

Omega-3 polyunsaturated fatty acid biomarkers and coronary heart disease: pooling project of 19 cohort studies

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Abstract

Importance: The role of omega-3 polyunsaturated fatty acids for primary prevention of coronary heart disease (CHD) remains controversial. Most prior longitudinal studies evaluated self-reported consumption, rather than biomarkers.

Objective: To evaluate biomarkers of seafood-derived eicosapentaenoic (EPA; 20:5n-3), docosapentaenoic (DPA; 22:5n-3), and docosahexaenoic acid (DHA; 22:6n-3), and plant-derived α -linolenic acid (ALA; 18:3n-3), and incident CHD.

Data Sources: A global consortium of 19 studies.

Study Selection: Available prospective (cohort, nested case-control) or retrospective studies with circulating or tissue omega-3 biomarkers and ascertained CHD.

Data Extraction and Synthesis: Each study conducted standardized individual-level analysis using harmonized models, exposures, outcomes, and covariates. Findings were centrally pooled using inverse-variance meta-analysis. Heterogeneity was examined by age, sex, race, diabetes, statins, aspirin, omega-6 levels, and *FADS*-desaturase genes.

Main Outcome(s) and Measure(s): Incident total CHD, fatal CHD, and nonfatal myocardial infarction (MI).

Results: The 19 studies comprised 16 countries, 45,637 unique individuals, and 7973 total CHD, 2781 fatal CHD, and 7157 non-fatal MI events, with omega-3 measures in plasma, phospholipids, cholesterol esters, and adipose. In continuous (per 1 standard deviation increase) multivariable-adjusted analyses, omega-3 biomarkers ALA, DPA, and DHA were associated with lower risk of fatal CHD, with RRs (95% CI): ALA 0.91(0.84-0.98), DPA 0.90(0.85-0.96), and DHA 0.90(0.84-0.96), with EPA showing a trend towards lower risk (0.91 (0.82-1.00)).

DPA, but not ALA, EPA, or DHA, was associated with lower risk of total CHD, with RRs (95% CI): 0.94(0.90-0.99), 1.00(0.95-1.05), 0.94(0.87-1.02), and 0.95(0.91-1.00), respectively.

Significant associations with nonfatal MI were not evident. Associations appeared generally stronger in phospholipids and total plasma. Restricted cubic splines did not identify evidence for nonlinearity in dose-responses.

Conclusions and Relevance: Based on available studies of free-living populations globally, biomarker concentrations of both seafood and plant-derived omega-3s are associated with a modestly lower incidence of fatal CHD.

Introduction

Experimental studies and randomized trials support a protective effect of omega-3 polyunsaturated fatty acids (omega-3 PUFA) on coronary heart disease (CHD) risk pathways and clinical risk factors (1). Yet, key controversies remain. First, randomized trials utilizing fish oil supplements have shown mixed effects on CHD events (2-8). However, most provided supplements for a few years or less and were conducted in patients with pre-existing CHD, or at high risk and taking multiple cardiovascular drugs. Further, the background dietary intake in these trials was not usually measured, and many participants may have had adequate intake from diet alone. Thus, their generalizability and relevance for long-term effects of dietary omega-3 PUFA for primary CHD prevention are uncertain. Second, while several prior observational studies have found inverse associations between seafood-derived omega-3 PUFA and CHD death, potential effects on nonfatal myocardial infarction (MI) or total CHD are less clear (9). Most prior studies also relied on self-reported dietary questionnaires, which may produce errors or bias in recall. A handful of studies have measured objective circulating or tissue omega-3 PUFA levels (1) but such findings could be limited by publication bias. In addition, prior individual biomarker studies were generally underpowered to explore potentially relevant differences in effects depending on underlying participant characteristics, medication use, or genetic variation. Thus, uncertainties remain about effects of omega-3 PUFA on CHD.

Most studies have evaluated combined intakes or biomarker levels of long-chain omega-3 PUFA. Yet, individual omega-3 PUFA including eicosapentaenoic acid (EPA; 20:5n-3), docosapentaenoic acid (DPA; 22:5n-3), and docosahexaenoic acid (DHA; 22:6n-3) may have both shared and complementary effects (10). Similarly, the majority of prior research has

focused on seafood-derived long-chain omega-3 PUFA, and potential CHD effects of plant-derived α -linolenic acid (ALA; 18:3n-3) are far less understood (11).

To address each of these important questions, we developed a global consortium including available studies with circulating or tissue omega-3 biomarkers and ascertained incident CHD. Our aims were to evaluate the associations of individual seafood- and plant-derived omega-3 PUFA with incident total CHD, fatal CHD, and nonfatal MI. We also explored dose-response and potential heterogeneity in effects according to key underlying participant characteristics, including medication use. Our primary hypothesis was that biomarkers of long-chain omega-3 PUFA would be associated with lower risk of incident fatal CHD, but not nonfatal MI.

Methods & Statistics

Consortium formation

The Fatty acids and Outcomes Research Consortium (FORCe) is an extension of the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) Fatty Acids Working Group (12, 13), originally developed to investigate effects of genetic variation on biomarker fatty acid levels. Using direct expert contacts and by reviewing the literature, we identified large studies that had measured circulating or tissue biomarkers of omega-3 PUFA in general populations and had ascertained incident CHD. We focused on prospective (cohort, nested case-control) studies; retrospective studies were included if fatty acids were measured in adipose tissue at the time of the first event given stability of adipose measures. Studies were asked to join the consortium and participate in standardized pooled analyses of biomarker fatty acids and clinical events. Of 20 identified studies by November 2014, only 1 study declined to join the consortium (14). All cohort participants gave written informed consent according to their local guidelines. All studies received approval from ethical oversight committees.

Analysis plan

A standardized analysis protocol was developed and provided to each participating study, including harmonized definitions of populations, exposures, outcomes, covariates, effect modifiers, and methods for pooling across studies. These specific methods are detailed below. An experienced analyst from each study performed the analysis for that study using individual-level data and provided all results to the lead investigator (L.D.) in standardized electronic forms.

Population

To understand potential effects on primary prevention and minimize reverse causation, participants from each study were excluded if they had a prior history of myocardial infarction (MI), angina, coronary revascularization, or stroke. All remaining participants with measured omega-3 PUFA biomarker levels were included in the analyses.

Fatty acid ascertainment

Fatty acid biomarker concentrations were assessed in study-specific lipid compartments, including total plasma, erythrocyte and plasma phospholipids, cholesterol esters, triglycerides, and adipose tissue, all reported as weight percent of total fatty acids. Details of fatty acid measurement methods for each study are provided in the **Online-Only Supplement (pg 27)**.

Assessment of CHD

We evaluated total incident CHD, defined as fatal or nonfatal MI, CHD death, or sudden cardiac death; nonfatal MI, generally defined as chest pain with abnormal cardiac enzyme concentrations or serial electrocardiogram changes; and fatal CHD, defined as fatal MI, CHD death, or sudden cardiac death. Details of the methods for assessing and defining these outcomes in each study are provided in **eTable 1**. We excluded soft CHD endpoints (e.g., angina, revascularization) to minimize bias in ascertainment of these events.

Covariates

Covariates were standardized across studies based on pre-specified, harmonized definitions and categorizations, including age (continuous), sex (male, female), race (Caucasian, black, and by other study-specific subgroups, if available), clinical center, if applicable (study-

specific categories), body mass index (continuous), education (less than high school graduate, high school graduate, some college or vocational school, college graduate), smoking (current, former, never; or current vs. not current, if former not assessed), physical activity (quartiles of metabolic equivalents (METs); or if METs unavailable, four categories of physical or leisure activity as defined in the study), alcohol intake (none, 1-6 drinks/week, 1-2 drinks/day, >2 drinks/day; with 14 grams alcohol=1 standard drink), diabetes mellitus (yes, no; defined as treatment with oral hypoglycemic agents, insulin, or fasting glucose; or study-specific definitions otherwise); treated hypertension (yes, no; defined as treatment with anti-hypertensive drugs; diagnosed hypertension otherwise), treated hypercholesterolemia (yes, no; defined as treatment with lipid-lowering drugs; or diagnosed hypercholesterolemia otherwise), regular aspirin use [yes, no; categorized as 3+ times/week], and biomarker concentrations of omega-6 PUFA linoleic acid (LA; 18:2n-6), arachidonic acid (AA; 20:4n-6), and total trans fatty acids (each continuous). For categorical variables, missing covariate data were included as an indicator category.

Statistical analysis and pooling

Standardized study-specific analyses were performed for each study using individual-level data. Due to variability in the total number of fatty acids assessed across the different assays (from 12 to 58), and to permit comparison and pooling of findings across the different biomarker compartments, each fatty acid was analyzed continuously per 1 standard deviation increase.

For prospective cohorts, Cox proportional hazards models estimated the hazard ratio for incident total CHD, nonfatal MI, and fatal CHD, with follow-up from the date of biomarker

measurement to date of event, end of follow-up, loss to follow-up, or death, whichever occurred first. For prospective and retrospective matched case-control studies, conditional logistic regression was used to estimate the hazard ratio (if matched on time to event) or odds ratio. All analyses used robust standard errors.

For pooled meta-analyses, hazard ratios and odds ratios were considered to approximate relative risks (RRs). Meta-analysis was performed using random effects models using the method of DerSimonian & Laird, with the estimate of heterogeneity obtained from the Mantel-Haenszel model (15). Results were first pooled separately for each biomarker compartment and then pooled together. If more than one biomarker had been measured in a given study, main results included the biomarker which might best represent diet: for long-chain omega-3s, total plasma/serum > phospholipids (erythrocyte or plasma) > adipose > other; and for ALA, adipose > total plasma/serum > phospholipids (16).

To assess potential non-continuous relationships, we also evaluated study-specific quintiles for each biomarker compartment, performing meta-analysis within each quintile and comparing each quintile-specific pooled result to the first quintile. To statistically test potential nonlinear dose-responses, we performed multivariate meta-regression, modeling restricted cubic splines (17). These analyses evaluated total plasma and phospholipids; cholesterol esters and adipose tissue fatty acids were not evaluated using splines due to fewer numbers of studies in these compartments.

Overall heterogeneity was assessed using the I^2 statistic, with heterogeneity considered low if $I^2 < 35\%$. Heterogeneity by pre-specified subgroups was evaluated by pooling individual study results based on defined strata, including age (< 60 , ≥ 60 years), sex (male, female), race/ethnicity (Caucasian, Black, Hispanic, Chinese), LA and AA biomarker concentrations ($<$ or

$\geq$ median value in each study), type 2 diabetes (yes, no), statin use (yes, no), regular aspirin use (yes, no), year of biomarker sampling (<2000 , ≥ 2000), and (for ALA) EPA+DPA+DHA biomarker concentration ($<$ or $\geq$ median value in each study). Effect estimates in each study-specific strata were pooled, and statistical significance of differences between subgroups of potential sources of heterogeneity was assessed using meta-regression. In 7 studies with available genetic data, we also examined potential interaction by variants in *FADS* desaturase genes (rs174546, rs968567) (13), pooling study-specific estimates using an additive genetic model (Online-Only Supplement pg 39). Within each study, interaction terms for each SNP were constructed as a cross-product term of the omega-3 PUFA biomarker (continuous) by the SNP (ordinal: 0, 1, or 2 T alleles), including the main effects in the model; these interaction terms were pooled using meta-analysis.

Meta-analyses were performed using STATA 12 (StataCorp, College Station, Tex).. We considered two-tailed p values <0.05 to be statistically significant.

Sensitivity analyses

We performed several sensitivity analyses. We performed continuous meta-analysis using absolute (percent of fatty acids), rather than study-specific (90th vs. 10th percentile), units. To minimize potential reverse causation due to pre-existing subclinical disease, we excluded cases identified in first 2 years after biomarker sampling. To minimize exposure misclassification due to within-person changes in biomarker levels over time, we censored participants after the first 6 years of follow-up. We performed additional analyses limited to prospective studies only and those without self-reported CHD events.

Results

Characteristics of studies and participants

The 19 studies included 45,637 unique participants from 16 countries including the United States, Australia, Costa Rica, Finland, France, Germany, Ireland, Israel, Italy, Norway, Singapore, the Soviet Union, Spain, Sweden, Switzerland, and the United Kingdom (**Table 1**; see Online-Only Supplement for details of the participating studies). Most were prospective cohort (n=10 studies) or prospective nested case-control (n=7) studies; 2 were retrospective case-control studies using adipose tissue biomarkers. Biomarker types included phospholipids (plasma or erythrocyte) (n=10), total plasma (n=6), adipose (n=4), and cholesterol esters (n=1); two studies (NHS I and HPFS) measured both total plasma and erythrocyte phospholipids. As expected, omega-3 PUFA concentrations and distributions varied across biomarker types and study assays (**eTable 2, eTable 3**) with coefficients of variation <10% for most fatty acids and biomarkers (see Online-Only Supplement) For example, median ALA concentrations were generally higher in adipose (0.72% of total fatty acids) than in circulating biomarkers (0.20% of total fatty acids); while EPA, DPA and DHA concentrations were generally higher in phospholipids than other compartments, consistent with prior reports (16, 18).

Median age at baseline was 59 years (range: 18, 97 years), and 62% were male (**eTable 4**). During a median 10 years of follow-up (range: 1.3, 42), 7973 incident CHD events, 7157 nonfatal MIs, and 2781 fatal CHD events occurred. As not all studies ascertained every exposure and outcome (Table 1), cases of non-fatal MI and fatal CHD do not sum to the number of total CHD cases. Medication use varied across studies; by design, all participants in the analysis were free of prevalent CHD or stroke (**eTable 5**). Most participants were Caucasian, but CHS and MESA included relatively higher proportions of African-Americans (12.3% and 24.7%,

respectively); MESA and the Costa Rican adults study included Hispanics (24.9% and 100%, respectively), and MESA and SCHS included Chinese participants (25.0% and 100%, respectively). Across all studies, median BMI was 26 kg/m² and generally up to 30% of participants were current smokers, except for higher rates in SHHEC (44.6%) and ULSAM 50 (51.0%). Alcohol intake was modest, with most participants consuming up to 1 drink/day. Fish oil supplementation was infrequently assessed across studies; use was low (0-4% participants) in 5 of 6 studies with this data (**eTable 6**); only EPIC-Norfolk had higher prevalence (33%).

EPA, DPA, and DHA and incident CHD

In continuous (per 1 standard deviation increase) multivariable-adjusted analyses, each omega-3 PUFA was associated with about a 9% lower risk of fatal CHD, with RRs (95% CI) for EPA of 0.91 (0.82-1.00); DPA, 0.90 (0.85-0.96); and DHA, 0.90 (0.84-0.96) (**Table 2**). There was moderate heterogeneity in this association for EPA ($I^2 = 37\%$), and DHA and low heterogeneity for DPA and DHA ($I^2 = 0\%$ and 31%, respectively) (**Figure 1**). The sum of EPA + DPA + DHA was associated with a 11% lower risk of fatal CHD (**eFigure 1**). By contrast, none of the long-chain omega-3 PUFA were significantly associated with non-fatal MI. DPA, but not EPA, or DHA, was associated with a significantly lower risk of total CHD, with RRs (95% CI): 0.94(0.90-0.99), 0.94(0.87-1.02), and 0.95(0.91-1.00), respectively (Table 2).

Some differences were observed when EPA, DPA, and DHA were evaluated across quintiles (**eTable 7**). Upper quintiles of EPA and DHA were associated with a lower risk of non-fatal MI (Quintile 5 vs Quintile 1 comparison: 0.71 (0.56-0.90), 0.87 (0.78-0.97), respectively). Top quintiles of DPA and DHA were associated with a lower risk of fatal CHD (Quintile 5 vs Quintile 1 comparison: 0.76 (0.65-0.90), 0.77 (0.64-0.89), respectively) (**Figure 2**). Across

biomarker types, inverse associations were generally stronger in the phospholipid and total plasma compartments (Table 2); heterogeneity decreased when adipose tissue and cholesterol ester estimates were excluded from meta-analyses.

ALA and incident CHD

In continuous analysis, ALA was associated with a 9% lower risk of fatal CHD: RR (95% CI) 0.91 (0.84-0.98) (Figure 1), but not total CHD or nonfatal MI. For ALA, no single biomarker compartment consistently showed stronger associations across outcomes.

Restricted cubic spline analysis

Restricted cubic splines did not identify evidence for nonlinear associations of any of the omega-3 PUFA biomarkers and outcomes in total plasma or phospholipids (p -nonlinearity > 0.05 each; **eFigure 2**).

Effect modification

No significant differences in associations of omega-3 PUFA biomarkers with incident CHD events were observed by age, sex, omega-6 PUFA (LA or AA) concentrations, type 2 diabetes status, statin use, regular aspirin use, year of biomarker sampling, or (for ALA) EPA+DPA+DHA concentrations (p -heterogeneity > 0.05 each) (**eTable 8**). In a post-hoc meta-regression, we observed no significant differences in associations by median length of follow-up ($<$ or ≥ 10 y) (p -heterogeneity > 0.05 each). Effect modification was suggested by ethnicity: compared to Caucasians, ALA was associated with significantly lower risk of nonfatal MI among African-Americans (p -heterogeneity=0.001); and for EPA, with significantly lower risk

of total CHD and nonfatal MI among Chinese participants (p-heterogeneity= 0.02 and 0.01, respectively). Among 7 studies with SNP data (**eTable 9**), no significant interaction was identified by *FADS* desaturase gene variants (**eTable 10**).

Sensitivity Analyses

Compared to the main findings, no appreciable differences were observed after excluding cases identified in the first 2 years after biomarker sampling, censoring participants at the first 6 years of follow-up, excluding retrospective studies, or studies with self-reported events (**eTable 11**). For two studies that analyzed phospholipids in addition to total plasma (NHS I, HPFS), results were similar when phospholipid results from these studies were used in place of total plasma in the meta-analysis (data not shown).

Discussion

In this consortium pooling individual-level harmonized analyses from 19 studies including 45,637 unique participants and nearly 8,000 first CHD events in 16 countries, omega-3 biomarkers ALA, DPA, and DHA were associated with a modestly lower risk of fatal CHD, with EPA showing a trend towards lower risk. The magnitude of observed effect sizes for fatal CHD (~9% per 1 standard deviation increase) are consistent with findings for cardiac death from meta-analyses of trials (8). In contrast, associations with nonfatal MI were generally less robust. Across these diverse studies, findings were consistent by age, sex, omega-6 PUFA LA and AA levels, year of biomarker sampling, presence of absence of diabetes, statin use, and aspirin use. By contrast, effect modification by ethnicity was observed for select exposure-outcome pairings. Separate analyses by biomarker compartment provided further insights, with generally stronger inverse associations in phospholipids and total plasma for EPA, DPA and DHA. This investigation provides the most comprehensive estimates to date of the relationships between seafood and plant-based omega-3 PUFA, assessed using biomarkers, and primary incidence of CHD in generally healthy, free-living populations around the world.

In randomized controlled trials, long-chain omega-3 PUFA benefit multiple major cardiovascular risk factors, including triglyceride levels, blood pressure, heart rate, heart rate variability, endothelial function, and myocardial oxygen demand (1, 10). Compared with many other single nutrients, these demonstrated physiologic benefits provide strong biologic plausibility to support an impact on clinical events. EPA and DHA are also precursors to bioactive lipid metabolites including specialized pro-resolving mediators (SPMs) (18) and CYP450-generated mono-epoxides (MEFAs) (19) that could contribute to lower CHD risk. Our findings are consistent with prior experimental evidence that long-chain n-3 PUFA may have

membrane stabilizing actions in the setting of ischemia-induced ventricular fibrillation (1, 20) and observational evidence showing that benefits of fish consumption are most related to arrhythmic events (21) and fatal CHD (22).

Use of biomarkers allowed separate investigation of each individual omega-3 PUFA. Interestingly, EPA, DPA, and DHA were similarly associated with lower risk of fatal CHD. Biomarker levels of these fatty acids are only moderately interrelated, e.g. $r=0.43$, 0.51 and 0.13 for EPA and DHA, EPA and DPA, and DPA and DHA, respectively (23). Thus, observed associations for any one of these fatty acids are unlikely to be fully explained by the others. Compared with EPA and DHA, comparatively less is known about the molecular and physiologic effects of DPA; some studies suggest DPA may inhibit ex vivo collagen-stimulated platelet aggregation, thromboxane production, and cyclooxygenase-1 activity (10). In addition, whereas circulating and tissue levels of EPA and DHA are strongly influenced by dietary seafood consumption, DPA concentrations appear to be mainly derived from endogenous elongation of EPA; interconversion between DPA and DHA is very limited (10).

Given diverse global dietary sources, effects of ALA on CHD are of particular interest. However, prior reports of dietary and biomarker ALA and CHD risk have been inconsistent (11), perhaps owing to methodologic and analytic differences in these investigations. A key strength of our analysis was the pre-specified, harmonized analytic plan using individual-level data within each study, which allowed consistent assessment of how ALA relates to CHD. In addition, the inclusion of 19 studies minimized the impact of publication bias, wherein studies with positive findings are more likely reported. Compared with prior work (24), we also took advantage of a far larger number of events (e.g., over 4-fold larger for fatal CHD) and observed a similarly lower risk of fatal CHD for ALA as for long-chain omega-3 PUFA. Mechanistically, these

findings are supported by effects of ALA on thrombosis, inflammation, arrhythmia, and endothelial function (25-29). Our findings, combined with relative affordability, global accessibility, and sustainability of ALA (30), support the potential importance of ALA for improving global cardiovascular health.

The large number of cases in our analysis, and inclusion of multiple ethnic subgroups, allowed us to explore potential effect modification in omega-3 PUFA-CHD outcome associations by race/ethnicity. The stronger effects among African-American and Chinese for select n-3-outcome pairings could be due to differences in consumption patterns, true biologic diversity in pathophysiologic pathways, or chance. For instance, preparation methods among Chinese populations could differ from Caucasians, with raw/steamed fish in the former population, vs. more deep fried fish in the latter. Differential pathophysiologic pathways for cardiometabolic risk have also been documented among Asian vs. Caucasian populations; for example, Asians develop type 2 diabetes at a much lower BMI than Caucasians (31,32) and at least three meta-analyses have reported a protective effect of seafood-derived n-3s on incident diabetes among Asians, but not Caucasians (33-35). Finally, our findings of effect modification by race could be due to chance; few studies were available with African-American (n=2 studies: CHS, MESA) and Chinese subgroups (n=2 studies: SCHS, MESA). Our results highlight the need for further work to better understand differences in associations by race for omega-3s and incident CHD phenotypes.

Randomized trials of fish oil supplements have shown mixed effects, although overall pooled findings indicate benefit for lowering risk of cardiac death (8), consistent with our findings. Our investigation tests unique and separate questions from these trials due to differences in omega-3 PUFA source (in our study, dietary; vs. supplements in trials), population

(primary prevention vs. secondary prevention/high risk), and duration (habitual intake vs. short-term supplementation). In cohort-harmonized stratified analyses, we found little evidence for effect modification by statin or aspirin use, which have been hypothesized to reduce benefits of omega-3's (36). We also found little evidence of effect modification by circulating omega-6 PUFA, LA or AA, which have been hypothesized – but never shown in humans – to reduce cardiovascular benefits of omega-3 PUFA (37). Altogether, our findings suggest that both seafood and plant-derived omega-3 PUFA are beneficial for fatal CHD prevention across diverse population subgroups.

Our stratification by biomarker type provides new insights on which lipid compartments may most influence CHD. Adipose tissue has been suggested for measurement of fatty acids because it reflects long-term dietary intake (~1 yr) (38). Yet, EPA and DHA are more highly concentrated in phospholipids than other compartments, and phospholipids also respond rapidly to dietary changes (39, 40), and may best reflect effects on membrane receptors (1). The best compartment for assessing ALA has also been unclear, with possible advantages to adipose tissue (16) but relatively little prior evaluation of other compartments. Our findings suggest generally stronger inverse associations with CHD for EPA, DPA, and DHA in phospholipids and total plasma, although fewer estimates and cases were available for adipose and cholesterol esters compartments, decreasing precision. Our results support use of phospholipids or total plasma for long-chain omega-3 PUFA exposure, and a need for further investigation of optimal biomarkers for assessing ALA.

Our analysis has several important strengths. Use of biomarkers provided measures of exposure free from recall error, and allowed separate evaluation of different omega-3 PUFA as well as different circulating and tissue lipid compartments. We used standardized definitions and

modeling for the populations, exposures, outcomes, covariates, effect modifiers, and analysis, reducing heterogeneity and potential investigator bias. The majority of studies employed centralized adjudication processes or registry linkage rather than self-report in ascertaining events, reducing information bias. Findings were similar in several sensitivity analyses, suggesting robustness of results to varying assumptions. Our inclusion of all available studies substantially reduces potential for publication bias. The studies included both sexes, multiple races, and a range of socioeconomic statuses across 16 countries, increasing generalizability.

Potential limitations should be considered. Relatively few studies were available for some lipid compartments (e.g., adipose, cholesterol esters), limiting inference for these specific compartments. All cohorts assessed omega-3 PUFA exposure once at baseline; and changes over time would attenuate findings toward the null, causing underestimation of associations. Reduced statistical power in quintile analyses and spline analyses made it difficult to ascertain the specific shape of dose-responses. As we analyzed observational studies, biomarkers and risk factors were not evenly distributed by randomization of study participants and hence, unmeasured or residual confounding cannot be excluded; omega-3 biomarker measures may in part be markers of a long-term healthy lifestyle. However, our findings were independent of a range of major cardiovascular risk factors, and the relative specificity for fatal CHD and known biologic effects of omega-3 PUFA also argues against residual confounding as the sole explanation for our findings. As our findings reflect habitual omega-3 exposure for primary CHD prevention, our results do not imply that fish oil supplementation will necessarily reduce CHD events, particularly in the context of short-term treatment or in those with pre-existing CHD. Both awareness of omega-3 PUFA, laboratory ascertainment methods, and medical treatment of hypertension, hypercholesterolemia, diabetes, angina, and MI have changed over time and could

contribute to heterogeneity of results; however, we observed no significant differences results for studies assaying omega-3 PUFA levels prior to vs. after the year 2000.

In conclusion, our pooled collaboration of global studies using biomarkers of omega-3 PUFA in participants without prevalent CHD shows that habitual consumption of both seafood and plant-based omega-3 PUFA is associated with a modestly lower risk of fatal CHD.

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Figure Legends

Figure 1. Relative risk (RR) of fatal CHD per 1 standard deviation (SD) increase in biomarker (A) alpha-linolenic acid (ALA; 18:3n3), B) eicosapentaenoic acid (EPA; 20:5n3), (C) docosapentaenoic acid, (DPA; 22:5n3), and (D) docosahexaenoic acid (DHA; 22:6n3). Estimates were pooled using random effects meta-analysis

Figure 2. Relative risk (RR) of fatal CHD by quintile (Q) of biomarker alpha-linolenic acid (ALA; 18:3n3), eicosapentaenoic acid (EPA; 20:5n3), docosapentaenoic acid, (DPA; 22:5n3), and docosahexaenoic acid (DHA; 22:6n3). Categorical estimates were pooled using random effects meta-analysis

Table 1. Baseline characteristics of 19 studies and 45,637 participants with biomarker measures of omega-3 fatty acids ¹

Study ²	Country	Study design ³	Age, y (mean)	Sex (% male)	Biomarker compartment	Year of blood sampling	Fatty acids assessed	Coronary heart disease (CHD) outcomes
ARIC	United States	PC	54	48	Plasma phospholipid	1987-1989	ALA, DPA, EPA, DHA	Total CHD
Costa-Rican adults	Costa Rica	RCC	59	72	Adipose tissue	1995-2004	ALA, DPA, EPA, DHA	Nonfatal MI
CHS	United States	PC	74	40	Plasma phospholipid	1992-1993	ALA, DPA, EPA, DHA	Total CHD, nonfatal MI, fatal CHD
EPIC-Norfolk	UK	PCC	63	49	Plasma phospholipid	2001-2004	ALA, DPA, EPA, DHA	Total CHD, nonfatal MI, fatal CHD
EURAMIC	Finland, Norway, Scotland, Ireland, Germany, Switzerland, Spain, Israel, Russia	RCC	54	100	Adipose tissue	1991-1992	ALA, DPA, DHA	Nonfatal MI
HPFS	United States	PCC	64	100	Total plasma; Erythrocyte phospholipid	1994, 1994	ALA, DPA, EPA, DHA	Total CHD, nonfatal MI, fatal CHD
InCHIANTI	Italy	PCC	65	45	Total plasma	1998-2000	ALA, EPA, DHA	Total CHD
KIHD	Finland	PC	52	100	Total plasma	1991-1992	ALA, DPA, EPA, DHA	Total CHD, nonfatal MI, fatal CHD
MCCS	Australia	PC	56	49	Plasma phospholipid	1990-1994	ALA, DPA, EPA, DHA	Fatal CHD
MESA	United States	PC	62	47	Plasma phospholipid	2000-2002	ALA, DPA, EPA, DHA	Total CHD, nonfatal MI, fatal CHD
NSHDS I	Sweden	PCC	55	79	Plasma phospholipid	1995	ALA, DPA, EPA, DHA	Total CHD
NSHDS II	Sweden	PCC	55	62	Plasma phospholipid	2007	ALA, DPA, EPA, DHA	Total CHD, nonfatal MI, fatal CHD
NHS I	United States	PCC	60	0	Total plasma; Erythrocyte phospholipid	1989-1990, 1989-1990	ALA, DPA, EPA, DHA	Total CHD, nonfatal MI, fatal CHD
PHS	United States	PCC	69	100	Erythrocyte phospholipid	1995-2000	ALA, DPA, EPA, DHA	Total CHD, nonfatal MI, fatal CHD
SHHEC	Scotland	PC	49	52	Adipose tissue	1984-1986	DPA, DHA	Total CHD, nonfatal MI, fatal CHD
SCHS	Singapore	PCC	66	65	Total plasma	1994-2005	ALA, EPA, DHA	Total CHD, nonfatal MI, fatal CHD
3C Study	France	PC	75	39	Total plasma	1999-2000	ALA, DPA, EPA, DHA	Total CHD
ULSAM 50	Sweden	PC	50	100	Cholesterol esters	1970	ALA, EPA, DHA	Total CHD, nonfatal MI, fatal CHD
ULSAM 70	Sweden	PC	71	100	Adipose tissue	1990	ALA, DPA, EPA, DHA	Total CHD, nonfatal MI, fatal CHD

¹Characteristics described at time of fatty acid biomarker measurement

²ARIC: Atherosclerosis Risk in Communities; Costa-Rican adults study; CHS: Cardiovascular Health Study; EPIC-Norfolk: European Prospective Investigation into Cancer (Norfolk); EURAMIC: European study on Antioxidants, Myocardial Infarction and Cancer; HPFS: Health Professionals Follow-up Study; InCHIANTI: Invecchiare in Chianti; KIHD: Kuopio Ischaemic Heart Disease Risk Factor Study; MCCS: Melbourne Collaborative Cohort Study; MESA: Multi-Ethnic Study of Atherosclerosis; NSHDS I & II: Northern Sweden Health & Disease Study I & II; NHS I: Nurses' Health Study I; PHS: Physicians' Health Study; SHHEC: Scottish Heart Health Extended Cohort; SCHS: Singapore Chinese Health Study; 3C Study: Three City Study; ULSAM 50 & 70: Uppsala Longitudinal Study of Adult Men

³PC=prospective cohort; PCC=prospective nested case-control; RCC=retrospective case-control

Table 2. Pooled relative risks (RR) of total CHD, nonfatal MI, and fatal CHD per 1 standard deviation increase in alpha-linolenic acid (ALA; 18:3n3), eicosapentaenoic acid (EPA; 20:5n3), docosapentaenoic acid, (DPA; 22:5n3), and docosahexaenoic acid (DHA; 22:6n3)¹

Exposure	Biomarker	Total CHD			Nonfatal MI			Fatal CHD		
		<i>N</i> cases ²	<i>N</i> studies	<i>RR</i> (95% <i>CI</i>)	<i>N</i> cases	<i>N</i> studies	<i>RR</i> (95% <i>CI</i>)	<i>N</i> cases	<i>N</i> studies	<i>RR</i> (95% <i>CI</i>)
ALA	Total plasma	2286	6	1.05 (0.91-1.20)	1578	4	1.07 (0.87-1.31)	640	4	0.76 (0.63-0.91)
	Phospholipids	3719	7	0.98 (0.92-1.06)	1790	5	0.95 (0.87-1.03)	1519	6	0.90 (0.82-0.98)
	Adipose tissue	206	1	1.09 (0.74-1.62)	2385	3	0.71 (0.26-1.95)	83	1	1.03 (0.58-1.82)
	Cholesterol esters	749	1	1.00 (0.92-1.08)	364	1	0.90 (0.80-1.01)	398	1	1.02 (0.92-1.13)
	Overall	6960	15	1.00 (0.95-1.05)	6117	13	0.95 (0.87-1.05)	2640	12	0.91 (0.84-0.98)
EPA	Total plasma	2194	6	0.93 (0.97-1.11)	1499	4	0.89 (0.69-1.14)	627	4	0.80 (0.51-1.25)
	Phospholipids	3703	7	0.89 (0.81-0.99)	1790	5	0.91 (0.79-1.04)	1519	6	0.85 (0.74-0.97)
	Adipose tissue	181	1	1.19 (1.05-1.33)	1734	2	0.85 (0.67-1.09)	74	1	1.08 (0.86-1.37)
	Cholesterol esters	749	1	0.97 (0.88-1.07)	364	1	0.95 (0.83-1.09)	398	1	0.97 (0.83-1.14)
	Overall	6827	15	0.94 (0.87-1.02)	5387	12	0.92 (0.83-1.01)	2613	12	0.91 (0.82-1.00)
DPA	Total plasma	1412	4	0.93 (0.84-1.02)	1111	3	0.94 (0.81-1.08)	348	3	0.84 (0.71-1.01)
	Phospholipids	3703	7	0.92 (0.86-0.97)	1767	5	0.95 (0.86-1.06)	1542	6	0.91 (0.80-1.02)
	Adipose tissue	1092	2	1.01 (0.94-1.08)	3137	4	0.98 (0.88-1.11)	222	2	0.96 (0.83-1.12)
	Cholesterol esters	-	-	-	-	-	-	-	-	-
	Overall	6207	13	0.94 (0.90-0.99)	6015	12	0.97 (0.93-1.02)	2112	11	0.90 (0.85-0.96)
DHA	Total plasma	2425	6	0.91 (0.82-1.02)	1866	4	0.91 (0.84-0.98)	640	4	0.78 (0.69-0.90)
	Phospholipids	3703	7	0.93 (0.89-0.97)	1790	5	0.97 (0.91-1.03)	1519	6	0.88 (0.83-0.93)
	Adipose tissue	1096	2	1.05 (0.95-1.16)	3137	4	0.89 (0.68-1.18)	224	2	0.99 (0.86-1.15)
	Cholesterol esters	749	1	1.01 (0.93-1.09)	364	1	1.03 (0.81-1.15)	398	1	1.02 (0.91-1.15)
	Overall	7973	16	0.95 (0.91-1.00)	7157	14	0.96 (0.92-1.01)	2781	13	0.90 (0.84-0.96)

¹Continuous estimates were pooled using random effects meta-analysis

²As not all studies ascertained every exposure and outcome (Table 1), cases of non-fatal MI and fatal CHD do not sum to the number of total CHD cases

