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Plasma phospholipid fatty acids and CHD in older men: Whitehall study of London civil servants

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Dietary fatty acids (FA) are the major determinants of blood lipids, and measurements of plasma phospholipid FA (PL-FA) composition that reflect the dietary intake of FA may provide insights into the relationships between diet and CHD. We assessed CHD mortality associations with PL-FA (SFA, PUFA and MUFA) levels measured in a nested case–control study of 116 cases of CHD death and 239 controls that were frequency-matched for age and employment grade. The participants had plasma levels of total cholesterol, LDL-cholesterol (LDL-C), HDL-cholesterol, apo B and apo A₁, C-reactive protein (CRP) and fibrinogen recorded. SFA levels were significantly positively correlated with total cholesterol, LDL-C, apo B, CRP protein and fibrinogen. By contrast, phospholipid-PUFA were inversely associated with CRP, but not with any of the lipids. A higher SFA content (top v. bottom quarter) was associated with a 2-fold higher risk of CHD (OR and 95% CI: OR 2.12; 95% CI: 1.13, 3.99), and an equivalent difference in PUFA was associated with a halving in CHD risk (OR and 95% CI: 0.26, 0.94), but MUFA was unrelated to CHD risk. These associations were substantially attenuated, after additional adjustment for lipids and inflammatory markers. Higher levels of saturated fat and lower levels of polyunsaturated fats were each associated with a higher risk of CHD in elderly men, and these associations were partly explained by their effects on blood lipids and biomarkers of inflammation.

Saturated fat: Polyunsaturated fat: Monounsaturated fat: Heart disease mortality: Elderly: Nested case–control studies

Much of our knowledge about the importance of dietary fats for the prevention of CHD has been derived from the metabolic ward studies, which assessed the effects of varying intakes of dietary fatty acids (FA) on plasma levels of total cholesterol and its fractions (LDL-cholesterol (LDL-C) and HDL-cholesterol (HDL-C))^(1,2). Since the dietary intake of FA is difficult to assess reliably in free-living populations, it is likely that observational epidemiological studies relating the dietary intake of FA in individuals may have underestimated the importance of FA for CHD risk^(3,4). Plasma phospholipid FA (PL-FA) proportions are a valid marker of cell membrane FA proportions that is closely correlated with the intake of dietary FA and can be measured directly^(5,6). Hence, the measurements of PL-FA proportions may provide useful insights into the relationships between diet and CHD risk. In a 6.8-year follow-up of cohort study of older men who were free of CHD, stroke or statin use at baseline, we examined the strength of the associations between plasma PL-FA proportions and (i) plasma lipids (total, LDL-C and HDL-C, and apo B and apo A₁); (ii) inflammatory biomarkers (C-reactive protein (CRP), albumin and fibrinogen); (iii) subsequent risk of death from CHD.

Methods

Study population

Male civil servants working in London and aged 40–69 years (*n* 19 019) were recruited between 1967 and 1970 into the Whitehall study, details of which have been reported previously^(7,8). Following the success of a pilot study of the feasibility of recontacting participants in 1995, all 8448 surviving participants were sought for resurvey during 1997–1998^(9,10). A postal questionnaire asked details of diagnoses (e.g. ever told by a doctor that they had angina, heart attack or diabetes), medications taken in the last month, smoking status and the last known civil service employment grade⁽¹⁰⁾. The 7044 participants who responded to the resurvey (83%) were subsequently sent a blood collection kit and asked to attend their local surgery to have a blood sample collected, blood pressure measured, and height and weight recorded. Non-fasting blood samples were obtained from 5434 men (77% of the respondents). Medical history, medication use and mortality follow-up status (see later) were available for 5360 of these men (98.6%). The resurvey was approved by the ethics committees of the participating institutions.

Abbreviations: CRP, C-reactive protein; FA, fatty acid; HDL-C, HDL-cholesterol; ICD, International Classification of Disease; LDL-C, LDL-cholesterol; PL-FA, plasma phospholipid fatty acid; PL-PUFA, phospholipid-PUFA.

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Mortality follow-up and design of the nested case-control study

The participants who died before October 2005 were identified at the Office for National Statistics, which provided the date and cause of all deaths (including International Classification of Disease (ICD) codes). The mean follow-up period was 6.8 years (maximum 8.4 years). Cause-specific mortality was coded using ICD-9 up to August 2002 and ICD-10 subsequently. CHD deaths were pre-defined as those allocated ICD-9 codes 410–414 and ICD-10 codes I20–I25 as the underlying cause of death. All CHD deaths among individuals with no prior history of heart attack, angina or stroke or use of statins and available plasma were selected as cases. From the remaining men with no prior history of CVD or use of statins, two controls were selected for each case and were frequency-matched to obtain the same 5-year age group by employment grade distribution as that of the cases. A total of 122 cases and 244 controls were selected from which the PL-FA results were available on 116 incident cases and 239 controls.

Laboratory methods

Blood was collected into a 10 ml vacutainer containing potassium EDTA with 0.34 mmol/l aprotinin. These whole-blood samples were mailed in sealed transport tubes at room temperature to the laboratory in Oxford. The mean time in transit was 1.3 d (range 0–7 d), with 78 % arriving within 24 h and 96 % arriving within 48 h of blood collection. On arrival in the laboratory, the blood was centrifuged, and the plasma was aliquoted for storage at -40°C . All lipid analyses were performed on Beckman Synchron CX4 and CX5 auto-analysers (Beckman Coulter UK Limited, High Wycombe, England, UK), which were programmed to subtract a sample blank absorbance reading from the final reaction absorbance to correct for any interference from haemolysis. The total cholesterol was measured enzymatically using Beckman reagent and HDL-C was measured directly using N-geneous reagents (Bio-Stat Limited, Stockport, England, UK). Details of the methods used to measure the other lipid indices (apo A₁, apo B and directly measured LDL-C) have been previously published⁽⁶⁾. The intra-assay coefficients of variation, based on repeat assays of laboratory control material, were 4 % for apo A₁ and apo B, 5 % for HDL-C and LDL-C, and 2 % for total cholesterol. Previous studies had indicated that minor changes in blood lipids arose due to delayed separation of mailed blood samples, so blood lipid values were adjusted for the time spent in transit^(11,12), and also for the date of assay to avoid assay drift. Blood lipids were available in 348 out of a total of 355 men with PL-FA results.

Plasma phospholipid fatty acids

Plasma lipids were extracted with methanol, methylene chloride and saline (1:5:10:15) as previously described⁽¹³⁾. The lipid fraction was removed, the solvent evaporated under nitrogen and resuspended in 1.5 ml of chloroform. The phospholipid fraction was isolated by solid-phase extraction after first removing the neutral lipids and NEFA. Once isolated, the phospholipids were heated with boron trifluoride (14 %) in methanol for 10 min at 100°C to generate FA methyl esters. These were extracted with hexane and water, and then analysed by GC with flame ionisation detection. The GC analysis was carried

out with an Agilent 6890 (Agilent Technologies, Palo Alto, CA, USA) equipped with a capillary column (SP2560, 100 m, Supelco, Bellefonte, PA, USA). The column conditions were as follows: initial temperature, 200°C for 1.8 min; ramp from $19^{\circ}\text{C}/\text{min}$ to 240°C ; hold to 16 min; carrier gas, hydrogen. A mixture of FA (GLC 673b, Nuchek Prep, Elysian, MN, USA) was included with each run as an external standard for peak identification and response factor adjustment. The response factor for palmitic acid was assumed to be 1.0, and that, for all other FA, the response factor was calculated based on this assumption. The FA peak areas were adjusted on a daily basis using these response factors, and adjusted area per cents were calculated. The coefficients of variation for PL-FA composition for SFA, PUFA and MUFA, as well as for total *n*-6 and *n*-3 FA, were 5 % or less. Among the 355 participants, the mean time in post for blood samples before the separation of plasma from whole blood was 1 d or less for 273 (77 %), 2 d for 61 (17 %), 3 d for 13 (4 %), 4 d for 3 (1 %) and 5 d for 5 (1 %), and no attempt was made to correct for days in post on FA proportions.

Statistical methods

Spearman's correlation coefficients were used to examine the associations between PL-FA and plasma levels of lipids and inflammatory biomarkers in all participants using stored blood samples collected at enrolment in both controls and cases. The ANOVA was used to assess the associations of plasma lipids with individual PL-FA and various PL-FA classes. The logistic regression was used to estimate OR (and 95 % CI) of CHD mortality for quartiles of PL-FA among controls after adjustment for the matching factors, age and civil service employment grade. These analyses were repeated with further adjustment for smoking habit, diabetes, treatment for high blood pressure, BMI, systolic blood pressure, apo A₁, HDL-C, apo B and LDL-C, CRP, albumin and fibrinogen. The stepwise change in the χ^2 statistic after making these adjustments provided a quantitative indication of potential confounding effects of these factors⁽¹⁴⁾. In order to preserve all participants in the analyses, multiple imputations were used to generate five datasets containing imputed data in place of the missing values⁽¹⁵⁾. The analyses conducted on each of these datasets gave very similar results and the mean of these estimates is presented. The standard errors for these means are computed as the average standard error across the five datasets plus a term that allows for the variation in the estimates across the five analyses. All analyses were carried out using Statistical Analysis Systems version 8.1 (SAS Institute, Cary, NC, USA).

Results

Characteristics of the study participants

The mean age of cases was 79.2 (SD 4.5) years, and cases and controls were well matched for age, employment grade, smoking, blood pressure, BMI and diabetes mellitus. Approximately 40 % of the cases and 30 % of the controls gave a history of hypertension. The majority of men were non-smokers, mean blood pressure was 145/80 mmHg and mean BMI was $24.8 \text{ kg}/\text{m}^2$. Over 90 % of men had achieved a high socio-economic class (as determined by the last known employment grade).

Case-control differences

Table 1 shows the distribution of proportions of PL-FA markers at enrolment in men who subsequently died of CHD (cases) compared with survivors during the follow-up (controls). Among PL-FA in controls, PL-SFA accounted for 43.5%, polyunsaturated accounted for 40.4%, monounsaturated accounted for 15.6% and *trans*-FA accounted for less than 0.5% of the total PL-FA. CHD cases had higher levels of stearic acid and lower levels of total PUFA and *n*-6 FA arachidonate when compared with controls, but, otherwise, the case-control differences in the FA distributions at enrolment were not statistically significant.

Associations with blood lipids, inflammatory biomarkers and BMI

Table 2 shows the mean plasma levels of blood lipids, CRP and BMI by quarters of the PL-FA. Individuals in the top compared with the bottom quarter of the PL-SFA had higher levels of total

cholesterol, LDL-C and apo B and lower levels of HDL-C compared with those in the bottom quarter of the PL-SFA. Table 2 also shows the Spearman correlation coefficients for the plasma FA with plasma lipids and biomarkers of inflammation. Among the 348 individuals with available data, PL-SFA were positively correlated with apo B (r 0.15, $P=0.004$), LDL-C (r 0.16, $P=0.004$) and total cholesterol (r 0.16, $P=0.003$), respectively. PL-SFA were also positively correlated with CRP (r 0.12, $P=0.02$) and fibrinogen (r 0.11, $P=0.05$), but not with albumin (r 0.07, $P=0.09$). By contrast, phospholipid-PUFA (PL-PUFA) was not significantly associated with any of the lipids, but was inversely correlated with CRP (r -0.13, $P=0.01$). PL-SFA was inversely associated with PL-PUFA (r -0.48, $P<0.001$), and to a lesser extent with PL-MUFA (r -0.15, $P<0.001$).

Associations with IHD mortality

Higher PL-SFA was positively associated with CHD mortality and higher PL-PUFA was inversely associated with CHD

Table 1. Plasma phospholipid fatty acid composition (%) in CHD cases and matched controls at enrolment (Mean values and standard deviations)

	CHD cases (n 116)		Controls (n 239)		P value
	Mean	SD	Mean	SD	
History					
Age (years)	79.2	4.5	78.8	4.9	0.44
Smoking, current smoker (%)	9.2		7.1		0.72
Hypertension (%)	38.8		28.9		0.06
Diabetes (%)	2.7		4.3		0.48
Employment grade, low (%)	6.9		6.3		0.94
Clinical					
Systolic BP (mmHg)	148.6	23.0	144.9	19.9	0.15
Diastolic BP (mmHg)	80.3	11.5	79.8	10.5	0.71
BMI (kg/m ²)	25.2	3.6	24.8	3.1	0.26
Laboratory					
Total cholesterol (mmol/l)	5.68	1.01	5.49	1.03	0.10
LDL-cholesterol (mmol/l)	3.51	0.85	3.35	0.83	0.07
HDL-cholesterol (mmol/l)	1.09	0.40	1.10	0.36	0.94
apo B (g/l)	91.2	25.5	85.6	27.8	0.07
apo A ₁ (g/l)	95.8	18.5	93.7	13.9	0.29
CRP (mg/l)	2.32	1.03	1.55	1.09	<0.001
Fibrinogen (g/l)	3.73	0.81	3.49	0.84	0.01
Albumin (g/l)	39.3	2.7	39.6	3.0	0.25
Saturated (%)					
Palmitic acid (16:0)	29.3	1.6	29.1	1.3	0.27
Stearic acid (18:0)	14.5	1.6	14.2	1.6	0.05
All saturated	43.8	2.0	43.5	1.9	0.11
C18 <i>trans</i> (%)					
Elaidic acid (18:1 <i>n</i> -9 <i>trans</i>)	1.5	1.1	1.4	0.4	0.28
Linoelaidic (18:2 <i>n</i> -6 <i>trans</i> , <i>trans</i>)	0.1	0.1	0.1	0.1	0.28
Monounsaturated (%)					
Oleic acid (18:1 <i>n</i> -9)	12.8	2.2	12.6	2.1	0.44
All monounsaturated	15.9	2.0	15.6	2.4	0.20
Polyunsaturated (%)					
Linoleic acid (18:2 <i>n</i> -6)	21.8	3.4	22.0	3.2	0.54
Arachidonic acid (20:4 <i>n</i> -6)	7.2	1.3	7.5	1.4	0.04
Eicosapentaenoic acid (20:5 <i>n</i> -3)	1.5	1.0	1.5	0.8	0.99
<i>n</i> -6-Docosapentaenoic (22:5 <i>n</i> -6)	0.1	0.1	0.1	0.1	0.85
<i>n</i> -3-Docosapentaenoic (22:5 <i>n</i> -3)	1.1	0.3	1.2	0.2	0.07
DHA (22:6 <i>n</i> -3)	4.4	1.3	4.5	1.4	0.32
Total <i>n</i> -3	7.6	2.2	7.7	2.1	0.51
Total <i>n</i> -6	32.2	3.4	32.7	3.2	0.13
All polyunsaturated	39.7	3.0	40.4	2.0	0.02

BP, blood pressure.

Table 2. Age-adjusted mean values of blood lipids and other risk factors by quarters of plasma fatty acids and associated correlation coefficients

Quarters of fatty acids	Plasma fatty acid mean (%)	apo B (g/l)	apo A ₁ (g/l)	Total cholesterol (mmol/l)	HDL-C (mmol/l)	LDL-C (mmol/l)	CRP* (mg/l)	BMI (kg/m ²)
Number of subjects		348	348	348	348	348	348	330
Saturated fat								
I (lowest)	41.5	0.80	0.93	5.28	1.12	3.21	1.65	24.1
II	42.8	0.87	0.95	5.54	1.10	3.39	1.45	25.0
III	43.9	0.87	0.94	5.52	1.07	3.39	1.98	25.5
IV (highest)	46.1	0.90	0.95	5.79	1.10	3.59	2.07	25.1
Difference (IV – I)	4.6	0.10	0.02	0.51	–0.02	0.38	0.42	1.0
Spearman's correlation		0.15	0.02	0.16	–0.07	0.16	0.12	0.13
P value		0.004	0.73	0.003	0.20	0.004	0.02	0.02
Monounsaturated fat								
I (lowest)	12.8	0.88	0.94	5.62	1.08	3.45	1.62	24.9
II	14.9	0.83	0.97	5.55	1.18	3.43	1.46	24.5
III	16.3	0.86	0.92	5.45	1.05	3.35	2.05	24.9
IV (highest)	18.8	0.87	0.95	5.51	1.08	3.36	2.01	25.4
Difference (IV – I)	6.0	–0.01	0.01	–0.11	0.00	–0.09	0.39	0.5
Spearman's correlation		–0.02	–0.06	–0.05	–0.05	–0.04	0.09	0.04
P value		0.72	0.30	0.37	0.40	0.45	0.11	0.46
Polyunsaturated fat								
I (lowest)	36.5	0.92	0.95	5.72	1.08	3.52	2.12	25.6
II	39.5	0.83	0.94	5.34	1.09	3.29	2.04	25.0
III	41.3	0.84	0.94	5.51	1.09	3.38	1.50	24.8
IV (highest)	43.4	0.86	0.95	5.55	1.13	3.39	1.51	24.3
Difference (IV – I)	6.9	–0.06	0.00	–0.17	0.05	–0.13	–0.61	–1.3
Spearman's correlation		–0.07	0.03	–0.05	0.07	–0.07	–0.13	–0.11
P value		0.17	0.55	0.32	0.18	0.22	0.01	0.04

* Geometric means presented.

mortality, but PL-MUFA was not associated with CHD mortality (Table 3). Specifically, men with PL-SFA in the top quarter had a 2-fold higher risk of CHD (OR 2.12; 95% CI: 1.13, 3.99), compared with men in the bottom quarter, after adjustment for age and employment grade. For PL-PUFA, men in the top compared with the bottom quarter had a 2-fold lower risk of CHD mortality (OR 0.49; 95% CI: 0.26, 0.94), after adjustment for age and grade. By contrast, the differences in PL-MUFA were unrelated to the risk of CHD. The associations with CHD risk for either PL-SFA or PL-PUFA were only slightly attenuated after adjustment for smoking, systolic blood pressure, diabetes and employment grade, but were no longer significant after additional adjustment for lipids and inflammatory biomarkers, respectively. After making these adjustments, the stepwise reductions by almost 50% in the χ^2 statistic for PL-SFA and CHD from 5.4 to 2.6 and for PL-PUFA from 4.9 to 2.9 provided a quantitative indication of potential confounding effects of these factors. These reductions indicate that almost half of the effects of PL-SFA and PL-PUFA were explained through their effects on blood lipids and inflammatory markers.

Discussion

Elderly men with plasma SFA in the top quarter of the population distribution had twice the risk of dying from CHD compared with those in the bottom quarter, and men with higher plasma levels of PUFA in the top quarter were half as likely to die from CHD compared with those in the bottom quarter. This suggests that even at 70 and 80 years of age, FA proportions appear to influence the cardiovascular health. The results of the present study differ from the previous studies linking FA proportions to CHD risk, in that the present

study allowed for adjustment of risk with lipids and biomarkers of inflammation. As such, the study could address the extent to which the link between PL-FA and risk was mediated by known risk factors for CHD. The substantial attenuation in the risk of CHD death in the multivariate model, which included lipids and lipoproteins and biomarkers of inflammation when compared with the simpler age-class model which did not, suggests that the adverse effects of PL-SFA and the beneficial effects of PL-PUFA are partly mediated by the traditional CHD risk factors.

The present study is consistent with previous reports^(16,17) from other studies that reported protective effects of higher levels of PUFA on the prospective risk of CVD; however, it was not consistent with several other studies in which higher PUFA biomarkers were not independently associated with CHD risk^(18–20). The precise reasons for the discrepant findings of different studies of the same topic remain unclear, but may reflect the differences in the distribution of PUFA, prevalent disease or incomplete adjustment for the effects of other CVD risk factors. In the large US Nurses Health study involving 939 CHD events during a 14-year period among 80082 middle-aged women, a higher intake of SFA was associated with increased IHD risk and a higher intake of unsaturated fat (PUFA and MUFA) was associated with a lower IHD risk⁽²⁾. The discrepancy between the studies of dietary intake of unsaturated FA compared with those of blood markers of such FA may reflect the poor correlation of dietary intake with blood markers. The replacement of SFA by either PUFA or MUFA was associated with a further reduction in CHD, but the substitution of SFA by carbohydrate was associated with only small and non-significant differences in the risk of CHD⁽²⁾. The results of the present study based on the plasma PL-FA levels are generally consistent with these

Table 3. Association of CHD mortality with plasma fatty acids (Odds ratios and 95% confidence intervals for quarters of fatty acids after adjustment for other known risk factors)

Fatty acids	Adjustment for covariates	Quartile 1 (lowest)		Quartile 2		Quartile 3		Quartile 4 (highest)		Test for trend (P value)	χ^2
		OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI		
Saturated fat	Age and grade	1.0	Ref.	1.01	0.52, 1.95	1.06	0.55, 2.04	2.12	1.13, 3.99	0.02	5.4
	Above + SBP, BMI, smoking, diabetes	1.0	Ref.	0.96	0.48, 1.91	1.02	0.52, 1.98	2.04	1.06, 3.93	0.03	4.8
	Above + apo A ₁ , HDL-C, apo B, LDL-C	1.0	Ref.	0.86	0.43, 1.73	0.93	0.47, 1.84	1.81	0.92, 3.54	0.07	3.3
Monounsaturated fat	Above + CRP, fibrinogen, albumin	1.0	Ref.	0.91	0.45, 1.87	0.91	0.45, 1.81	1.77	0.89, 3.50	0.11	2.6
	Age and grade	1.0	Ref.	0.97	0.52, 1.82	0.75	0.39, 1.42	1.03	0.55, 1.93	0.86	0.0
	Above + SBP, BMI, smoking, diabetes	1.0	Ref.	1.03	0.54, 1.96	0.71	0.37, 1.39	1.00	0.52, 1.93	0.74	0.1
Polyunsaturated fat	Above + apo A ₁ , HDL-C, apo B, LDL-C	1.0	Ref.	1.03	0.54, 1.98	0.73	0.37, 1.43	0.99	0.51, 1.92	0.75	0.1
	Above + CRP, fibrinogen, albumin	1.0	Ref.	1.08	0.55, 2.10	0.68	0.34, 1.35	0.95	0.49, 1.86	0.59	0.3
	Age and grade	1.0	Ref.	0.65	0.35, 1.21	0.56	0.30, 1.05	0.49	0.26, 0.94	0.03	4.9
	Above + SBP, BMI, smoking, diabetes	1.0	Ref.	0.66	0.35, 1.25	0.58	0.30, 1.12	0.49	0.25, 0.95	0.03	4.5
	Above + apo A ₁ , HDL-C, apo B, LDL-C	1.0	Ref.	0.70	0.37, 1.35	0.62	0.32, 1.20	0.51	0.26, 1.02	0.05	3.7
	Above + CRP, fibrinogen, albumin	1.0	Ref.	0.69	0.35, 1.35	0.65	0.33, 1.30	0.54	0.27, 1.08	0.09	2.9

Ref., reference; SBP, systolic blood pressure; HDL-C, HDL-cholesterol; HDL-C, LDL-cholesterol; CRP, C-reactive protein.

findings and suggest that replacing SFA by either MUFA or preferably PUFA is likely to be a cardioprotective dietary strategy⁽²⁾. However, it must be recognised that neither PL-SFA nor PL-MUFA necessarily reflect the dietary intake since both of these classes of FA can be synthesised *de novo*; PL-PUFA, on the other hand, is reflective of PUFA intakes. While the proportions of PUFA may be influenced by the presence of diabetes or glucose intolerance, it was not possible to exclude the effects of undiagnosed diabetes on the PUFA levels in this population.

In contrast to other studies, there was no relationship between the PL *n*-3 PUFA levels and the risk of CHD death. It is possible that the prospective matching on employment grade (a surrogate for the socio-economic status) may have contributed to this failure. In addition, it must be appreciated that the subjects in this study may have been unusually resistant to CHD, being a mean age of seventy-nine at entry and having no history of CHD, hypercholesterolaemia or statin use. Arachidonic acid levels were lower in cases than in controls. This observation does not support the view that arachidonic acid is pro-atherogenic (since it serves as the substrate for the synthesis of pro-inflammatory eicosanoids), but is consistent with a recent meta-analysis⁽²¹⁾ showing no increase in the risk of CHD events associated with higher arachidonic acid levels.

The results of the present study support expert recommendations in the UK, where there has been a shift in dietary advice away from reducing the total fat intake (and replacing with carbohydrate) towards replacing SFA by PUFA and MUFA^(17,22,23). In addition, higher intakes of *n*-3 FA from fish are recommended, with a target of approximately 450 mg/d. Several large-scale trials are presently underway, assessing the effects on the vascular risk of higher intake of PUFA and the results of these trials are now required to assess the independent relevance of *n*-3 FA and *n*-6 FA for vascular and non-vascular mortality.

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