal and non-fatal CHD or stroke (52).

Our findings are also consistent with those of two prospective studies (46,47) that found no statistically significant association of circulating ALA with the risk of fatal and non-fatal CHD or stroke (52). On the other hand, the results of the meta-analysis showed a modest inverse association of dietary ALA with the risk of fatal CHD. The results of the present study are inconsistent with those of two studies in which higher dietary ALA intake was found to be associated with a lower risk of fatal IHD (54) and sudden cardiac death (55) among women who participated in the Nurses' Health Study.
n-3 fatty acid. The present study also has several limitations. Plasma phospholipid ALA and dietary ALA were assessed once, and levels may have changed during the follow-up. Nevertheless, terminating follow-up at 8 years, which would minimise effects of misclassification, did not alter the results. For the purposes of the present study, dietary ALA and plasma phospholipid ALA were considered as complimentary measures of ALA exposure. However, plasma phospholipid ALA levels were low (<1% total fatty acids), and the correlation between plasma phospholipid ALA and dietary ALA was modest. It is possible that other tissue compartments with higher proportions of ALA (e.g. adipose tissue) may be a better marker of dietary ALA intake, and more studies are needed to examine the association of other biomarkers of ALA with the risk of mortality, CHD and stroke. Additional limitations include errors in dietary ALA measurement, which if random could attenuate findings towards the null. Although we included several major risk factors as covariates in our analyses of the association of ALA with the risk of mortality, CHD and stroke, residual confounding by unknown or poorly measured factors is possible. As ALA competes with LA for elongation/desaturation, the absolute intakes of ALA, LA and LA are important determinants of ALA metabolism, and the association of ALA with the risk of mortality, CHD and stroke may differ in populations following other diets (e.g. populations with very low intakes of LA). Similarly, all study participants were aged ≥65 years, and the generalisability of these findings to younger populations is not known. On the other hand, we found little evidence for effect modification by LA intake or age.

In summary, in this large prospective cohort of older adults, neither plasma phospholipid nor dietary ALA was associated with CVD mortality, CHD or stroke. The inverse association

Table 4. Hazard ratios (HR) for the association of dietary α-linolenic acid with the risk of incident stroke and CHD among 2583 adults

<table>
<thead>
<tr>
<th>Quintiles</th>
<th>II</th>
<th>HR</th>
<th>95% CI</th>
<th>III</th>
<th>HR</th>
<th>95% CI</th>
<th>IV</th>
<th>HR</th>
<th>95% CI</th>
<th>V</th>
<th>HR</th>
<th>95% CI</th>
<th>P for trend</th>
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<tbody>
<tr>
<td>Total stroke</td>
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<td></td>
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<tr>
<td>No. of cases</td>
<td>4691</td>
<td>4785</td>
<td>4891</td>
<td>4997</td>
<td>5380</td>
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<td></td>
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<tr>
<td>Age–sex- and energy-adjusted</td>
<td>1-0 (ref.)</td>
<td>0.86</td>
<td>0.61-1.21</td>
<td>1.00</td>
<td>0.72-1.39</td>
<td>1.05</td>
<td>0.76-1.45</td>
<td>0.84</td>
<td>0.60-1.17</td>
<td>0.69</td>
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<tr>
<td>Additionally adjusted model*</td>
<td>1-0 (ref.)</td>
<td>0.89</td>
<td>0.64-1.26</td>
<td>0.97</td>
<td>0.70-1.35</td>
<td>1.09</td>
<td>0.78-1.51</td>
<td>0.86</td>
<td>0.60-1.21</td>
<td>0.80</td>
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<tr>
<td>Ischaemic stroke</td>
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<tr>
<td>No. of cases</td>
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<tr>
<td>Age–sex- and energy-adjusted</td>
<td>1-0 (ref.)</td>
<td>0.85</td>
<td>0.59-1.23</td>
<td>0.85</td>
<td>0.59-1.24</td>
<td>1.03</td>
<td>0.72-1.47</td>
<td>0.67</td>
<td>0.45-1.01</td>
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<tr>
<td>Additionally adjusted model*</td>
<td>1-0 (ref.)</td>
<td>0.89</td>
<td>0.61-1.30</td>
<td>0.84</td>
<td>0.58-1.22</td>
<td>1.08</td>
<td>0.75-1.54</td>
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<td>0.47-1.04</td>
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<td>Haemorrhagic stroke</td>
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<tr>
<td>No. of cases</td>
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<tr>
<td>Age–sex- and energy-adjusted</td>
<td>1-0 (ref.)</td>
<td>1.26</td>
<td>0.44-3.63</td>
<td>2.36</td>
<td>0.91-6.08</td>
<td>1.57</td>
<td>0.57-4.36</td>
<td>2.29</td>
<td>0.88-5.99</td>
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<td>1-0 (ref.)</td>
<td>1.19</td>
<td>0.41-3.44</td>
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<td>0.54-4.24</td>
<td>1.96</td>
<td>0.73-5.27</td>
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<tr>
<td>Total CHD</td>
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<tr>
<td>Person-years</td>
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<td>4815</td>
<td>4960</td>
<td>5321</td>
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<td>No. of cases</td>
<td>70</td>
<td>64</td>
<td>75</td>
<td>81</td>
<td>68</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Age–sex- and energy-adjusted</td>
<td>1-0 (ref.)</td>
<td>0.92</td>
<td>0.67-1.27</td>
<td>0.87</td>
<td>0.63-1.21</td>
<td>1.25</td>
<td>0.92-1.70</td>
<td>0.92</td>
<td>0.66-1.28</td>
<td>0.67</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Additionally adjusted model*</td>
<td>1-0 (ref.)</td>
<td>0.97</td>
<td>0.70-1.34</td>
<td>0.88</td>
<td>0.63-1.23</td>
<td>1.25</td>
<td>0.91-1.70</td>
<td>0.93</td>
<td>0.67-1.30</td>
<td>0.75</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ref., Reference.

* Additionally adjusted for race, enrolment site, education, smoking status, diabetes, BMI, alcohol consumption and treated hypertension.
of dietary ALA with the risk of total mortality and non-CVD mortality requires further investigation and replication in other studies.

Supplementary material
To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0007114514001925

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The authors' contributions are as follows: A. M. F. performed the literature review and data analysis as well as wrote the manuscript; R. N. L., D. M. and D. S. S. were the senior investigators of the project and supervised all activities and aided in all aspects of the project, including development of the research questions and writing of the manuscript; C. S., B. M. and D. S. were the biostatisticians of the project and supervised the statistical methods used in the study, as well as reviewed all drafts of the manuscript; I. B. K., B. M. P., E. B. R. and X. S. obtained funding, collected the data, and reviewed and edited all drafts of the manuscript.

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References