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Polyunsaturated Fat Intake Estimated by Circulating Biomarkers and Risk of Cardiovascular Disease and All-Cause Mortality in a Population-Based Cohort of 60-Year-Old Men and Women

Running title: *Marklund et al.; Serum PUFA, CVD and Mortality*

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Abstract

***Background*—**High intake of polyunsaturated fatty acids (PUFA) may reduce the risk of cardiovascular disease (CVD) and mortality. Large prospective studies including both sexes and circulating PUFA as dietary biomarkers are needed. We investigated sex-specific associations of major dietary PUFA; eicosapentaenoic acid (EPA), docohexaenoic acid (DHA), linoleic acid (LA), and α-linolenic acid (ALA), with incident CVD and all-cause mortality in a population-based cohort.

***Methods and Results*—**PUFA in serum cholesterol esters were measured at baseline in 2193 Swedish 60-year old women and 2039 men. Using national registers, 484 incident CVD events (294 men and 190 women) and 456 all-cause deaths (265 men and 191 women) were identified during follow-up (median 14.5y), in individuals without prior CVD at baseline. Associations of PUFA with CVD and mortality were evaluated using Cox proportional hazard models. In multivariable adjusted models, 1-SD increments of EPA and DHA were associated with lower risk of incident CVD among women (hazard ratios 0.79 [95% CI 0.64-0.97] and 0.74 [0.61-0.89], respectively). ALA was associated with moderately increased CVD risk in women (1.16 [1.02-1.32]). Inverse associations with all-cause mortality was observed for EPA and DHA among all participants (0.81 [0.72-0.91] and 0.80 [0.72-0.89], respectively) and for LA in men (0.73 [0.64-0.83]).

***Conclusions*—**Serum LA and very long-chain n-3 PUFA, partly reflecting vegetable oil and fish intake, respectively, were inversely associated with all-cause mortality. Inverse associations of EPA and DHA with incident CVD were only observed in women.

Key words: fatty acid, prospective cohort study, cardiovascular disease, mortality, biomarker

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Introduction

Data from observational and clinical studies have shown that dietary fat quality, rather than quantity, is more important in altering atherogenic blood lipids and influencing cardiovascular disease (CVD) risk[1-4]. Traditionally, observational studies have mainly relied on self-reported dietary intake data, which are limited by e.g., reporting bias and inaccurate food databases. However, the use of tissue fatty acid (FA) composition (e.g., in plasma fractions or adipose tissue) as biomarkers of dietary fat has increased in recent years. As most FAs are endogenously synthesized in humans, only a few can be considered reliable biomarkers of dietary fat composition. Among these are two very long-chain (VLC) n-3 polyunsaturated fatty acids (PUFA) of mainly marine origin, eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), as well as the two essential PUFA, linoleic acid (LA; 18:2n-6) and α-linoleic acid (ALA; 18:3n-3), abundant in non-tropical vegetable oils. LA and, to lesser extent, ALA lower LDL cholesterol when replacing saturated fats[5]. However, ALA, unlike LA, may decrease HDL concentrations[5]. EPA and DHA on the other hand have potential anti-arrhythmic effects and may improve endothelial function[6] and lower blood pressure[7] and triglycerides[8]. Thus, different PUFA seem to have selective roles in the development of CVD, whereas possible influences on non-CVD outcomes are less clear[9]. Dietary intake of EPA, DHA, LA, and to a lesser extent ALA are reflected in the FA composition in serum cholesteryl esters (CE), and thus the proportions of these PUFA in CE can be utilized as biomarkers to assess the intake of the respective PUFA[10-12]. Accordingly, FA composition (especially EPA, DHA, and LA) in CE correlates well with self-reported FA intake in both Swedish[13] and other cohorts[14].

Several prospective cohort and nested case-control studies have utilized serum CE FA as biomarkers of dietary fat and investigated possible relationships with risks of all-cause and CVD

mortality[15], fatal coronary heart disease (CHD)[16], and incidences of CHD[17], heart failure[18], as well as stroke[19, 20]. Among middle aged Swedish men, one SD-increase (5.2%) of serum CE LA was associated with 13 and 15 % lower risk of all-cause and CVD mortality, respectively, while no significant associations were observed for serum CE EPA, DHA, or ALA. In addition, a recent meta-analysis suggested that CE LA is associated with a lower risk of fatal CHD, while no such associations have been observed for n-3 PUFA[16]. However, previous studies investigating relationships between serum CE PUFA and CVD-related outcomes have predominantly included men, although men and women may differ in CVD risks and mortality[21]. Thus, identification of possible sex-specific associations might have been hampered by the limited number of women.

Furthermore, recent studies have reported inverse associations of both n-6 and n-3 PUFA in plasma phospholipid with all-cause and cause-specific mortality as well as incident CVD in a US cohort of older men and women[22, 23].

This study investigated associations between biomarkers of PUFA intake (EPA, DHA, LA, and ALA) in serum CE and incident CVD and all-cause mortality in men and women, separately and combined, in a large population-based cohort.

Material and methods

Study population

The current cohort has been utilized for prospective studies on cardiovascular risk, as previously described[24]. In brief, a Swedish population register was used to identify all men and women living in Stockholm County who turned 60 between July 1, 1997 and June 30, 1998. Invitations were sent out between August 1997 and March 1999 asking every third individual identified to participate in health screening, including blood sampling and completion of an extensive

questionnaire regarding disease history, health status, medication, and lifestyle (e.g., nutritional habits, physical activity, alcohol intake, and smoking). Among 5460 individuals invited, 4232 (78%) agreed to participate (2039 men and 2193 women). All study participants underwent physical examinations where anthropometric measures and blood pressure were recorded. Blood samples were collected after overnight fasting. Serum was stored at -80 °C until analysis.

From the questionnaire, alcohol intake was classified as <1 drink (=14 g ethanol) per wk, <1 drink/d, <2 drinks/d, and ≥2 drinks/d. Education level was categorized as primary school (<9 y), secondary school (≤12 y), and university education (>12 y). Physical activity in leisure-time was classified as sedentary, light-intensity, regular moderate-intensity, and regular high-intensity. Smoking was categorized as never, former, and current smoker. Diabetes was registered based on self-reported diabetes diagnosis, medication-treated diabetes, or fasting serum glucose ≥7 mmol/L. Drug-treated hypertension and hypercholesterolemia were identified based on self-reported use of blood pressure-lowering and lipid-lowering drugs, respectively. The study was approved by the Ethics Committee at Karolinska Institutet and all participants gave their informed consent to participate.

Serum cholesteryl ester fatty acid measurement

Fatty acid composition in serum CE was measured by gas chromatography as described earlier[25]. In brief, serum CE were methylated, extracted in petroleum ether, evaporated, and redissolved in hexane before analysis by gas chromatography with flame ionization detection. Thirteen different FA were quantified (14:0-22:6n3) and the proportion of each was expressed as a percentage of all measured FA. Fatty acid composition in one serum sample was repeatedly analyzed in duplicates in all batches for quality control and the intra- and interassay coefficient of variations were ≤0.24 and ≤2.49 %, respectively, for the fatty acids utilized for statistical

analyses (EPA, DHA, LA, and ALA). Data regarding serum FA profile were available for 2133 women and 2017 men. Participants (n=82) without relevant FA data were excluded from statistical analyses.

Endpoint definition

The main outcomes, incident CVD and all-cause mortality, were identified using the Swedish Hospital Discharge and Cause of Death registers. Incident CVD comprised first-time ischemic CVD events including fatal and non-fatal myocardial infarction, fatal and non-fatal ischemic stroke, and hospitalization due to angina pectoris (International Classification of Disease 10th revision (ICD-10) codes: I20, I21, I25, I46, and I63-I66). In order to elucidate whether potential associations between serum FA and all-cause mortality was determined by CVD-related deaths, CVD mortality was included as an additional outcome defined as death due to CVD (primary cause of death specified as ICD-10 codes I10-I99). All participants were followed up regarding incidence of CVD and death until December 31, 2012. Among participants with measured serum FA composition at baseline, 139 women and 229 men with prevalent CVD at baseline (self-reported or identified by register data) or uncertain disease history were excluded from further analyses.

Statistical analysis

Median and interquartile range (IQR) or mean and SD of FA proportions, blood lipids, blood glucose, blood pressure, and BMI were calculated separately for men and women. For skewed variables, Wilcoxon-Mann-Whitney test was utilized to assess differences between sexes[26], while differences in frequencies of baseline characteristics were assessed by χ^2-tests. For variables with normal distribution, sex differences in means were evaluated by two-tailed Student's t-test. Relationships between the selected serum PUFA (EPA, DHA, LA, and ALA) were investigated

by calculating Pearson's correlation coefficients.

Crude and multivariate-adjusted Cox proportional hazard models were employed to calculate hazard ratio (HR) and 95% CI for incident CVD and all-cause mortality in men and women separately and together. In the adjusted models, BMI, smoking, physical activity, education, alcohol intake, diabetes, drug-treated hypertension, and drug-treated hypercholesterolemia at baseline were included as covariates. Serum FA (EPA, DHA, LA, and ALA) were investigated as continuous (per 1-SD increase) and categorical (quartiles) variables. The proportional hazard assumption was not violated according to the Schoenfeld residuals. To retain study power, missing indicator categories were used for missing covariates; the frequency of missing values in each covariate was <1%, and <3% of the study population had missing values in ≥1 covariate. In order to evaluate the impact of fish oil supplementation on associations of EPA and DHA with incident CVD and mortality, analyses of such associations were repeated after exclusion of participants with reported fish oil supplement use. Restricted cubic splines (mkspline command) with 5 knots (located at percentiles 5, 27.5, 50, 72.5, and 95) as previously suggested[27] were utilized for evaluation of potential nonlinear associations. Due to the low number of CVD-related deaths, analyses for CVD mortality were only performed on the total population. $P<0.05$ was considered significant. All statistical calculations were performed using STATA 11 (Stata Corporation, College Station, TX, USA).

Results

At baseline, men had slightly higher fasting glucose, fasting triglycerides, and blood pressure compared to women, who instead had somewhat higher concentrations of total and HDL cholesterol and serum ALA (**Table 1**). In addition, the proportions of never-smokers, low/none-

consumers of alcohol (<1 drink/week), and non-diabetics were greater among women, while men were more physically active (**Table 1**). The use of fish oil supplementation was low (1%) and did not differ between men and women.

No differences between men and women were observed at baseline for the serum LA and EPA, while median serum CE proportions of ALA and DHA were greater in women than men (**Table 1**). Serum proportions of EPA and DHA were highly correlated (r=0.72), while weaker inverse correlations were observed between LA and the two VLC n-3 PUFA (**Supplementary Table 1**). Correlations between serum ALA and the other PUFA were absent or weak (r≤0.16).

During follow-up (median 14.5 y), 304 men and 180 women suffered a first CVD event. By the end of the follow-up period (December 31, 2012), 191 women and 265 men had died. Cancer (ICD10: C00-C99), CVD (ICD10: I00-I99), and respiratory diseases (ICD10: J00-J99), were the most common causes of deaths and were responsible for 47% (54% in women and 42% in men), 25% (17% in women and 30% in men), and 7.0% (8.9% in women and 5.7% in men), respectively, of all deaths.

Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)

Serum CE EPA was inversely associated with incident CVD in women (**Table 2, Supplementary Table 2, Figure 1A-B**), while DHA was associated with lower risk of incident CVD in women as well as in the whole population (**Table 2, Supplementary Tables 3, Figure 1C-D**). After adjustment for potential confounders, a 1-SD increase in EPA and DHA (i.e., 1.0% and 0.2% of total FA, respectively) among women resulted in 21% and 26% lower risk of CVD, respectively (**Table 2**). When extreme quartiles were compared, women in the highest quartiles of EPA and DHA had 41% (adjusted HR 0.59, 95% CI 0.39-0.91) and 52% (adjusted HR 0.48, 95% CI 0.31-0.73) lower risk of CVD, respectively (**Supplementary Tables 2-3**).

EPA and DHA were inversely associated with all-cause mortality in the total population and in men and women separately (**Table 2, Supplementary Tables 2-3, Figure 2A-D**). In the adjusted model, a 1-SD increment in EPA and DHA (approximately 1.0 % and 0.2% of total FA, respectively) was associated with approximately 20% lower mortality risk in men and women (**Table 2**). In the total study population, individuals in the highest EPA and DHA quartiles had 42% (adjusted HR 0.58, 95% CI 0.43-0.77) and 40% (adjusted HR 0.60, 95% CI 0.46-0.79), respectively, decreased mortality risk than those in the lowest quartiles (**Supplementary Tables 2-3**). In the total population, DHA was associated with lower risk of CVD mortality after adjustments for potential confounders, HR 0.75 (95% CI 0.59-0.94); and EPA tended to be inversely associated with CVD mortality, adjusted HR 0.80 (95% CI 0.64-1.00). Exclusion of participants with reported use of fish oil supplements (1% of the study population) did not affect any observed associations.

Linoleic acid (LA)

In the crude model, 1-SD (i.e., 4% of total FA) increment in LA was associated with lower risk of incident CVD in men (crude HR 0.88, 95% CI 0.78-0.99), and although the point estimate of association did not change substantially after adjustment for confounders, the association was no longer significant due to wider confidence interval (**Table 2**). After adjustment, no association between LA and CVD was observed in women and men, either when analyzed separately or combined (**Table 2, Supplementary Table 4**).

However, LA was associated (after adjustments) with decreased all-cause mortality in the total population and in men, but not in women (**Table 2**, **Supplementary Table 4, Figure 2E-F**). Men in the highest LA quartile had 41% lower (adjusted HR 0.59, 95% CI 0.41-0.85) mortality risk than those in the lowest quartile (**Supplementary Table 4**). A 1-SD increment in LA (i.e.,

4% of total FA) was associated with 16% lower risk of CVD mortality in the total study population, adjusted HR 0.84 (95% CI 0.70-1.00, P=0.046).

A-linolenic acid (ALA)

After adjustments for potential confounders, ALA was associated with higher risk of CVD among women (multivariable-adjusted HR 1.16; 95% CI 1.02-1.32 per 1-SD increment, i.e., 0.2% of total FA), but not men or the total population (**Table 2, Supplementary Table 5, Figure 1E-F**). Women in the highest ALA quartile had 72% higher risk compared with those in the lowest quartile, adjusted HR 1.72 (95% CI 1.12-2.66) (**Supplementary Table 5**).

ALA was not associated with all-cause mortality (**Table 2**, **Supplementary Table 5**) or CVD mortality (data not shown).

Discussion

In this population-based cohort study, VLC n-3 PUFA (i.e., EPA and DHA) in serum CE was inversely associated with incident CVD in women and all-cause mortality in both sexes. The major dietary PUFA, LA, was inversely associated with all-cause mortality in men but not in women, and not associated with incident CVD. ALA was somewhat unexpectedly associated with increased risk of incident CVD among women. To our knowledge, this is the largest prospective cohort study with data on both serum PUFA and mortality in men and women.

There were sex-specific associations between serum n-3 PUFA and CVD, i.e., EPA and DHA were inversely associated with incident CVD only in women. Lower CVD risk at higher levels of circulating VLC n-3 PUFA derived from fish intake agrees with previous prospective studies (primary prevention) and with evidence from experimental studies and some clinical trials[8], although the latter evidence from secondary prevention trials using fish oil supplements

are somewhat inconsistent[28]. In one US cohort, serum DHA was inversely associated with heart failure in women only[18], and in a recent meta-analysis, VLC n-3 PUFA biomarkers were inversely associated with CHD in women but not men[29]. In addition, another meta-analysis reported inverse associations between dietary VLC n-3 PUFA and stroke only in women[30]. Discrepancies between men and women could be due to differences in overall dietary patterns[31] or fish intake, the latter currently reflected by higher serum DHA in women. However, previous studies have reported higher DHA concentrations in women than men, independent of intake[32, 33]. Diverse protective effects in men and women may also involve e.g. platelet aggregation which may be inhibited in a sex-specific manner by VLC n-3 PUFA supplementation[34, 35]. Further research is needed to establish whether the sex-specific associations observed between various n-3 PUFA and outcomes are explained by differences in diet, biology (e.g., estrogens), PUFA metabolism, CVD etiology, or a combination of these.

Serum EPA and DHA showed inverse associations with all-cause mortality in both sexes. In accordance, VLC n-3 PUFA in total plasma and plasma phospholipids were inversely associated with sudden cardiac death and with all-cause mortality, respectively, in two separate American cohorts[22, 36]. Interestingly, individual PUFA displayed different associations with diverse CVD-related deaths, e.g., DHA, but not EPA, was inversely associated with cardiac arrhythmia[22]. In serum CE, EPA and DHA were inversely associated with all-cause mortality risk in individuals with CHD[37]. However, no associations between serum VLC n-3 PUFA and all-cause or CVD mortality was observed in Swedish men with baseline measurements ~20 y before the present study. Apart from CVD protective effects of EPA and DHA, inverse associations of VLC n-3 PUFA and all-cause mortality may involve non-CVD diseases, which caused 75% of all deaths in the present study. For example, possible anti-inflammatory effects of

both EPA and DHA[38] may be involved in reducing fatal events in many diseases[22].

LA was not associated with incident CVD, but was strongly associated with lower risk of all-cause mortality in men. In a US cohort, plasma phospholipid LA was inversely associated with total and CVD mortality, with the strongest link to non-arrhythmic CHD mortality[23]. Such findings are in line with LDL-cholesterol lowering effects of LA, a likely contributing mechanism behind the inverse associations between circulating LA and CVD-related mortality observed in other cohorts[15, 16, 23]. Two studies have reported inverse associations between serum LA and mortality (all-cause and CVD-related) among men in Northern Europe[15, 39]. Such data also accord with an inverse association between dietary LA intake and CHD (events and deaths) as reported in a recent meta-analysis, although sex-specific associations were not directly investigated[4]. Although LA was associated with lower risk of CVD mortality in the total study population, the observed inverse association between LA and all-cause mortality in men may indicate a possible protection also of non CVD-related deaths, e.g., cancer[40] or respiratory disease[23].

The observed moderately increased risk of CVD in women with the highest proportions of ALA does not accord with the overall literature and is for several reasons unlikely to reflect a detrimental effect of ALA per se on CVD risk. First, controlled secondary prevention trials have reported protective effects of increased ALA intake[41, 42], or a trend towards decreased CVD risk in women[40]. A recent meta-analysis also reported a non-significant trend toward lower CVD risk at higher levels of ALA biomarkers[43]. Second, ALA in serum CE seems to be a weaker biomarker of dietary intake compared to LA, EPA, and DHA, as reported in Swedish men who was investigated during the same time period as in the present study[13]. A weak correlation between dietary and serum CE ALA could partly be attributed to the extensive beta-oxidation of

ALA and conversion to VLC n-3 PUFA[44]. It is possible that high levels of circulating ALA indicate limited beta-oxidation due to low muscle mass[44], and/or poor conversion of ALA to VLC n-3 PUFA[45]. Notably, such endogenous VLC n-3 PUFA synthesis is generally more efficient in women compared to men[46]. Finally, it is also possible that the current association between ALA and incident CVD is partly explained by high trans-FA or saturated fat content in ALA-containing margarines and spreads. Although reduced in the Nordic countries since the mid-1990's[47], intake of trans-FA from margarines has been suggested to confound associations between adipose ALA and non-fatal MI among Norwegians[48]. Also, ALA in serum CE correlated with butter intake among young Finns[49]. Unfortunately, neither data on trans-FAs or saturated fat intake are available in the present study.

This study has several strengths. To our knowledge, it is the largest study to utilize PUFA in a specific serum lipid fraction as nutritional biomarkers in a prospective cohort with comparable numbers of women and men. The high participation rate (78%) increases generalizability and reduces the risks of selection bias, while the prospective design decreases the risk of recall bias.

A potential limitation was that serum fatty acid assessment was performed once at baseline and temporal fluctuations in biomarker levels may have increased misclassification of exposure during follow-up. However, serum CE FA measured in Swedish men 20 y apart indicated fairly stable FA proportions[50]. Moreover, the results were similar on presenting serum PUFA by quartiles or as continuous PUFA proportions. The relatively few CVD deaths limited the statistical power for this additional endpoint, especially when investigated in men and women separately. Although the Swedish hospital discharge and death registers utilized for determination of CVD events and mortality causes have traditionally been considered accurate[15],

some deaths may have been misclassified. We cannot exclude residual confounding from imprecisely measured or non-measured factors (e.g., dietary patterns and trans-FA). However, adjustments for dietary factors did not substantially affect associations of plasma phospholipid VLC n-3 PUFA with mortality and CVD in a recent US study[22].

In summary, this population-based cohort study of 60-years-old individuals suggests that individual PUFA in serum are associated with lower risks of incident CVD and mortality, associations that are partly sex-specific. Overall, the results support current dietary recommendations to increase n-3 PUFA (EPA and DHA) and n-6 PUFA (LA) from fish and non-tropical vegetable oils, respectively. Future studies should address whether sex-specific recommendations on PUFA intake are warranted.

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Table 1. Baseline characteristics of participants.

	Total (n=3671)	Men (n=1733)	Women (n=1938)	P^*
BMI, kg/m^2	26.7 ± 4.1	26.8 ± 3.7	26.5 ± 4.5	0.0152
Serum LA; % of total fatty acids	48.5± 4.1	48.5± 4.2	48.4 ± 4.1	0.54
Serum ALA; % of total fatty acids	0.88 ± 0.20	0.87 ± 0.20	0.90 ± 0.20	0.0002
Serum EPA; % of total fatty acids	1.88 (1.46-2.47)	1.87 (1.44-2.46)	1.89 (1.47-2.48)	0.40
Serum DHA; % of total fatty acids	0.92 ± 0.25	0.91 ± 0.25	0.93 ± 0.20	0.0095
Total cholesterol, mmol/L	5.99 ± 1.06	5.82 ± 1.01	6.13 ± 1.09	<0.0001
HDL cholesterol, mmol/L	1.50 ± 0.39	1.33 ± 0.33	1.64 ± 0.39	<0.0001
LDL cholesterol, mmol/L	3.89 ± 0.93	3.86 ± 0.90	3.92 ± 0.96	0.0419
Total-to-HDL cholesterol ratio	4.0 (3.3-5.0)	4.5 (3.7-5.3)	3.7 (3.1-4.5)	<0.0001
Fasting glucose, mmol/L	5.2 (4.8-5.7)	5.4 (5.0-5.8)	5.1 (4.7-5.5)	<0.0001
Triglycerides, mmol/L	1.1 (0.8-1.6)	1.2 (0.8-1.7)	1.1 (0.8-1.5)	<0.0001
Systolic blood pressure, mmHg	138 ± 22	143 ± 20	134 ± 22	<0.0001
Diastolic blood pressure, mmHg	84 ± 11	88 ± 10	82 ± 10	<0.0001
Smoking, %				<0.001
Current	21	20	22	
Former	38	45	31	
Never	40	34	46	
Education, %				0.092
≤9 y	28	26	29	
≤12 y	48	44	43	
>12 y%	28	29	27	
Physical activity, %				<0.001
Sedentary	11	10	11	
Light intensity	57	54	60	
Medium intensity	23	25	21	
High intensity	7	9	6	
Alcohol intake†, %				<0.001
<1 drink wk^{-1}	21	11	30	
<1 drink d^{-1}	43	34	50	
<2 drinks d^{-1}	22	32	14	
≥2 drinks d^{-1}	14	23	6	
Diabetes‡, %	6.3	8.5	4.7	<0.001
Drug-treated hypertension, %	16	16	16	0.99
Drug-treated hypercholesterolemia, %	3.5	4.4	2.8	0.012
Use of fish oil supplements, %	1.1	1.0	1.2	0.55

Values are mean± SD, median (Q1-Q3), or percentages by categories

*Sex differences were tested by t-tests for normally distributed variables, by χ^2 in categorical variables, and by Wilcoxon-Mann-Whitney test if the variable was skewed.

†Categorized from questionnaires as 1 drink = 14 g ethanol.

‡Defined as either self-reported diabetes diagnosis, medication-treated diabetes, or fasting serum glucose ≥7 mmol/L.

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Table 2. Cox proportional hazard ratios for serum fatty acids in relation to incident cardiovascular disease (CVD) and all-cause mortality in Swedish men and women.

				Total (n=3671)	Men (n=1733)	Women (n=1938)
Incident CVD	Events (person-years)			484 (48,743)	304 (22,274)	180 (26,469)
	Hazard ratio (95% CI)*	EPA	Crude	0.91 (0.82, 1.01)	0.99 (0.88, 1.12)	0.78 (0.64, 0.96)
			Adjusted†	0.94 (0.84, 1.04)	1.02 (0.90, 1.16)	0.79 (0.64, 0.97)
		DHA	Crude	0.83 (0.75, 0.92)	0.93 (0.83, 1.05)	0.70 (0.59, 0.84)
			Adjusted†	0.87 (0.79, 0.97)	0.96 (0.85, 1.09)	0.74 (0.61, 0.89)
		LA	Crude	0.91 (0.82, 1.00)	0.88 (0.78, 0.99)	0.94 (0.80, 1.09)
			Adjusted†	0.93 (0.84, 1.03)	0.90 (0.79, 1.03)	0.99 (0.84, 1.18)
		ALA	Crude	1.03 (0.94, 1.12)	0.99 (0.88, 1.11)	1.15 (1.01, 1.31)
			Adjusted†	1.07 (0.99, 1.17)	1.02 (0.91, 1.14)	1.16 (1.02, 1.32)
Total mortality	Deaths (person-years)			456 (51,335)	265 (23,917)	191 (27,418)
	Hazard ratio (95% CI)	EPA	Crude	0.78 (0.70, 0.87)	0.79 (0.69, 0.91)	0.78 (0.65, 0.94)
			Adjusted†	0.81 (0.72, 0.91)	0.82 (0.71, 0.95)	0.79 (0.65, 0.96)
		DHA	Crude	0.75 (0.68, 0.84)	0.78 (0.68, 0.89)	0.75 (0.63, 0.89)
			Adjusted†	0.80 (0.72, 0.89)	0.82 (0.71, 0.94)	0.78 (0.66, 0.93)
		LA	Crude	0.81 (0.74, 0.90)	0.73 (0.65, 0.83)	0.93 (0.80, 1.07)
			Adjusted†	0.81 (0.73, 0.90)	0.73 (0.64, 0.83)	0.95 (0.81, 1.12)
		ALA	Crude	1.04 (0.95, 1.13)	1.10 (0.98, 1.23)	0.99 (0.87, 1.13)
			Adjusted†	1.05 (0.97, 1.15)	1.10 (0.99, 1.22)	0.98 (0.86, 1.12)

ALA, α-linolenic acid; CVD, cardiovascular disease; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; LA, linoleic acid.
*Per 1 SD increment: EPA (1.0%), DHA (0.2%), LA (4.1%, 4.2%, and 4.1% in the total population, men, and women, respectively), ALA (0.2%).
†Adjusted for sex (only in analyses of the total study population), BMI, smoking, physical activity, education, alcohol intake, diabetes, drug-treated hypertension, and drug-treated hypercholesterolemia.

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Figure Legends:

Figure 1. Multivariate-adjusted associations of serum eicosapentaenoic acid [A, B], docosahexaenoic acid [C, D], and α-linolenic acid [E, F] with incident CVD among 1938 women [A, C, E] and 1733 men [B, D, F]. Hazard ratios (circles) and 95% CI (error bars) of quartiles of serum fatty acid concentrations (% of total fatty acids) were estimated using Cox models adjusted for BMI, smoking, physical activity, education, alcohol intake, diabetes, drug-treated hypertension, and drug-treated hypercholesterolemia, and plotted against the median fatty acid concentration of each quartile, with the lowest quartile as reference. Linear trends across quartiles were evaluated by assigning participants the median FA concentration of each quartile and assessing this as a continuous variable. Non-linearity was evaluated using restricted cubic splines with five knots located at percentiles 5, 27.5, 50, 72.5, and 95.

Figure 2. Multivariate-adjusted associations of serum eicosapentaenoic acid [A, B], docosahexaenoic acid [C, D], and linoleic acid [E, F] with all-cause mortality among 1938 women [A, C, E] and 1733 men [B, D, F]. Hazard ratios (circles) and 95% CI (error bars) of quartiles of serum fatty acid concentrations (% of total fatty acids) were estimated using Cox models adjusted for BMI, smoking, physical activity, education, alcohol intake, diabetes, drug-treated hypertension, and drug-treated hypercholesterolemia, and plotted against the median fatty acid concentration of each quartile, with the lowest quartile as reference. Linear trends across quartiles were evaluated by assigning participants the median FA concentration of each quartile and assessing this as a continuous variable. Non-linearity was evaluated using restricted cubic splines with five knots located at percentiles 5, 27.5, 50, 72.5, and 95.

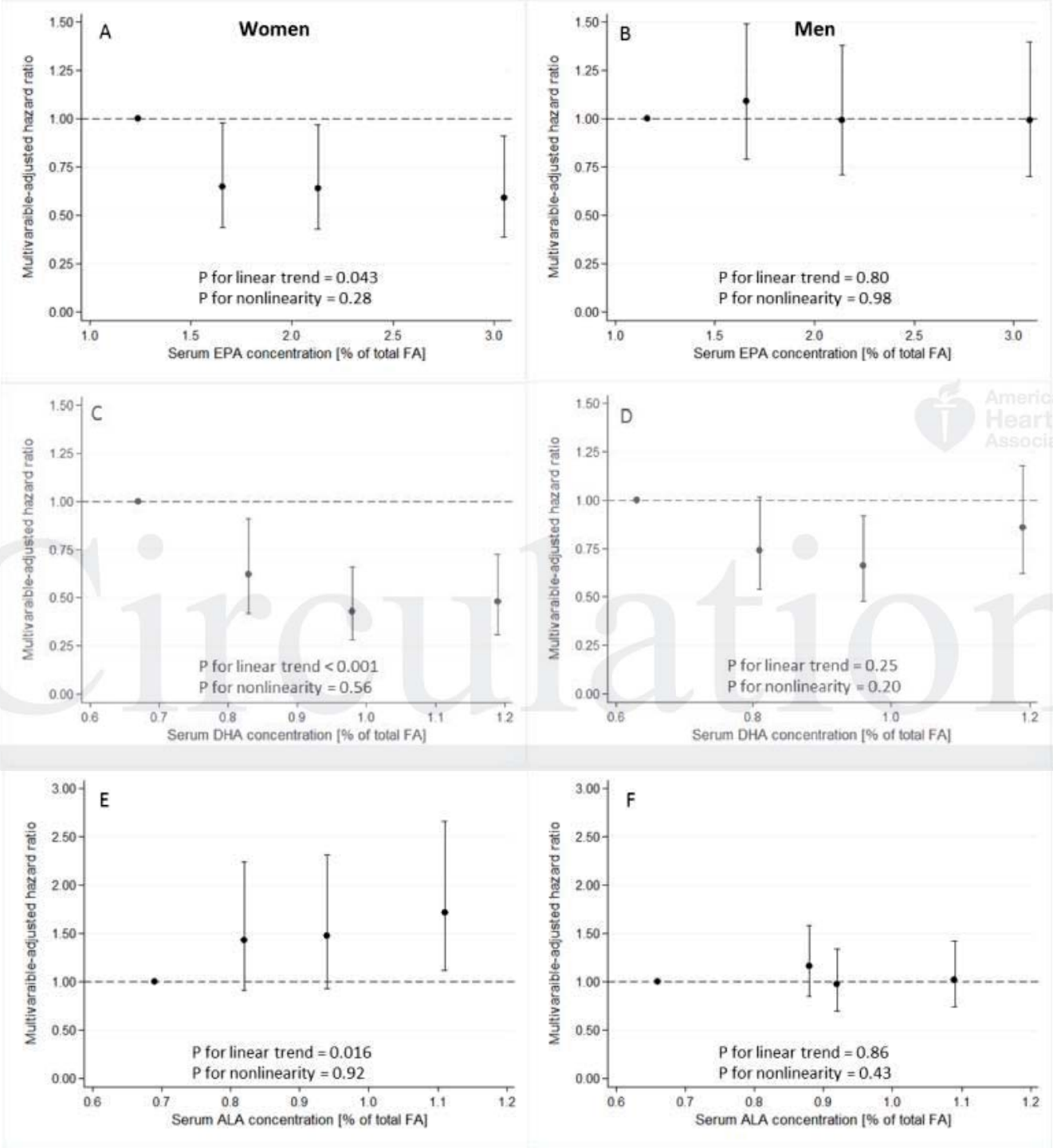
A
Women
Multivaraible-adjusted hazard ratio
1.50
1.25
1.00
0.75
0.50
0.25
0.00
P for linear trend = 0.043
P for nonlinearity = 0.28
1.0
1.5
2.0
2.5
3.0
Serum EPA concentration [% of total FA]
B
Men
Multivaraible-adjusted hazard ratio
P for linear trend = 0.80
P for nonlinearity = 0.98
Serum EPA concentration [% of total FA]
C
Multivaraible-adjusted hazard ratio
P for linear trend < 0.001
P for nonlinearity = 0.56
0.6
0.7
0.8
0.9
1.0
1.1
1.2
Serum DHA concentration [% of total FA]
D
Multivaraible-adjusted hazard ratio
P for linear trend = 0.25
P for nonlinearity = 0.20
Serum DHA concentration [% of total FA]
E
Multivaraible-adjusted hazard ratio
3.00
2.50
2.00
1.50
1.00
0.50
0.00
P for linear trend = 0.016
P for nonlinearity = 0.92
Serum ALA concentration [% of total FA]
F
Multivaraible-adjusted hazard ratio
P for linear trend = 0.86
P for nonlinearity = 0.43
Serum ALA concentration [% of total FA]

Figure 1

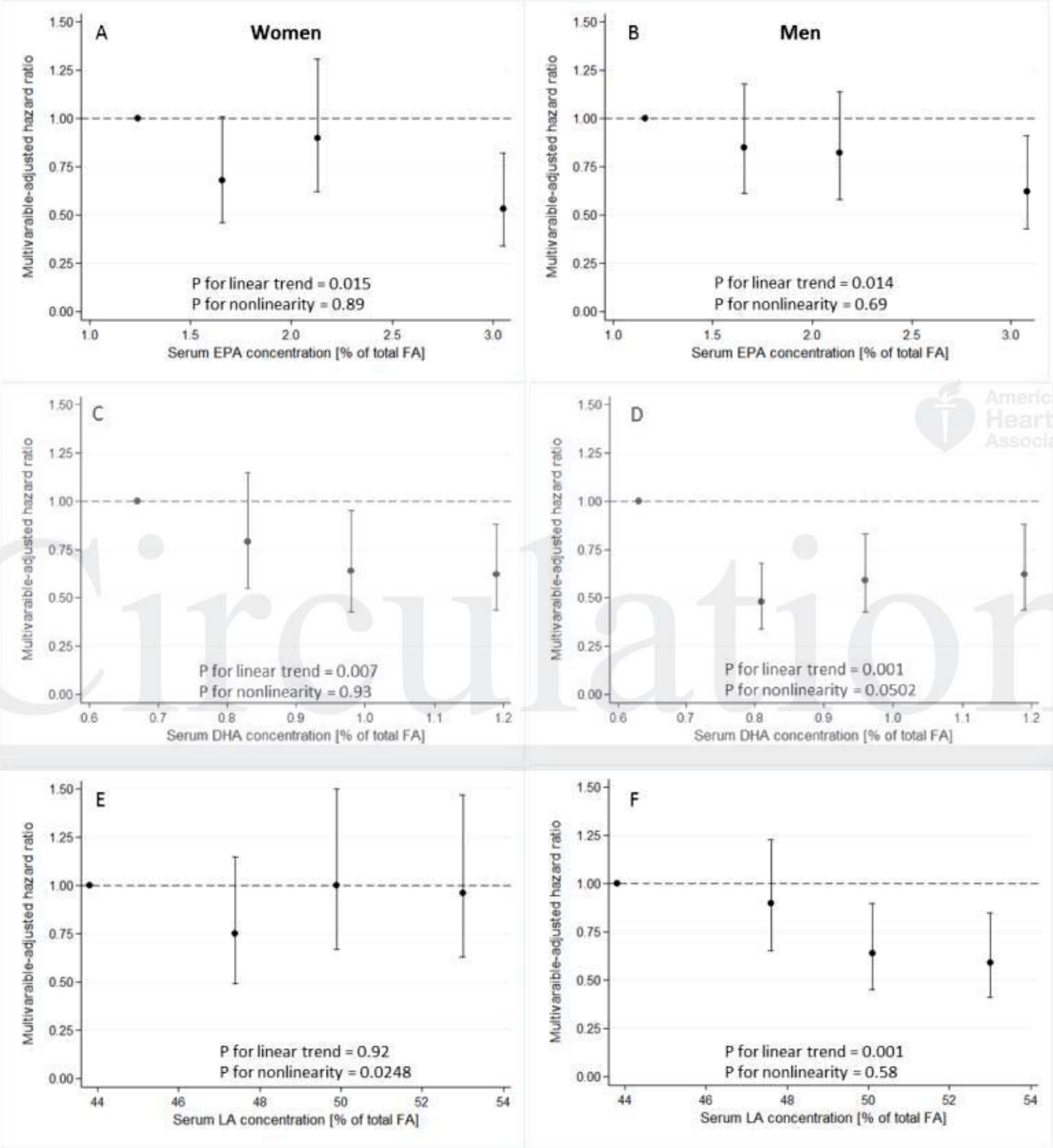
A
Women
Multivaraible-adjusted hazard ratio
1.50
1.25
1.00
0.75
0.50
0.25
0.00
P for linear trend = 0.015
P for nonlinearity = 0.89
1.0
1.5
2.0
2.5
3.0
Serum EPA concentration [% of total FA]
B
Men
Multivaraible-adjusted hazard ratio
P for linear trend = 0.014
P for nonlinearity = 0.69
Serum EPA concentration [% of total FA]
C
Multivaraible-adjusted hazard ratio
P for linear trend = 0.007
P for nonlinearity = 0.93
0.6
0.7
0.8
0.9
1.0
1.1
1.2
Serum DHA concentration [% of total FA]
D
Multivaraible-adjusted hazard ratio
P for linear trend = 0.001
P for nonlinearity = 0.0502
Serum DHA concentration [% of total FA]
E
Multivaraible-adjusted hazard ratio
P for linear trend = 0.92
P for nonlinearity = 0.0248
44
46
48
50
52
54
Serum LA concentration [% of total FA]
F
Multivaraible-adjusted hazard ratio
P for linear trend = 0.001
P for nonlinearity = 0.58
Serum LA concentration [% of total FA]

Figure 2

Supplemental Material

Supplementary Table 1. Correlations between individual fatty acids in serum cholesteryl esters among men (n=1733), *women* (n=1938), *and the total study population.*

		DHA		LA		ALA	
		r	P	r	P	r	P
EPA	Total	0.72	<0.0001	-0.39	<0.0001	0.04	0.0129
	Women	0.70	<0.0001	-0. 39	<0.0001	0.04	0.0541
	Men	0.74	<0.0001	-0.39	<0.0001	0.04	0.1331
DHA	Total			-0.24	<0.0001	-0.15	<0.0001
	Women			-0.25	<0.0001	-0.16	<0.0001
	Men			-0.23	<0.0001	-0.15	<0.0001
LA	Total					0.04	0.0227
	Women					0.05	0.0189
	Men					0.02	0.3585

ALA, α-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; LA, linoleic acid. Values represent Pearsons's correlation coefficients and p-values indicate the significance of correlations.

Supplementary Table 2. Hazard ratios (HR) of Incident Cardiovascular Disease (CVD) and All-Cause Mortality According to Sex-Specific Quartiles of Serum Cholesteryl Ester Eicosapentaenoic acid (EPA) in Swedish Women (n=1938) and Men (n=1733).

		Quartiles of EPA*				
		1	2	3	4	P_{trend}†
Total	Median EPA, % of total FA	1.21	1.66	2.13	3.07	
CVD incidence	Events (person-years)	137 (11,837)	123 (12,201)	113 (12,247)	111 (12,458)	
	Crude HR (95% CI)	1.00 (reference)	0.89(0.68-1.11)	0.79(0.62-1.02)	0.76(0.60-0.98)	0.041
	Adjusted HR (95% CI)‡	1.00 (reference)	0.89(0.70-1.14)	0.83(0.64-1.07)	0.81(0.62-1.06)	0.14
All-cause mortality	Deaths (person-years)	142 (12,586)	113 (12,864)	119 (12,831)	82 (13,053)	
	Crude HR (95% CI)	1.00 (reference)	0.78 (0.61-0.99)	0.82 (0.64-1.04)	0.55 (0.42-0.72)	<0.001
	Adjusted HR (95% CI)‡	1.00 (reference)	0.77 (0.60-1.00)	0.84 (0.66-1.09)	0.58 (0.43-0.77)	<0.001
Female	Median EPA, % of total FA	1.24	1.66	2.13	3.05	
CVD incidence	Events (person-years)	60 (6371)	42 (6653)	42 (6648)	39 (6797)	
	Crude HR (95% CI)	1.00 (reference)	0.67(0.45-0.99)	0.62(0.41-0.93)	0.60(0.40-0.90)	0.032
	Adjusted HR (95% CI)§	1.00 (reference)	0.65(0.44-0.98)	0.64(0.43-0.97)	0.59(0.39-0.91)	0.043
All-cause mortality	Deaths (person-years)	61 (6708)	43 (6893)	54 (6841)	33 (6976)	
	Crude HR (95% CI)	1.00 (reference)	0.68 (0.46-1.01)	0.86 (0.60-1.25)	0.52 (0.34-0.79)	0.007
	Adjusted HR (95% CI)§	1.00 (reference)	0.68 (0.46-1.01)	0.90 (0.62-1.31)	0.53 (0.34-0.82)	0.015
Male	Median EPA, % of total FA	1.16	1.66	2.14	3.08	
CVD incidence	Events (person-years)	77 (5,466)	81 (5,548)	74 (5,599)	72 (5,661)	
	Crude HR (95% CI)	1.00 (reference)	1.04(0.76-1.42)	0.94(0.68-1.29)	0.90(0.65-1.24)	0.41
	Adjusted HR (95% CI)§	1.00 (reference)	1.09(0.79-1.49)	0.99(0.71-1.38)	0.99(0.70-1.40)	0.80
All-cause mortality	Deaths (person-years)	81 (5,879)	70 (5,971)	65 (5,990)	49 (6,077)	
	Crude HR (95% CI)	1.00 (reference)	0.85 (0.62-1.17)	0.78 (0.56-1.08)	0.59 (0.41-0.83)	0.002
	Adjusted HR (95% CI)§	1.00 (reference)	0.85 (0.61-1.18)	0.82 (0.58-1.14)	0.62 (0.43-0.91)	0.014

CVD, cardiovascular disease; HR, hazard ratio; EPA, eicosapentaenoic acid; FA, fatty acids

*Sex-specific quintiles. Prior to analyses of the total population, categorization of quintiles was performed separately in men and women before combining the two sexes. †Linear trend across quartiles was evaluated by assigning participants the median EPA proportion of each sex-specific quartile and assessing this as a continuous variable. ‡Adjusted for sex, BMI, smoking, physical activity, education, alcohol intake, diabetes, drug-treated hypertension, and drug-treated hypercholesterolemia. §Adjusted for BMI, smoking, physical activity, education, alcohol intake, diabetes, drug-treated hypertension, and drug-treated hypercholesterolemia.

Supplementary Table 3. Hazard ratios (HR) of Incident Cardiovascular Disease (CVD) and All-Cause Mortality According to Sex-Specific Quartiles of Serum Cholesteryl Ester Docosahexaenoic Acid (DHA) in Swedish Women (n=1938) and Men (n=1733).

		Quartiles of DHA*				
		1	2	3	4	P_{trend}†
Total	Median DHA, % of total FA	0.65	0.82	0.97	1.19	
CVD incidence	Events (person-years)	164 (11,660)	116 (12,244)	93 (12,449)	111 (12,390)	
	Crude HR (95% CI)	1.00 (reference)	0.67(0.53-0.85)	0.53(0.41-0.68)	0.63(0.49-0.80)	<0.001
	Adjusted HR (95% CI)‡	1.00 (reference)	0.68(0.54-0.87)	0.56(0.43-0.72)	0.69(0.53-0.89)	<0.001
All-cause mortality	Deaths (person-years)	165 (12,534)	101 (12,956)	98 (12,904)	92 (12,941)	
	Crude HR (95% CI)	1.00 (reference)	0.59 (0.46-0.75)	0.57 (0.44-0.73)	0.53 (0.41-0.69)	<0.001
	Adjusted HR (95% CI)‡	1.00 (reference)	0.60 (0.47-0.78)	0.61 (0.47-0.78)	0.60 (0.46-0.79)	<0.001
Female	Median DHA, % of total FA	0.67	0.83	0.98	1.19	
CVD incidence	Events (person-years)	72 (6320)	44 (6589)	31 (6807)	33 (6754)	
	Crude HR (95% CI)	1.00 (reference)	0.58(0.40-0.85)	0.39(0.26-0.60)	0.42(0.28-0.64)	<0.001
	Adjusted HR (95% CI)§	1.00 (reference)	0.62(0.42-0.91)	0.43(0.28-0.66)	0.48(0.31-0.73)	<0.001
All-cause mortality	Deaths (person-years)	65 (6720)	51 (6854)	40 (6923)	35 (6921)	
	Crude HR (95% CI)	1.00 (reference)	0.76 (0.53-1.10)	0.59 (0.40-0.88)	0.52 (0.34-0.78)	0.001
	Adjusted HR (95% CI)§	1.00 (reference)	0.79 (0.55-1.15)	0.64 (0.43-0.95)	0.62 (0.44-0.88)	0.007
Male	Median DHA, % of total FA	0.63	0.81	0.96	1.19	
CVD incidence	Events (person-years)	92 (5,340)	72 (5,655)	62 (5,643)	78 (5,636)	
	Crude HR (95% CI)	1.00 (reference)	0.73(0.60-1.00)	0.63(0.46-0.87)	0.80(0.59-1.08)	0.11
	Adjusted HR (95% CI)§	1.00 (reference)	0.74(0.54-1.02)	0.66(0.48-0.92)	0.86(0.62-1.18)	0.25
All-cause mortality	Deaths (person-years)	100 (5,814)	50 (6,103)	58 (5,980)	57 (6,020)	
	Crude HR (95% CI)	1.00 (reference)	0.47 (0.33-0.66)	0.56 (0.40-0.77)	0.54 (0.39-0.75)	<0.001
	Adjusted HR (95% CI)§	1.00 (reference)	0.48 (0.34-0.68)	0.59 (0.43-0.83)	0.62 (0.44-0.88)	0.001

CVD, cardiovascular disease; HR, hazard ratio; DHA, docosahexaenoic acid; FA, fatty acids

*Sex-specific quintiles. Prior to analyses of the total population, categorization of quintiles was performed separately in men and women before combining the two sexes. †Linear trend across quartiles was evaluated by assigning participants the median DHA proportion of each sex-specific quartile and assessing this as a continuous variable. ‡Adjusted for sex, BMI, smoking, physical activity, education, alcohol intake, diabetes, drug-treated hypertension, and drug-treated hypercholesterolemia. §Adjusted for BMI, smoking, physical activity, education, alcohol intake, diabetes, drug-treated hypertension, and drug-treated hypercholesterolemia.

Supplementary Table 4. Hazard ratios (HR) of Incident Cardiovascular Disease (CVD) and All-Cause Mortality According to Sex-Specific Quartiles of Serum Cholesteryl Ester Linoleic Acid (LA) in Swedish Women (n=1938) and Men (n=1733).

		Quartiles of LA*				
		1	2	3	4	P_{trend}†
Total	Median LA, % of total FA	43.8	47.5	50.0	53.0	
CVD incidence	Events (person-years)	131 (11,984)	125 (12,178)	106 (12,402)	122 (12,180)	
	Crude HR (95% CI)	1.00 (reference)	0.94 (0.73-1.20)	0.78 (0.60-1.01)	0.91 (0.71-1.17)	0.32
	Adjusted HR (95% CI)‡	1.00 (reference)	1.01 (0.79-1.30)	0.86 (0.66-1.12)	1.02 (0.78-1.33)	0.86
All-cause mortality	Deaths (person-years)	139 (12,679)	113 (12,856)	106 (12,937)	98 (12,863)	
	Crude HR (95% CI)	1.00 (reference)	0.80 (0.62-1.02)	0.74 (0.58-0.96)	0.69 (0.53-0.90)	0.005
	Adjusted HR (95% CI)‡	1.00 (reference)	0.84 (0.65-1.08)	0.79 (0.59-1.00)	0.73 (0.55-0.95)	0.015
Female	Median LA, % of total FA	43.8	47.4	49.9	53.0	
CVD incidence	Events (person-years)	52 (6,583)	41 (6,661)	42 (6,694)	45 (6,531)	
	Crude HR (95% CI)	1.00 (reference)	0.78 (0.52-1.17)	0.79 (0.53-1.19)	0.87 (0.59-1.30)	0.50
	Adjusted HR (95% CI)§	1.00 (reference)	0.86 (0.57-1.32)	0.94 (0.61-1.44)	1.08 (0.70-1.66)	0.74
All-cause mortality	Deaths (person-years)	55 (6,828)	39 (6,910)	50 (6,872)	47 (6,807)	
	Crude HR (95% CI)	1.00 (reference)	0.70 (0.46-1.05)	0.90 (0.62-1.32)	0.86 (0.58-1.27)	0.63
	Adjusted HR (95% CI)§	1.00 (reference)	0.75 (0.49-1.15)	1.00 (0.67-1.50)	0.96 (0.63-1.47)	0.92
Male	Median LA, % of total FA	43.8	47.6	50.1	53.0	
CVD incidence	Events (person-years)	79 (5,401)	84 (5,517)	64 (5,708)	77 (5,649)	
	Crude HR (95% CI)	1.00 (reference)	1.04 (0.76-1.41)	0.76 (0.55-1.06)	0.93 (0.68-1.27)	0.32
	Adjusted HR (95% CI)§	1.00 (reference)	1.11 (0.81-1.51)	0.83 (0.59-1.17)	1.03 (0.73-1.44)	0.77
All-cause mortality	Deaths (person-years)	84 (5,851)	74 (5,946)	56 (6,065)	51 (6,056)	
	Crude HR (95% CI)	1.00 (reference)	0.86 (0.63-1.18)	0.63 (0.45-0.89)	0.58 (0.41-0.82)	<0.001
	Adjusted HR (95% CI)§	1.00 (reference)	0.90 (0.65-1.23)	0.64 (0.45-0.90)	0.59 (0.41-0.85)	0.001

CVD, cardiovascular disease; HR, hazard ratio; LA, linoleic acid, FA, fatty acids

*Sex-specific quintiles. Prior to analyses of the total population, categorization of quintiles was performed separately in men and women before combining the two sexes. †Linear trend across quartiles was evaluated by assigning participants the median LA proportion of each sex-specific quartile and assessing this as a continuous variable. ‡Adjusted for sex, BMI, smoking, physical activity, education, alcohol intake, diabetes, drug-treated hypertension, and drug-treated hypercholesterolemia. §Adjusted for BMI, smoking, physical activity, education, alcohol intake, diabetes, drug-treated hypertension, and drug-treated hypercholesterolemia.

Supplementary Table 5. Hazard ratios (HR) of Incident Cardiovascular Disease (CVD) and All-Cause Mortality According to Sex-Specific Quartiles of Serum Cholesteryl Ester A-linolenic Acid (ALA) in Swedish Women (n=1938) and Men (n=1733).

		Quartiles of ALA*				
		1	2	3	4	P_{trend}†
Total	Median ALA, % of total FA	0.68	0.81	0.93	1.10	
CVD incidence	Events (person-years)	110 (12,206)	128 (12,164)	118 (12,212)	128 (12,162)	
	Crude HR (95% CI)	1.00 (reference)	1.17 (0.91-1.51)	1.07 (0.83-1.39)	1.17 (0.91-1.51)	0.67
	Adjusted HR (95% CI)‡	1.00 (reference)	1.23 (0.95-1.59)	1.13 (0.87-1.47)	1.25 (0.96-1.61)	0.16
All-cause mortality	Deaths (person-years)	107 (12,806)	104 (12,857)	122 (12,854)	124 (12,817)	
	Crude HR (95% CI)	1.00 (reference)	0.97(0.74-1.27)	1.14 (0.88-1.48)	1.15 (0.89-1.50)	0.30
	Adjusted HR (95% CI)‡	1.00 (reference)	0.99 (0.75-1.30)	1.15 (0.88-1.49)	1.15 (0.89-1.50)	0.19
Female	Median ALA, % of total FA	0.69	0.82	0.94	1.11	
CVD incidence	Events (person-years)	33 (6637)	45 (6606)	46 (6626)	56 (6601)	
	Crude HR (95% CI)	1.00 (reference)	1.38 (0.88-2.16)	1.40 (0.90-2.20)	1.72 (1.12-2.64)	0.015
	Adjusted HR (95% CI)§	1.00 (reference)	1.43 (0.91-2.24)	1.47 (0.93-2.31)	1.72 (1.12-2.66)	0.016
All-cause mortality	Deaths (person-years)	45 (6840)	46 (6824)	52 (6878)	48 (6876)	
	Crude HR (95% CI)	1.00 (reference)	1.03 (0.68-1.55)	1.16 (0.78-1.72)	1.07 (0.71-1.60)	0.67
	Adjusted HR (95% CI)§	1.00 (reference)	1.02 (0.67-1.55)	1.15 (0.79-1.72)	1.02 (0.68-1.55)	0.82
Male	Median ALA, % of total FA	0.66	0.88	0.92	1.09	
CVD incidence	Events (person-years)	77 (5,569)	83 (5,558)	72 (5,586)	72 (5,561)	
	Crude HR (95% CI)	1.00 (reference)	1.08 (0.79-1.47)	0.93 (0.68-1.29)	0.94 (0.68-1.29)	0.52
	Adjusted HR (95% CI)§	1.00 (reference)	1.16 (0.85-1.58)	0.97 (0.70-1.34)	1.02 (0.74-1.42)	0.86
All-cause mortality	Deaths (person-years)	62 (5,967)	58 (6,034)	70 (5,976)	75 (5,940)	
	Crude HR (95% CI)	1.00 (reference)	0.92 (0.64-1.31)	1.13 (0.80-1.59)	1.22 (0.87-1.71)	0.15
	Adjusted HR (95% CI)§	1.00 (reference)	0.97 (0.67-1.39)	1.12 (0.79-1.58)	1.23 (0.88-1.73)	0.16

CVD, cardiovascular disease; HR, hazard ratio; ALA, α-linolenic acid, FA, fatty acids

*Sex-specific quintiles. Prior to analyses of the total population, categorization of quintiles was performed separately in men and women before combining the two sexes. †Linear trend across quartiles was evaluated by assigning participants the median ALA proportion of each sex-specific quartile and assessing this as a continuous variable. ‡Adjusted for sex, BMI, smoking, physical activity, education, alcohol intake, diabetes, drug-treated hypertension, and drug-treated hypercholesterolemia. §Adjusted for BMI, smoking, physical activity, education, alcohol intake, diabetes, drug-treated hypertension, and drug-treated hypercholesterolemia.